Biochemical and functional characterization of cell-free expressed G-protein coupled receptors of the human endothelin system

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„Fantasie ist wichtiger als Wissen. Wissen ist begrenzt, Fantasie aber umfasst die ganze Welt.“

Albert Einstein

Table of contents

Abbreviations

4-Ala-ET-1	4 – alanine 1,3,11,15 mutant ET-1
°	degree
∞	for ever
µ	micro
A	Ampère
aa	amino acid(s)
AcP	acetyl phosphate
APS	ammoniumperoxodisulfate
AR	adrenergic receptor
aso-PC	L-α-phosphatidylcholine from soybean, type IV-S
ATP	adenosine triphosphate
b-ET-1	biotinylated ET-1
BN-PAGE	Blue Native polyacrylamide gel electrophoresis
β-OG	n-β-octyl-D-glucopyranoside
bp	base pairs
B35 (Brij 35)	polyoxyethylene-(23)-lauryl-ether
B58 (Brij 58)	polyoxyethylene-(20)-cetyl-ether
B78 (Brij 78)	polyoxyethylene-(20)-stearylether
B98 (Brij 98)	polyoxyethylene-(20)-oleyl-ether
BSA	bovine serum albumin
c	concentration
C	control, Celsius
C_6F-TAC	$C_6F_{13}C_2H_4$-S-poly[tris(hydroxymethyl)aminomethane]
C_8F-TAC	$C_8F_{17}C_2H_4$-S-poly[tris-(hydroxylmethyl)aminomethane]
CCM	cholesterol consensus motif
CD	circular dichroism
CECF	continuous-exchange cell-free
CF	cell-free
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid
CHS	cholesteryl hemisuccinate
CMC	critical micellar concentration
Co-NTA	cobalt-nitrilotriacetic acid
CRF	corticotropin releasing factor receptor
CV	column volume
Da	Dalton
D-CF	cell-free protein synthesis in presence of detergents
DDM	n-dodecyl-β-D-maltoside
DHPC, diC_6PC	1,2-dihexanoyl-sn-glycero-3-phosphocholine
DMPC	1,2-dimyristoyl-sn-phosphatidylcholine
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleinacid
DOPE	1,2-dioleyl-sn-glycero-3-phosphoethanolamine
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
dNTP	desoxyribonucleitriphosphate
DTT	dithiothreitol
ECL	extracellular loop
E. coli	*Escherischia coli*
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
EM	electron microscopy
ER	endoplasmic reticulum
ESI	electrospray ionisation
ET	endothelin

ETA	endothelin A receptor
ETB	endothelin B receptor
et al.	and others
EtBr	ethidium bromide
f-4-Ala-ET-1	fluorescein labelled 4 – alanine 1,3,11,15 mutant ET-1
FM	feeding mix
FPLC	fast performance liquid chromatography
Fos-12	n-dodecylphosphocholine
Fos-16	n-hexadecylphosphocholine
FRET	fluorescence resonance energy transfer
g	gram
GFP	green fluorescence protein
GPCR	G-protein coupled receptor
h	hour(s)
HDM	n-hexadecyl-β-D-maltoside
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
HRH 1	hitamine H1 receptor
HRP	horseradish peroxidase
HSQC	heteronuclear single quantum coherence
IB	inclusion body
IC50	half maximal inhibitory concentration
IL	intracellular loop
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl-β-D-thiogalactopyranosid
K_D	Equilibrium dissociation constant
KP	potassium phosphate
L	liter(s)
LB	Luria-Bertani
L-CF	cell-free protein synthesis in presence of lipids
LMPC	1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine
LMPG	1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)]
LPPG	1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)]
m	milli
M	molar (mol/L)
MALS	multi angle light scattering
MAM	monomeric avidin matrix
MBP	maltose binding protein
mdeg	millidegree
mg	milligram(s)
min	minute
mL	milliliter(s)
mRNA	messenger RNA
MP	membrane protein
MW	molecular weight
MWCO	molecular weight cut-off
n	nano
NaP	sodiumphosphate
NC	negative control
Ni-NTA	nickel-nitrilotriacetic acid
nL	nanoliter(s)
nm	nanometer
NMR	nuclear magnetic resonance
NTP	nucleotide triphosphate
NTR	neurotensin receptor
o. n.	over night
P	pellet
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine

P-CF	precipitate generating cell-free protein synthesis without addition of hydrophobic substances
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PEI	polyethyleneimine
PEP	phosphoenol pyruvate
PG	phosphatidylglycerol
PI	phosphatidylinositol
POPS	1-pamitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt)
PR	proteorhodopsin
PS	phosphatidylserine
PTM	post-translational modification
RM	reaction mix
RNA	ribonucleic acid
RI	refractive index
ppm	parts per million
rpm	round per minute
RT	room temperature
s	second
S	supernatant
SEC	size exlusion chromatography
SDS	sodium dodecylsulfate
SPR	surface plasmon resonance
T7RNAP	T7 RNA polymerase
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
TM	transmembrane
TMS	transmembrane segment
TMV	tobacco mosaic virus
Tris	tris(hydroxymethyl)methylamine
Triton X-100	polyethylene-glycol P-1,1,3,3-tetra-methyl-butylphenyl-ether
tRNA	transfer RNA
Trx	thioredoxin
Tween 20	polyoxyethylene-sorbitane-monolaurate 20
UTR	untranslated region
V2R	vasopressin 2 receptor
WGE	wheat germ extract

Summary

G-protein coupled receptors (GPCRs) are the most abundant class of cell surface receptors encoded by the human genome. They are the key players in signal perception and transduction which makes them consequently to one of the currently most important class of drug targets. Approximately 50 % of market pharmaceuticals are directed against them. The majority of GPCRs is expressed at very low levels in native tissues rendering it virtually impossible to obtain amounts sufficient for the determination of their three-dimensional structure. Heterologous overexpression in cell-based systems as an indispensable prerequisite for structural analysis of most GPCRs has therefore been approached but remains a challenge now for decades. The recent few successful structural approaches result from sophisticated and elaborated strategies solely adapted to single targets. The major bottleneck for structural determination of GPCRs is hence to obtain milligram quantities of highly pure, homogenous and stable protein.

Instead of using conventional *in vivo* expression hosts, I focused on the alternative cell-free (CF) expression strategy for the production and characterization of the human endothelin (ET) receptom in this study. The receptor associated system is one of the major modulators in cardiovascular regulation and plays a crucial role in mitogenesis. Many civilisatory disorders are associated with its dysfunction and ET antagonism is considered to be a possible treatment of diseases like heart failure, hypertension, diabetes, artherosclerosis and even cancer.

CF expression has emerged as a highly competitive, powerful technique to overcome major restrictions of classical *in vivo* MP production as it eliminates the complexity of a living host cell. Its open nature and high versatility offers in addition a variety of valuable advantages that can be adapted to the needs of the target protein.

As initial comparisons between self-made eukaryotic wheat germ CF extracts and a self-made prokaryotic *E. coli* CF expression system did not show any benefit of the eukaryotic background with regard to the productivity of the human endothelin B receptor (ETB) I chose the easier to handle prokaryotic source for further studies.

The major task of this thesis was the development of preparative scale expression protocols for the endothelin A receptor (ETA) in qualities sufficient for structural analyses along with the set-up of target specific optimization protocols. The versatility of the CF expression system was used to modulate its sample quality by alteration of detergents hence presenting different solubilization environments to the synthesized protein at different stages of the production process. Sample properties after co- and post-translational solubilization have

been analysed by the evaluation of homogeneity, protein stability and receptor ligand binding competence.

After basic optimization I was able to produce up to 2-3 mg/mL of ETA in the precipitate generating continuous exchange (P-CF) mode. The resolubilization in LPPG was quantitative and resulted in a homogenous sample preparation as evaluated by BN-PAGE and size exclusion chromatography (SEC). Detergent-based expression (D-CF) in contrast showed an apparently lower homogeneity. Analysis of the initial P-CF ETA precipitate by solid-state NMR (Karsten Mörs, group of Prof. Clemens Glaubitz) already revealed an estimated amount of 30-40 % of α-helical secondary structure elements, only slightly lower than the determined and predicted values and supported further the use of P-CF expressed receptor.

Highest purity with over 90 % along with the highest yield of up to 1 mg/mL were achieved upon purification by metal chelate chromatography coupled to a detergent exchange or to a reduction of detergent concentration from LPPG in LPPG, B35 and Fos-16. In those conditions, ETA showed a high degree of apparent homogeneity and stability at 4 °C for at least one week evaluated by its elution profiles in SEC analyses. By circular dicroism (CD) spectroscopy at 222 nm, the secondary structure of ETA was further characterized by a transition at ~85 °C under optimal conditions, indicating a very high thermostability in B35. Monodispersity of ETA was most prominent in B35 as supported by electron micrographs of negatively stained samples. BN-PAGE and SDS-PAGE analysis hinted to a potential formation of SDS-resistant dimers/oligomers of ETA which was supported by ESI mass spectrometry. A similar behaviour has been detected in native receptors in literature before. The equilibrium in between monomeric and dimeric CF expressed ETA could obviously be triggered by the surrounding micelles like multi angle light scattering (MALS) experiments suggested. Whereas in Fos-16 ETA seemed to be mostly monomeric, coexistent dimer and monomer fractions were obtained in B35. Moreover, first hints of a potential homo- or hetero-oligomerization behaviour of CF expressed ETA and ETB receptors were obtained by co-expression of full-length receptors with various designed truncated derivatives and subsequent pull-down assays. Negative controls which resulted in false positives pointed however at the need of complementary approaches to support these initial findings.

Functional folding of ETA was evaluated by different approaches in present thesis. Classical post-translational *in vitro* reconstitution in artificial liposomes revealed homogenously distributed particles by freeze-fracture analysis. Equilibrium dissociation constants (K_D) of 1.4 nM for its circular ligand ^{125}I-endothelin 1 (ET-1) were determined and IC50 values of 2.17 µM for homologous competition were obtained. Lipid-based CF (L-CF) expressed ETA used for co-translational reconstitution further showed preliminary ligand binding capacity.
The ligand binding competence of detergent-solubilized ETA receptor was investigated by ligand affinity chromatography and fluorescence anisotropy. An efficient and reliable protocol

for the purification of ligand-bound CF expressed ETA was therefore developed based on a biotinylated ligand variant (b-ET-1) immobilized on monomeric avidin agarose beads. Dependent on different production modes and diverse micellar environments, different degrees of ligand binding competence were obtained. Binding of ETA to its ligand ET-1 at levels of up to 50 % in B35 resulted in the isolation of up to 0.5 mg/mL binding competent receptor. Fluorescence anisotropy of ETA in B35 further supported functional folding. A K_D of the linear fluorescein-labelled mutant of ET-1 (f-4-Ala-ET-1) was determined at around 1.2 µM which is comparable to published values of crude membrane fractions.

X-ray crystallization trials were started with ETA in B35 but in initial condition screens no crystals have been obtained yet. Tendencies towards the formation of microcrystals might however hint to a future potential under optimized conditions.

As a basis to alternative structural analyses using nuclear magnetic resonance (NMR) spectroscopy the use of self-splicing split inteins for the segmental labelling of the transmembrane segment 1 was successfully approached by CF synthesis. However, as the final yield of the end product was very low, optimization of constructs as well as of the splicing reaction itself should be prime parameters before setting up NMR studies.

The obtained results document the CF production of the ETA receptor in two different modes as an example of a class A GPCR in ligand-binding competent and non-aggregated form in quantities sufficient for structural approaches. The presented strategy could serve as basic guideline for the production of related receptors in similar systems.

Zusammenfassung

G-Protein gekoppelte Rezeptoren (GPCRs), bilden mit etwa 3 % die größte Klasse an Zelloberflächenrezeptoren im menschlichen Proteom. Ihre Hauptaufgabe besteht in der Aufnahme einer Vielzahl externer Stimuli. Infolgedessen, meist durch dynamische Interaktion mit heterotrimeren G-Proteinen ausgelöst, werden diese Stimuli zu einer spezifischen intrazellulären Antwort umgesetzt. Durch ihre bedeutende Funktion bei der Verarbeitung vielfältiger Umweltsignale und bei der Steuerung physiologischer Abläufe im menschlichen Organismus ist es nicht verwunderlich, dass GPCRs mit einem Anteil von rund 50 % zu den wichtigsten Angriffspunkten heutiger Arzneimittel gehören und daher im Fokus der biochemischen und pharmakologischen Forschung stehen.

Hochaufgelöste dreidimensionale Strukturen stellen nach wie vor die Grundlage für die Aufklärung der Funktionsweise dieser Proteine auf molekularer Ebene dar. Sie sind essentiell, um wirksame und spezifisch abgestimmte Medikamente zu entwickeln. Die Mehrheit der GPCRs wird allerdings auf sehr geringem Niveau in nativen Geweben produziert. Somit ist es nahezu unmöglich, ausreichende Mengen homogener und stabiler Proteine zu ihrer strukturellen Aufklärung zu gewinnen. Heterologe Überexpression wird daher als unabdingbare Voraussetzung für die strukturelle Analyse der meisten GPCRs angesehen und viele Systeme wurden intensiv auf ihre Anwendbarkeit untersucht. Die Überexpression der Zielproteine in konventionellen zell-basierenden Systemen ist aber seit Jahrzehnten schon eine Herausforderung, da sie oft mit einer zytotoxischen Wirkung auf den Organismus einhergeht und die funktionelle Extraktion aus den Zellmembranen in präparativen Mengen häufig große Schwierigkeiten bereitet.

Die vorliegende Arbeit konzentrierte sich daher nicht auf die Anwendung klassischer *in vivo* Expressionsysteme für die Überproduktion von GPCRs, sondern auf die Entwicklung effizienter Syntheseprotokolle in alternativen selbst hergestellten zellfreien Expressionssystemen. Als Zielproteine wurden die humanen Endothelin (ET)-Rezeptoren gewählt, die sich durch eine hohe pharmakologische Relevanz auszeichnen. Das humane ET-System ist einer der wichtigsten Modulatoren des kardiovaskulären Systems und spielt zudem eine essentielle Rolle bei Zellproliferation und Mitogenese. Es besteht aus zwei Rhodopsin-ähnlichen GPCRs, dem Endothelin A (ETA) und dem Endothelin B Rezeptor (ETB). Der prinzipielle Agonist des kardiovaskulären Systems ist das zyklische und hochkomplexe Peptid ET-1, das zu beiden Rezeptoren gleiche Affinität besitzt. Trotz einer Identität ihrer Aminosäuresequenz von 59 % unterscheiden sich die beiden Rezeptoren hinsichtlich ihres physiologischen Wirkspektrums. Eine Stimulation von ETA führt beispielsweise zur Vasokonstriktion während ETB eine vasodilatierende Wirkung zugeschrieben wird. Viele bekannte Zivilisationskrankheiten werden

mit endothelialer Fehlfunktion assoziiert. ET-Rezeptor-Antagonismus wird daher unter anderem als potente Methode zur Behandlung von Herzinsuffizienz, Bluthochdruck, Diabetes, Atherosklerose und sogar von Krebs aufgefasst.

Die zellfreie Expression hat sich zu einer hochkompetitiven, potenten Technik, insbesondere auch zur Produktion von Membranproteinen, entwickelt. Durch die Arbeit mit Zellextrakten wird die Komplexität lebender Zellen beseitigt, der Produktionsprozess vereinfacht, und somit einige Hauptprobleme konventioneller *in vivo* Membranprotein-Expression umgangen. Toxische Effekte der synthetisierten Proteine auf den Expressions-organismus treten nicht auf und die Geschwindigkeit der zellfreien Reaktion mit hohen Produktionsraten macht sie interessant für strukturelle Untersuchungen. Zusätzlich ermöglicht die zellfreie Synthese durch direkte Zugänglichkeit zur Reaktion eine individuelle, auf das jeweilige Zielprotein abgestimmte Optimierung. Frisch translatierte Proteine können durch eine Reihe fördernder Additive zu jedem Zeitpunkt des Syntheseprozesses stabilisiert werden.

Das in dieser Arbeit verwendete zellfreie Expressionssystem beruht auf einem optimierten *E. coli* S30-Extrakt, der alle wichtigen hochmolekularen Komponenten zur Translation enthält. Zusätzliche Bestandteile und Vorstufen für eine gekoppelte Transkriptions/Translations-Reaktion werden noch hinzugefügt. Ein effizientes System zur Energieregeneration ist ebenfalls integraler Bestandteil der zellfreien Synthese. Zur Herstellung der ET-Rezeptoren wurde die „continuous-exchange cell-free“ Konfiguration verwendet, die durch ein Dialyseverfahren eine Akkumulation inhibierender Nebenprodukte verhindert und gleichzeitig die weitere Versorgung der Reaktionslösung mit Substraten gewährleistet. Dieser Aufbau, der durch lange Reaktionszeiten charakterisiert ist, erlaubt eine effiziente Produktion der Zielproteine sowohl im analytischen (~50 µL) als auch im präparativen Maßstab von ≥1 mL.

Die Hauptaufgabe der vorliegenden Dissertation bestand in der Entwicklung Zielprotein-spezifischer, effizienter Syntheseprotokolle für GPCRs am Beispiel des humanen ET-Rezeptoms in zellfreien Expressionssystemen. Da initiale Mengenanalysen des zellfrei exprimierten ETB Rezeptors und Hinweise auf eine ähnliche Qualität keine ersichtlichen Vorteile in selbst hergestellten eukaryotischen Weizenkeimextrakten aufzeigten, wurde für weitere Untersuchungen das verlässlich etablierte auf *E. coli* basierende prokaryotische Zellfrei-System angewandt.

Im Speziellen sollte der ETA Rezeptor in diesem System in präparativen Mengen gewonnen und seine Qualität für funktionelle und strukturelle Charakterisierung optimiert werden. Die Vielseitigkeit der zellfreien Proteinsynthese wurde dazu genutzt, die Probenqualität von ETA durch Variation von Detergenzien zu beeinflussen, indem um ETA verschiedene mizellare Umgebungen zu unterschiedlichen Phasen des Produktionsprozesses generiert wurden. Die Probenqualität nach ko- und post-translationeller Solubilisierung wurde

anschließend anhand von Parametern wie Homogenität, Proteinstabilität sowie Liganden-bindungs-Kompetenz analysiert. Zur funktionellen Analyse von zellfrei exprimiertem Rezeptor wurden hier verschiedene Methoden etabliert.

Alternativ sollte ETA in artifizielle Liposomen rekonstituiert werden, um einen Vergleich zu *in vivo* exprimierten ET-Rezeptoren zu ermöglichen, deren Funktionalität üblicherweise in Membranfraktionen bestimmt wird. Entsprechende Radioliganden-Experimente wurden zu diesem Zweck eingeführt. Einerseits wurde hierzu die klassische post-translationelle Rekonstitution angewandt und deren Effizienz durch Optimierung spezifischer Parameter anhand von Messung der Funktionalität evaluiert. Zusätzlich wurde ein dritter zellfrei-spezifischer Modus zur Rekonstitution von ETA untersucht. Die direkte Zugabe von artifiziellen Liposomen im Lipid basierenden Modus (L-CF) wurde für ETA implementiert und vorläufige Untersuchungen zur Insertionseffizienz unternommen.

Abschließend wurden Untersuchungen zum Oligomerisierungs-Potential der zellfrei synthetisierten ET-Rezeptoren durchgeführt. Da in der Oligomerisierung von GPCRs eine hohe pharmakologische Relevanz vermutet wird, sollte Aufschluss über das native Verhalten der zellfrei hergestellten Rezeptoren erhalten und mittels verkürzter Varianten der Rezeptoren Hinweise auf die mögliche Interaktionsfläche erlangt werden.

Im P-CF Modus wurden nach Optimierung der Grundparameter präparative Mengen von ETA von bis zu 2-3 mg/mL erhalten. Die Resolubilisierung des Präzipitats im Detergenz LPPG erwies sich mit 100 % Löslichkeit als am erfolgreichsten. Analytische Gelfiltrations-experimente der Direktsolubilisate zeigten zudem die höchste Homogenität in LPPG. Blau-native Polyacrylamid-Gelelektrophorese ließ zusätzlich mono- und dimeren Charakter des Proteins in diesem Detergenz vermuten.

Für die lösliche Produktion von ETA im D-CF Modus wurden der Polyoxyethylen-Alkyl-Ether Brij 35 (B35) und das Steroid-Derivat Digitonin als geeignet gefunden. Allerdings erreichte Digitonin nur eine Solubilisierungseffizienz von 60 %. B35 erlaubte eine Produktionsmenge von ETA von maximal 1 mg/mL und lag demnach um durchschnittlich 50 % niedriger als die P-CF Expression. Eine Evaluierung der Homogenität der D-CF Probe nach Aufreinigung in B35 durch Gelfiltration und Negativfärbung zeigte eine geringere Probenqualität, so dass ETA infolgedessen bevorzugt im P-CF Modus synthetisiert wurde.

Festkörper-Kernmagnetische Resonanz Analysen des P-CF exprimierten ETA Rezeptors (Karsten Mörs, Arbeitskreis Prof. Clemens Glaubitz) offenbarten bereits einen ungefähren Anteil von 30-40 % α-helikaler Sekundärstrukturelemente in den generierten Präzipitaten, so dass sich diese von den klassischen Einschlusskörperchen der *in vivo* Expression abgrenzen ließen. Der geschätzte Prozentsatz lag nur geringfügig niedriger als die vorhergesagten Werte von etwa 50 % und die durch Fern-UV CD-Spektroskopie ermittelten

Werte von 46 % im Detergenz B35, die sich in den charakteristischen Minima bei 208 und 222 nm widerspiegelten.
Detergenzaustausch oder Verringerung der Detergenzkonzentration durch Reinigung mittels immobilisierter Metallchelat-Affinitätschromatographie wurde im Folgenden zur Modulation der mizellaren Umgebung von ETA verwendet, um deren Einfluss auf die Qualität des Rezeptors zu untersuchen. Die höchste Reinheit in Verbindung mit der höchsten Ausbeute von durchschnittlich 1 mg/mL wurde bei der Aufreinigung von ETA von LPPG zu LPPG (geringere Konzentration), B35 oder dem Phosphocholin-Derivat Fos-16 erreicht. Unter diesen Bedingungen zeigten Gelfiltrationsanalysen von ETA schlanke Elutionsprofile, die einen hohen Grad an Homogenität und zusätzlich eine Stabilität des Zielproteins von mindestens einer Woche bei 4 °C erkennen ließen. Negativfärbung unterstützte des Weiteren die Monodispersität der P-CF synthetisierten ETA Proben in B35 und LPPG.

Thermostabilität als eine der Voraussetzungen für Röntgenstrukturanalyse wurde für ETA in unterschiedlichen Detergenzien mittels Fern-UV CD-Spektroskopie bei der festgelegten Wellenlänge von 222 nm untersucht. Hierbei wurde die Änderung der Elliptitizät während des Aufschmelzens der Sekundärstruktur im Temperaturbereich von 4-90 °C untersucht. Eine hohe thermische Sekundärstrukturstabilität von ~85 °C zeigte sich bei P-CF exprimiertem und in B35 aufgereinigtem ETA. Selbst D-CF synthetisiertes ETA in B35 wies eine hohe Stabilität seiner Sekundärstrukturelemente mit einer klaren Transition bei 72 °C auf.

Analysen durch native und denaturierende Gelelektrophorese deuteten des Weiteren auf die potentielle Bildung SDS-resistenter Dimere/Oligomere des ETA Rezeptors hin. Diese Annahme wurde durch ESI Massenspektrometrie von B35 solubilisiertem Protein unterstützt und ist übereinstimmend mit der in der Literatur diskutierten Theorie, dass in nativ gefalteten Rhodopsin-ähnlichen GPCRs Oligomere durch robuste hydrophobe Wechselwirkungen ausgebildet werden. Das Gleichgewicht zwischen monomerem und dimerem zellfrei exprimiertem ETA Rezeptor konnte offenbar zudem durch die den Rezeptor umgebende Mizelle gesteuert werden. Vielwinkel-Lichtstreuung (MALS: multi angle light scattering) zeigte beispielsweise, dass Fos-16 vorwiegend monomeres ETA generierte, während in B35 vornehmlich monomere und dimere Formen des Rezeptors koexistierten.

Erste Anzeichen auf ein natives Verhalten in Bezug auf Homo- und Heterodimerisierung beider ET-Rezeptoren wurden durch die lösliche Koexpression der kompletten Rezeptoren mit verschiedenen verkürzten Rezeptorderivaten mittels anschließenden Affinitäts-‚Pull-Down' Analysen erlangt. Hierbei wurde einerseits auf die von Dr. Christian Klammt in seiner Dissertation hergestellten ETB Fragmente zurückgegriffen. Andererseits wurden zusätzlich ETA Fragmente unterschiedlicher Länge erstellt und erfolgreich zellfrei synthetisiert. Allerdings konnten mit dieser Methode bisher keine eindeutigen Aussagen zur Interaktionsfläche getroffen werden, da in Detergenzumgebung auch die gewählten

Negativkontrollen mit den ET-Rezeptoren interagierten. Somit konnte nicht zwischen unspezifischer Assoziation und spezifischer Interaktion differenziert werden.

Klassische post-translationelle *in vitro* Rekonstitution in artifizielle Liposomen zeigten in elektronenmikroskopischen Gefrierbruchanalysen zwar eine sehr geringe, aber dennoch homogene Partikelverteilung, welche auf eine funktionelle Faltung von ETA schließen lässt. Weitere Funktionsanalysen in Lipidumgebung erfolgten durch in dieser Arbeit etablierte Radioliganden-Experimente. Gleichgewichtsdissoziationskonstanten (K_D) von 1,4 nM für die Bindung des zirkulären Liganden ^{125}I-Endothelin 1 (ET-1) wurden bestimmt. Mittlere inhibitorische Konzentrationen (IC50) von 2,17 µM in homologer Kompetition wurden weiterhin erhalten, was für die funktionelle Faltung von zellfrei exprimiertem ETA spricht. Zusätzlich konnten mittels der Radioligandenanalysen vorläufige Daten zur Ligandenbindungsfähigkeit L-CF exprimierten ETA Rezeptors in Abhängigkeit der umgebenden Lipidzusammensetzung gewonnen werden.

Die Ligandenbindungs-Kompetenz Detergenz-solubilisierten ETAs wurde durch Liganden-Affinitätschromatographie und Fluoreszenz-Anisotropie untersucht. Ein effizientes und verlässliches Protokoll für die Reinigung des Liganden-gebundenen Rezeptors wurde hierfür entwickelt. In Abhängigkeit unterschiedlicher zellfreier Produktionsarten und mizellarer Umgebungen wurden verschiedene Bindungskompetenzen erhalten. Die Bindung von P-CF exprimiertem ETA an seine Liganden mit bis zu 50 % in B35 erzielte unter optimalen Bedingungen eine Ausbeute an funktionellem Rezeptor von 0.5 mg/mL. Fluoreszenz-Anisotropie von ETA in B35 deutete zusätzlich auf die funktionelle Faltung von ETA hin. Unter Verwendung einer Fluorescein markierten linearen Mutante von ET-1 wurde ein K_D von 1,2 µM bestimmt, der sich gut mit publizierten Daten aus nativen Membranfraktionen vergleichen lässt.

Initiale Röntenstrukturanalysen mit zellfrei exprimiertem ETA in B35 zeigten allerdings noch keinen Erfolg. Tendenzen zu der Ausbildung von Mikrokristallen deuten dennoch auf ein zukünftiges Potenzial unter optimierten Bedingungen hin.

Als Basis zur alternativen Strukturanalyse mittels kernmagnetischer Resonanz wurde zusätzlich die initiale Einführung selbst-spleißender Inteine in die zellfreie Reaktion zur selektiven Markierung des Transmembransegments 1 des ETB Rezeptors gezeigt. Allerdings wurden nur so geringe Mengen an Endprodukt gewonnen, dass erst eine Optimierung einen Einsatz zur strukturellen Analyse rechtfertigen würde.

Die erhaltenen Ergebnisse dokumentieren, dass der ETA Rezeptor mit wenigen mL Reaktionsvolumina sowohl im P-CF als auch im D-CF Modus in ausreichenden Mengen für strukturelle Analysen synthetisiert werden kann, wobei seine Qualität von der Expressionsart sowie von der finalen mizellaren Umgebung abhängig ist. Die präsentierte Strategie könnte als

Orientierungshilfe dienen, auch die Qualität verwandter Rezeptoren in zellfreien Systemen zu optimieren. Pharmakologische Studien an zellfrei synthetisierten Rezeptoren sollten aufgrund teilweise deutlich unterschiedlicher Kinetiken aber immer mit *in vivo* Daten abgeglichen werden.

1. Introduction

1.1. Selecting expression systems for the large scale production of functional integral membrane proteins

1.1.1. Basic characteristics of membrane proteins

Membrane proteins (MPs) represent essential mediators between cytosolic pathways and the cellular environment thus being vital to any living cell. They are responsible not only for numerous physiological but also for pathophysiological cell responses mediated by their diverse array of functions ranging from transport processes and transmission of signals to the control of intercellular contacts. Integral MPs are either spanning biological membranes completely with their transmembrane segments (TMSs) or are exposed to one side of the membrane. They are encoded by approximately 20–30 % of open reading frames of an averaged genome (Wallin and Von Heijne 1998) and are very diverse in their function since they include receptors, channels, pores, transporters, enzymes, proteins of energy transduction, accumulation and proteins for cell adhesion. They mostly follow the positive-inside rule as positively charged amino acids (aa) are more abundant in cytoplasmic regions (Von Heijne and Gavel 1988, Von Heijne 1992). Characterized by the structure of their transmembrane (TM) domains they can be classified into α-helical and β-barrel MPs. The latter class does predominantly exist in the outer membrane of Gram-negative bacteria, mitochondria, chloroplasts as well as in the cell wall of Gram-positive bacteria probably sharing an ancient evolutionary origin. α-helical MPs are prevalent as they are found in the inner and outer membranes of bacteria and the plasma membrane of eukaryotes and prokaryotes and constitute about 27 % of MPs in the human genome (Almen et al. 2009, Wallin and Von Heijne 1998). A typical α-helical TMS consists of 20–25 amino acids displaying an average tilt angle of 24° to the membrane normal as well as an intrinsic stability in their natural lipid environment. Hydrophobic amino acids such as alanine, isoleucine, leucine and valine have been shown to prevail in the TM domains with approximately 45 % abundancy (Ulmschneider et al. 2005).

If compared with soluble cytoplasmic proteins, the expression of MPs requires a variety of special considerations. Integral MPs are not just released into the cytosol but must rather be targeted and translocated to their final destinations in membranes (Dalbey and Chen 2004, Drew et al. 2003, Schnell and Hebert 2003, Wagner et al. 2006). In particular in eukaryotes, the directed targeting through the endoplasmic reticulum (ER) and early and late Golgi apparatus to the plasma membrane requires sophisticated recognition and sorting mechanisms. The insertion of MPs into membranes usually does not occur spontaneously. It requires the cooperation of translating ribosomes with translocation machineries like the eukaryotic Sec61 complex or the

homologous prokaryotic SecYEG machinery (White and Von Heijne 2008). Copy number and capacity of those systems are limited and translocation can be selective for distinct groups of MPs only (Drew et al. 2003, Facey and Kuhn 2004, Schnell and Hebert 2003).

1.1.2. Conventional cell-based expression of MPs

In some cells or organisms, one or few MPs are highly abundant, for instance certain rhodopsin derivatives in *Halobacterium salinarium* and in bovine eye retina. It has been attempted to use naturally evolved high level expression systems for the production of closely related MPs (Junge et al. 2008). However, drawbacks were numerous as the efficiency of the expression pathway in *H. salinarium* appeared to be rather specific for bacteriorhodopsin and some of its derivatives. Other eukaryotic MPs even under control of the bacteriorhodopsin promoter were expressed only in spurious amounts (Sohlemann et al. 1997, Turner et al. 1999). Consequently, the homologous expression of MPs in their natural cellular environments, preferentially with increased copy numbers after genetic recombination, appears promising in order to achieve optimal folding conditions, targeting and translocation of the target MPs as all necessary machineries should in principle be present. Almost all established expression systems with clear emphasis on *E. coli* have been used for homologous expression. In a number of further not commonly used prokaryotes or lower eukaryotes, MPs in crystallization-grade quality could also be obtained by homologous expression but this was limited to individual targets (Lancaster et al. 1999, Luecke et al. 2001, Stroebel et al. 2003). A promising option could also be the expression of MPs in hosts that are very closely related to their origin. Overall, an estimate of 45 % of the currently available MP structures originates from homologous expression systems (Junge et al. 2008).

More critical in particular with regard to the availability of efficient accessory helper systems are heterologous expression approaches of MPs in non-related hosts or cellular backgrounds. However, powerful and efficient expression systems are difficult to establish and the necessary technical developments, e.g. the construction of strains, vectors and controllable regulation mechanisms, are very demanding. Few elaborated and well documented systems like their expression in *E. coli*, yeasts or in insect cells are therefore prevalent for MP production as their versatility, efficiency and the availability of reliable protocols dominates over potential disadvantages of not providing the optimal expression background (Table 1).

The expression in *E. coli* is frequently the first and only choice for prokaryotic MPs, while eukaryotic MPs are often attempted to be produced in prokaryotic as well as in eukaryotic backgrounds. Reasons to switch to the latter more expensive and time consuming systems are almost exclusively the intention to improve functionality or the overall quality of the MP samples (Junge et al. 2008).

Table 1: General features of MP expression systems[1].

	E. coli	*L. lactis*	Yeast	Insect	Mammalian	E-CF/W-CF
Basic properties[2]						
Set up requirements	1	1	1	3	3	1/1
Time investment	1	1	2	3	3	1/3
Costs	1	1	2	3	3	2/2
Robustness	3	3	3	1	1	2/2
Preparative scale	3	3	3	2	1	3/3
Bio-safety risk	1	1	1	1-2	1-3	1/1
Characteristics						
Throughput option	+	+	+	–	–	+/+
Expression environment						
Native membranes	+	+	+	+	+	?/?
Micelles	–	–	–	–	–	+/+
Artificial liposomes	–	–	–	–	–	+/+
IBs/precipitate	+	?	(+)	(+)	–	+/+
MP labelling						
Uniform	+	+	+	–	–	+/+
Specific	+	+	+	–	–	+/+
Combinatorial	(+)	(+)	(+)	–	–	+/+
PTMs						
Glycosylation	–	–	+	+	+	–/?
Prenylation	–	–	+	+	+	–/?
Disulfid bonds	(+)	–	+	+	+	+/?

[1] IBs, inclusion bodies; PTMs, post-translational modifications; ?, not analysed yet, E-CF, *E. coli* cell-free, W-CF, wheat germ cell-free
[2] 1, low; 2, medium; 3, high

Central factors that could determine the yield, integrity, activity and stability of synthesized MPs are the availability of highly processive transcription and translation machineries, suitable folding environments, the lipid composition of cellular membranes, the presence of efficient targeting systems and appropriate pathways for post-translational modifications (PTMs) (Table 1). Differences in the codon usage of the target gene and the host's translation machinery could result in premature termination of the polypeptide chain or in mis-incorporation of amino acids, both having severe effects on sample quality and functionality of the final MP (Kurland and Gallant 1996, Sorensen et al. 2003). Most PTMs of recombinant eukaryotic MPs are unlikely to occur in prokaryotic expression hosts like *E. coli* and *Lactococcus lactis*, which sometimes may hamper functional folding but in other cases could be beneficial for crystallization studies. In addition, bacterial MPs typically have a cytoplasmic location for both the N- as well as the C-terminus (Daley et al. 2005). The prevalent requirement of an N-terminus-out topology for eukaryotic MPs like G-protein coupled receptors (GPCRs) could therefore cause problems in their correct targeting and membrane insertion.

Optimization of expression protocols is most demanding for the MP production in any system. Modifications of each little step like vector design and transfer, cultivation conditions, expression strategies, MP extraction or choice of detergents and buffer conditions can have

significant impacts on the final yield and sample quality. Expression protocols have thus to be individualized according to the requirements of each particular MP. It should be realized that highest yield expression and best sample quality often exclude each other and protocols allowing satisfying compromises have to be established. Optimization strategies are usually based on iterative processes of trial and error and they can take many months (Junge et al. 2008).

1.2. Cell-free expression of MPs

Cell-free (CF) systems do not require sophisticated hardware and can easily be set up in standard biochemical labs. The CF technique has initially been used for deciphering the genetic code (Nirenberg and Matthaei 1961) and later for the analytical scale production of soluble proteins (Zubay 1973). Their preparative scale expression just evolved in line with the development of efficient new reaction protocols (Kigawa and Yokoyama 1991, Spirin et al. 1988). Today, the expression and structural analysis of soluble proteins by CF systems is already routinely used with high success rates (Endo and Sawasaki 2003, Vinarov et al. 2004, Yokoyama 2003).

Preparative scale CF expression of MPs is a relatively new approach with first reports starting in 2004 (Berrier et al. 2004, Elbaz et al. 2004, Klammt et al. 2004). Comprehensive protocols for the CF production of MPs have been published and the preparation of essential compounds has been described (Kigawa et al. 1999, Kim et al. 2006, Schwarz et al. 2007a, Spirin et al. 1988, Swartz et al. 2004, Zubay 1973). Evident advantages are that CF expression reactions are open systems allowing easy access at any time point (Fig. 1). Furthermore, several principle problems of MP overproduction commonly occurring in living host cells are eliminated by using CF extracts. Most toxic or growth inhibitory effects upon overexpression can be avoided. No transport or selection mechanisms through cellular membranes have to be considered. Moreover, the cellular metabolism pathways are largely reduced in cell extracts and metabolic conversion problems of supplemented compounds are minimized (Junge et al. 2008).

A first X-ray structure involving a CF produced MP could be presented with the multidrug transporter EmrE (Chen et al. 2007) and most recently, crystals of the voltage-gated anion channel VDAC1 have be obtained from D-CF expressed protein (Deniaud et al. 2010, Nguyen et al. 2010). Furthermore, the structure of the CF produced C-terminal fragment of human presenilin 1 (CTF) has been solved by liquid-state nuclear magnetic resonance (NMR) spectroscopy (Sobhanifar et al. 2010), all reports pointing to the potential of CF expression for the production of MPs for structural approaches.

1.2.1. CF extract sources: Selection of the adequate expression environment

Preparative scale expression achieving mg amounts of protein per mL of reaction mixture (RM) is currently most efficient if using prokaryotic *E. coli* or eukaryotic wheat germ extracts (WGE) (Endo and Sawasaki 2005, Zubay 1973).

The quality of WGE highly depends on the origin of the wheat seeds and therefore enormous batch variations have to be considered. Preparing extracts from wheat germ embryos is relatively laborious and time-consuming (Endo and Sawasaki 2005, Erickson and Blobel 1983, Madin et al. 2000). However, these extracts are of eukaryotic origin which might be advantageous, are highly stable and protein expression can even proceed for weeks if substrates and mRNA are continuously supplied with final protein yields of up to 10 mg/mL (Sawasaki et al. 2002).

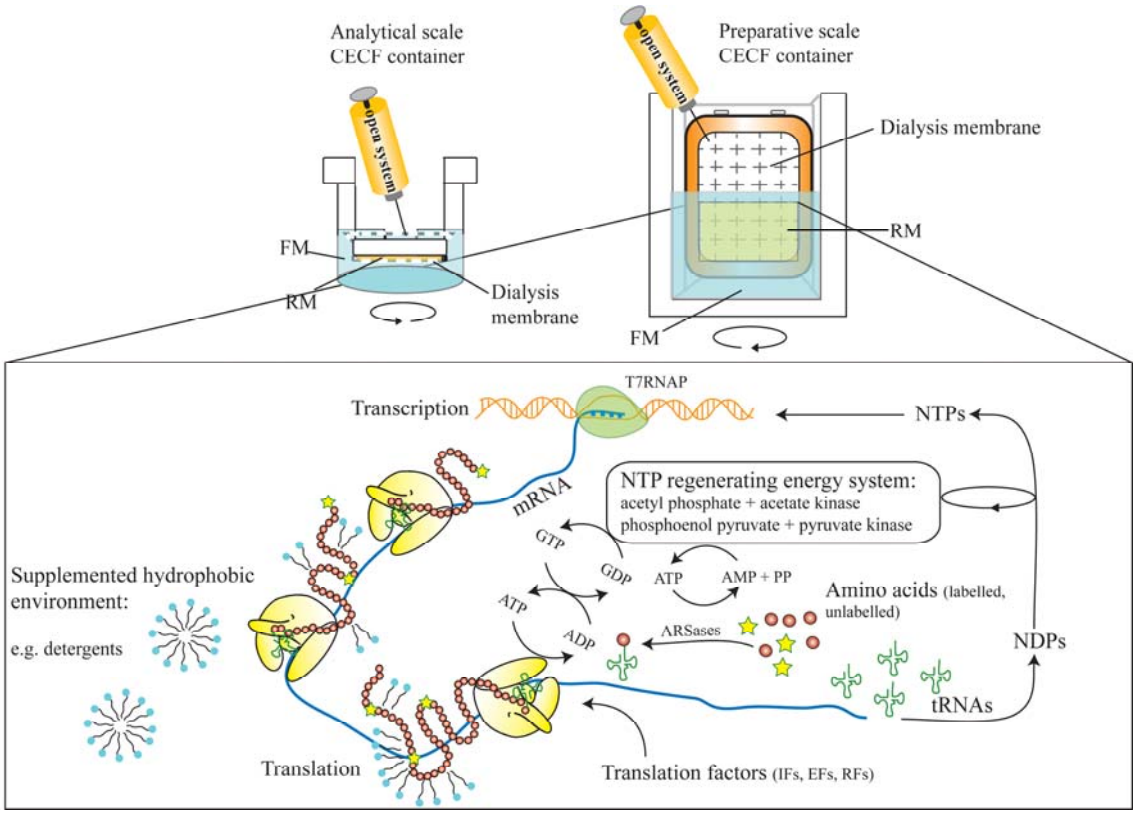


Fig. 1: Analytical and preparative scale configurations of transcription/translation CECF reactions. The S30 extract contains all necessary basic compounds for the translation process. The reactions can be incubated in individually designed dialyzers (Schneider et al. 2010) under vigorous stirring or shaking. Due to the open nature of the CF system any beneficial compound as well as labelled amino acids and necessary precursors can be added at any time point. CECF, continuous-exchange cell-free; FM, feeding mix; RM, reaction mix; T7RNAP, T7 RNA polymerase.

Various strains like BL21 derivatives or strains deficient in endogenous RNAses such as A19 or D10 have been used for preparation of the commonly used S30 *E. coli* extract and protocols can be completed witin a day (Kim et al. 2006, Swartz et al. 2004, Zubay 1973). Endogenous mRNA as well as amino acids and other low molecular weight compounds are eliminated during extract preparation which minimizes background expression and allows control over the

amino acid pool ensuring efficient labelling of the target proteins. *E. coli* CF systems are mostly operated as coupled transcription/translation systems and double stranded DNA or PCR products are applied as templates for the transcription (Fig. 1). *E. coli* as well as wheat embryo extracts have similar efficiencies in the production of prokaryotic and eukaryotic MPs and even proteins exceeding 100 kDa in size can be synthesized in both systems (Liguori et al. 2007, Schwarz et al. 2010).

Other eukaryotic systems based on rabbit reticulocyte extracts (RRL) (Craig et al. 1992), insect cells (Ezure et al. 2006, Katzen and Kudlicki 2006) or the parasitic protozoan *Leishmania* (Mureev et al. 2009) may perform a variety of PTMs (Arduengo 2007, Ren et al. 1993, Tie et al. 2005), but they can currently only be used for analytical scale productions.

Alternatively to complex cell extracts, the PURE (Protein Synthesis Using Recombinant Elements) system has been developed in which the complete *E. coli* translation machinery is assembled out of the purified recombinant components (Ohashi et al. 2010, Shimizu et al. 2001). The better defined expression environment could be beneficial in order to address specific aspects such as minimizing random proteolysis or the study of translation kinetics.

1.2.2. E. coli CF reaction compounds and major system configurations

E. coli systems are coupled transcription/translation systems that are based on transcription by the highly processive T7 or SP6 RNA polymerase (Klammt et al. 2004, Matveev et al. 1996, Sawasaki et al. 2005). The target gene has therefore to be cloned under control of an appropriate promoter (**see 3.1.3.**).

In contrast to *E. coli*, the wheat germ CF system does not work well as a coupled transcription/translation system since magnesium optima for transcription are higher (~15-18 mM Mg^{2+}) than those for translation (around 3 mM Mg^{2+}) (Sawasaki and Endo 2007). Translation systems are thus preferred with supplied mRNA as template. The translation efficiency of mRNA templates in wheat germ systems strongly depends on the nature of the 5' and 3' untranslated regions (UTR) and they represent prime targets for optimization strategies (Endo and Sawasaki 2003, Mignone et al. 2002, Sawasaki et al. 2002). Stable viral leaders mostly found in plant virus RNA genomes substitute for the otherwise required cap structures and poly(A)tails of eukaryotic mRNA (Endo and Sawasaki 2003). Popular 5' enhancing elements contain AC-rich sequence elements mostly derived from the tobacco mosaic virus (TMV) like the OMEGA leader (Gallie et al. 1987) or its derivatives (Endo and Sawasaki 2003). Alternatively, 5' obelin RNA leader sequences from the hydroid polyp *Obelia longissima* have been used. The length of the 3' UTRs is of primary importance in order to antagonize exosomes degrading mRNAs from their 3' ends (Beelman and Parker 1995).

CF extracts already contain all important factors for translation like ribosomes, aminoacyl-tRNA synthetases (ARSases) and translation factors as well as acetate kinase for

energy regeneration. Precursors for transcription and translation like amino acids, tRNAs, nucleoside triphosphates (NTPs), template nucleic acids and efficient energy regeneration systems have to be supplied to the CF reactions. Amino acids can be added in concentrations from 0.3 to 2 mM and highly unstable amino acids (R,C,W,M,D,E) are recommended to be supplemented in even higher concentrations to ensure productive expression. The efficient and long-lasting energy supply is one of the most critical parameters in CF expression. Commonly used components for the regeneration of ATP in continuous-exchange cell-free (CECF) reactions in *E. coli* extracts are phosphoenolpyruvate (PEP) (Zubay 1973), acetylphosphate (AcP) (Ryabova et al. 1995) or combinations of both (Klammt et al. 2006) together with their respective enzymes. Numerous modifications of *E. coli* CF reaction protocols have been published recently that mainly address variations in energy precursors and strategies for the extension of reaction times in batch systems (Calhoun and Swartz 2005, Jewett and Swartz 2004, Kim and Swartz 2001, Sitaraman et al. 2004).

The open nature and high versatility of CF expression allows a variety of new ways to rationally design and to optimize expression environments as well as to modulate folding kinetics for MPs independent of their origin, size, topology and function (Junge et al. 2010a). Stabilizers, protease inhibitors, ligands, chaperones and virtually any other compound that might improve the expression or folding of a particular MP can be directly added into the expression reaction (Fig. 1). Additional tRNA or the co-expression of rare codon tRNA might compensate for difficulties arising from heterologous codon usage. Disulfide bond formation in CF reactions might already occur during CF expression but can for instance also be modulated by providing appropriate redox systems or disulfide bond shuffling systems (Goerke and Swartz 2008). CF systems based on prokaryotic sources like *E. coli* most likely lack the ability to introduce co- and PTMs in eukaryotic proteins and are further characterized by the absence of eukaryotic chaperone systems which may be necessary for their functional folding or their stability in membranes. Approaches in order to address those potential problems are to supplement extracts with chaperones or microsomal fractions isolated from eukaryotic cells (Jiang et al. 2002, Kolb et al. 2000, Ryabova et al. 1997). The operator can further provide specified amino acid mixtures with regard to amino acid biases of the target MP or in view of selective labelling approaches. The easy implementation of labelled amino acids in any combination is of particular value for structural approaches by NMR and by X-ray crystallography. Selenomethionine, isotope labelled amino acids or any other labelled amino acid might be added and the complete control over the low-molecular weight compounds ensures up to 100 % label incorporation and minimized scrambling backgrounds (Kainosho et al. 2006, Ozawa et al. 2005, Reckel et al. 2008, Torizawa et al. 2004, Trbovic et al. 2005).

Two main configurations are commonly used in CF expression: batch or CECF formats (Fig. 2). Batch systems only contain one compartment and are particularly useful for analytical purposes as the reaction can be carried out in microplates with reaction volumes of just few µL.

The whole process can be automated and is therefore suited for high-throughput applications. The disadvantage of this configuration are the reaction times that are limited to few hours since essential precursors for translation are exhausted until then or by-products reach inhibitory concentrations. Consequences are moderate expression yields, whereas it may be possible to obtain significantly increased production yields with specialized protocols (Jewett and Swartz 2004, Kim et al. 2006, Sawasaki et al. 2002, Swartz et al. 2004).

Extended reaction times and highest averaged levels of MP production can be obtained with CECF systems (Shirokov et al. 2007, Spirin et al. 1988). In the CECF set up, the reaction is divided into two compartments holding a RM and a feeding mixture (FM) with constant volumes (Fig. 2). The two compartments are separated by a semi-permeable membrane with a molecular weight cut-off (MWCO) of 10-50 kDa. The RM contains all high molecular weight compounds necessary for transcription and translation like ribosomes, nucleic acids and enzymes whereas the FM contains low molecular weight precursors like amino acids and nucleotides.

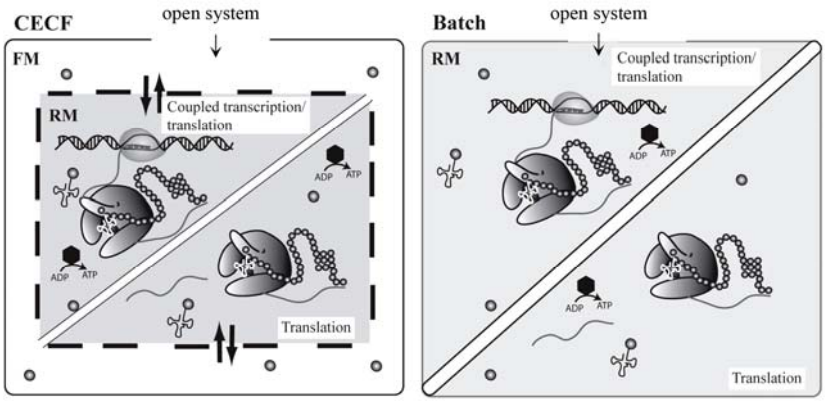


Fig. 2: Batch and CECF configuration. The RM reservoir (grey) contains all necessary high molecular weight compounds in the CECF format and is separated from the FM (white) by a semi-permeable membrane. Batch formats only have one compartment.

Volume ratios of RM to FM usually in between 1:10 and 1:50 are an important parameter for the final yield of the protein. Increasing the FM volume will also result in an increase of production efficiency. However, precursors are expensive and there is no linear correlation between ratio and yield since the capacity of CF extract is limiting. Therefore, compromises are found in between 1:10 and 1:20. Intensive stirring, rolling or shaking of the reaction device during incubation ensures an efficient exchange of fresh precursors from the FM against breakdown products from the RM through the membrane. CECF systems reach expression times of approximately 20 hours (h) and can yield amounts of up to several mg of target protein per mL of RM (Junge et al. 2008, 2010a).

1.2.3. CF reaction modes for the production of MPs

CF expression offers the opportunity to produce MPs in three new ways. The target proteins can be produced either in the precipitate forming (P-CF) mode without providing hydrophobic environments or in presence of supplied hydrophobic agents in the detergent based (D-CF) or in the lipid based (L-CF) mode (Fig. 3).

In the P-CF mode, MP precipitation occurs instantly after translation as CF extracts are almost completely devoid of membranes. Only residual amounts of lipids of 50-100 µg/mL extract have been detected and those lipids might not even be accessible for the target MP. The P-CF precipitates can usually be solubilized efficiently by the addition of particular detergents within few hours without applying classical refolding procedures known for inclusion bodies (IBs). Detergents especially suited for an efficient resolubilization which still might allow functional folding include the lipid-like n-dodecylphosphocholine (Fos-12) and the lyso-phosphoglycerols 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)] (LMPG) and 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)] (LPPG) (Klammt et al. 2004, 2005). These detergents also enable structural investigations by NMR (Krueger-Koplin et al. 2004). If initial MP precipitation should be prevented, selected detergents or lipids in various combinations can also be directly added into the reaction (Fig. 3).

In D-CF systems, the operator creates artificial hydrophobic environments of defined composition directly in the reaction chamber, in which the nascent protein chain can immediately be integrated during or shortly after translation. The emerging full-length MPs can then stay in a soluble form. Solubilization efficiencies are MP specific and optimal detergent/MP combinations and ratios have to be identified individually for every target (Ishihara et al. 2005, Klammt et al. 2005).

Few detergents like n-β-octyl-D-glucopyranoside (β-OG), Fos-12, sodium cholate or 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) appear to inhibit protein expression in D-CF systems already at low concentrations hardly exceeding their critical micellar concentration (CMC). Other commonly used detergents like n-dodecyl-β-D-maltoside (DDM) or Triton X-100 are tolerated by CF systems up to 10 times of their CMC. Exceptionally suitable for the D-CF expression of even larger α-helical MPs like GPCRs are Brij detergents, relatively mild polyoxyethylene-alkyl-ethers, and the steroid derivative digitonin. The tolerance of CF systems for Brij detergents is very high and exceeds 100 times CMC (Klammt et al. 2005).

Alternatively, amphipols (Pocanschi et al. 2006, Popot 2010) or fluorinated surfactants (Park et al. 2007, Polidori et al. 2006) may be used for D-CF solubilization. They differ from classical detergents as they are do not disintegrate membranes (Krafft and Riess 1998, Park et al. 2007), are therefore very mild MP solubilizing agents and may even promote the rapid partitioning of MPs into lipid bilayers. Different detergents (mixed micelles) as well as lipids

and detergents (lipomicelles) may be combined in order to create optimal individual hydrophobic environments (Fig. 3). These options might represent interesting alternatives to pure micelles since lipids often have chaperone-like effects on MPs (**see 1.3.3.** and **4.4.**).

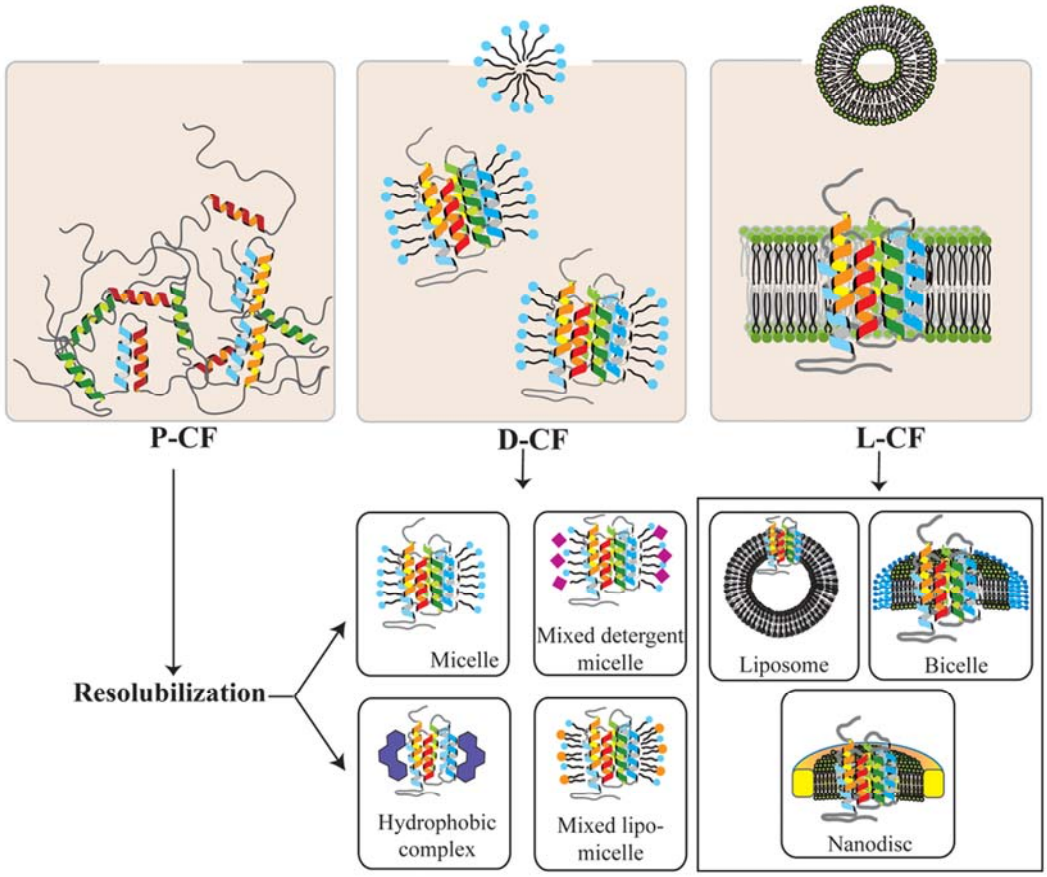


Fig. 3: Versatility of the CF expression technique. Basic expression modes for the CF synthesis of MPs are illustrated. The protein is either expressed without any hydrophobic agents (P-CF mode) or in presence of detergents (D-CF mode) or lipids (L-CF mode). Processing of the different samples (indicated by arrows) results in a variety of primary MP sample environments.

An important characteristic of D-CF expression is that the synthesized MPs are instantly available in detergent solubilized form. The critical and often inefficient step of MP extraction out of cellular membranes is therefore completely avoided (Klammt et al. 2005, 2007a, Opekarova and Tanner 2003, Schwarz et al. 2007b).

Membrane integration of CF expressed MPs can be operated in two different ways. The post-translational reconstitution of P-CF or D-CF solubilized MPs is based on routine protocols adapted from cellularly expressed MPs and many CF expressed proteins have already been reconstituted successfully by these conventional methods (Berrier et al. 2004, Elbaz et al. 2004, Kamonchanok et al. 2008, Keller et al. 2008, Klammt et al. 2007a). Alternatively, the L-CF expression mode in presence of supplied lipids offers a unique option for the co-translational insertion of MPs. Lipids are the natural environment of MPs and often essential modulators of folding, stability and function (Opekarova and Tanner 2003). It is necessary to adapt the lipid composition to the target protein as well as to the CF expression process as the charge of the

used lipids can clearly effect transcription and translation efficiencies. While transcription is usually not affected in presence of any lipids, translation may be inhibited by cationic lipids. Lipid concentration may furthermore influence the expression level (Bui et al. 2008, Umakoshi et al. 2009).

Lipids can be supplied into CF reactions in different formulations such as (I) preformed liposomes of defined compositions, (II) isolated fractions of cell membranes, (III) detergent solubilized lipo-micelles providing a mixed environment for MPs, (IV) bicelles consisting of planar bilayers surrounded by detergents and (V) nano-lipid particles or nanodiscs providing highly soluble and confined bilayer areas (Fig. 3). Mechanisms of co-translational MP translocation can thus be analysed in dependency of a large variety of lipid mixtures. MP integration into the provided bilayers may furthermore be more directed during L-CF mode expression if compared with the random orientation in classical post-translational reconstitution approaches which might support functional assays. In addition, the synthesized target will be the only MP inserted into the bilayers and measured activities could thus clearly be attributed. These characteristics can have considerable benefits for subsequent functional studies.

The L-CF expression mode and its diverse modifications are emerging techniques and their potential as approaches for the preparative scale expression of MPs still have to be explored. The co-translational membrane insertion of functional bacteriorhodopsin was shown by using different lipids and lipid compositions (Kalmbach et al. 2007). A further efficient approach was to add cholate solubilized phosphatidylcholines into the RM of CECF reactions which easily dialyzes out during the reaction resulting in bacteriorhodopsin proteoliposome formation (Shimono et al. 2009). Combining detergent and lipids in lipomicelles might generally be an interesting approach in order to improve functional CF expression (Marques et al. 2007, Nozawa et al. 2007). MP integration in the L-CF mode was analysed upon addition or co-expression of chaperones (Nishiyama et al. 2006) as well as by addition of microsomes (Joseph et al. 1997, Lyford and Rosenberg 1999) or crude inner membrane vesicles as shown for the bacterial outer membrane secretin PulD or for *E. coli* transporters (Guilvout et al. 2008, Wuu and Swartz 2008). However, added chaperones did not increase the integration rate of functional aquaporin Z into synthetic liposomes, although a higher association of the MP to the lipid bilayer was noted (Hovijitra et al. 2009). Variation of lipid composition, lipid head group polarity and alkyl chain length to alter bilayer thickness or using different types of bilayers such as nano-lipoprotein particles are tools in order to improve MP integration in L-CF expression mode approaches (Cappuccio et al. 2008, Katzen et al. 2008, Nishiyama et al. 2006).

1.3. G-protein coupled receptors – GPCRs

1.3.1. Diversity, evolution and topology of GPCRs

GPCRs belong to the largest classes of cell surface receptors encoded by the mammalian genome, mediate their complex cellular responses by a vast array of different external stimuli responding to hormones and neurotransmitters and play a role in olfaction, taste and vision (Fredriksson et al. 2003, Rosenbaum et al. 2009). Understandably, approximately 50 % of modern pharmaceuticals target GPCRs (Klabunde and Hessler 2002). A recently performed detailed phylogenetic classification of human GPCRs revealed over 800 single human GPCRs with approximately 460 olfactory receptors representing the major sub-class within this receptor superfamily. On the basis of similarity in the seven TMSs the receptors could be clustered into five families which are the rhodopsin-like family (class A, 701 members), the adhesion family (24 members), the frizzled/taste family (24 members), the glutamate family (15 members) and the secretin-like family (15 members) (Fredriksson et al. 2003). The receptors of the rhodopsin-like family are mostly characterized by specific highly conserved motifs such as the NPxxY(x)5,6F motif localized in TMS 7 (Fritze et al. 2003) or the E/DRY motif localized between TMS 3 and the intracellular loop (IL) 2 (Rovati et al. 2007). They furthermore often share a conserved disulfide bridge as receptor stabilizing element which links the first to the second extracellular loop (ECL) (Ji et al. 1998).

So far, a large fraction of these 800 GPCRs has no defined physiological ligand initiating their activation and is therefore termed as orphan. Deciphering their function known as deorphanization is a very promising tool for the generation of novel drug targets in pharmaceutical research (Howard et al. 2001).

GPCRs are structurally characterized by seven hydrophobic α-helical TMSs each commonly consisting of 20-27 amino acids. The TMSs are linked by three alternating ECLs and ILs, a fourth cytoplasmic loop arising upon palmitoylation at cysteine residues in the C-terminal segment. They furthermore share an extracellular N-terminal domain and a C-terminus which is exposed to the cytoplasm (Fig. 4).

The most prominent diversity of GPCRs can be found in the soluble domains. The C-terminus varies between 12-360 and the N-terminus between 7-600 aa, its length weakly correlated to the ligand size (Ji et al. 1998). In peptide as well as in monoamine transporters the amino terminal domain consists of only few amino acids (10-50 aa) whereas it is much larger for glycoprotein hormone receptors (350-600 aa) and the receptors of the glutamate family. Receptors of the adhesion family have the largest N-terminal domains (Kobilka 2007).

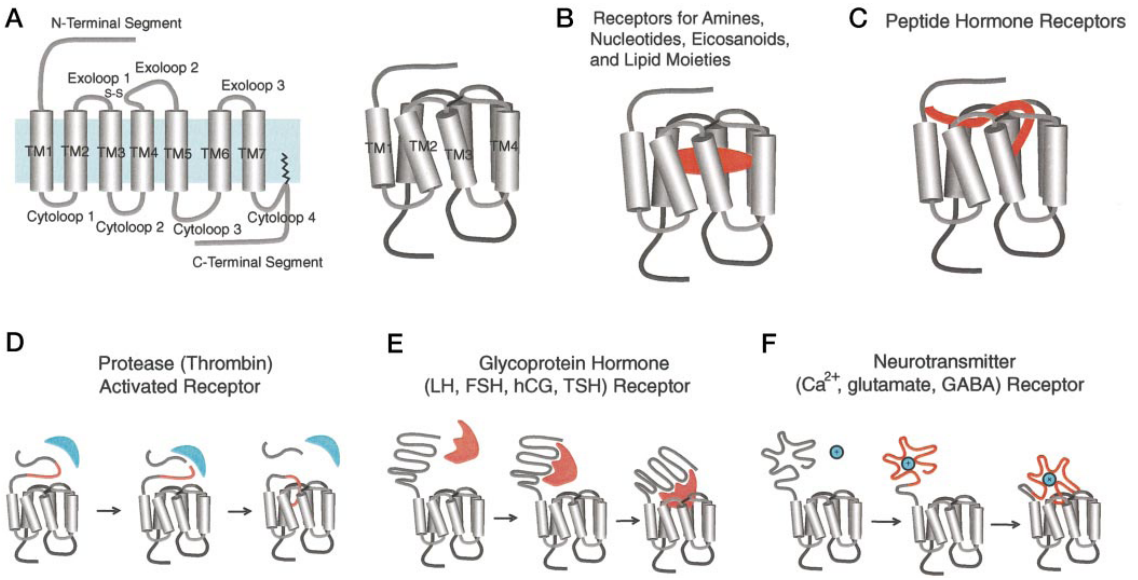


Fig. 4: Schematic representation of common structural features of GPCRs (A) and the proposed modes of receptor-ligand interactions including the location of ligand binding sites (B-F). Adapted from Ji et al. 1998.

1.3.2. Ligand binding and GPCR activation

Natural GPCR ligands are characterized by an immense structural diversity contrasting the structural similarity pattern of their receptors (Ji et al. 1998). The ligands range from subatomic particles (photons), ions (H^+ and Ca^{2+}), small organic molecules, to peptides and whole proteins (Kobilka 2007) and their respective binding domains have already been determined for a range of GPCRs (Fig. 4) (Ji et al. 1998). Ligand binding seems to be promoted by ion pairs, hydrogen bonds and hydrophobic contacts. Small organic molecules like biogenic amines, nucleosides as well as photons and some lipids tend to bind exclusively within the TMSs, peptides smaller than 40 aa mostly interact with the ECLs and the upper parts of the TMSs whereas larger peptides and proteins mostly bind either solely to the N-terminus (glycoproteins) or to ECLs and the N-terminal sequence (Ji et al. 1998, Kobilka 2007). It is however impossible to devise a universal rule for the location of the ligand binding site by the size of the ligand alone. For instance, receptors of the glutamate/pheromone family, such as $GABA_B$, are activated if the respective ligands such as the main neurotransmitters glutamate and γ-aminobutyric acid bind to the expanded N-terminal domain by interaction with the so-called Venus Flytrap module (Kristiansen 2004, Pin et al. 2005). It is also interesting to note that for a variety of GPCRs, although binding their agonists with their soluble extracellular domains, small molecular weight allosteric modulators have been identified which bind within the TMSs (Carroll et al. 2001, Knoflach et al. 2001).

The common signal transduction mechanism shared by the majority of GPCRs is the mediation of intracellular signalling through coupling to specific heterotrimeric guanyl

nucleotide-binding proteins (G-proteins) which consist of α-subunits plus the tightly associated heterodimeric βγ-complexes (Hepler and Gilman 1992).

The βγ-complex interacts with a hydrophobic pocket present in the Gα-GDP subunit. Its release is triggered by the binding of GTP to Gα, consequently removing this hydrophobic interaction interface (Lambright et al. 1994).

G-proteins can be classified into four major families based on the respective α-subunits. $G\alpha_s$ and $G\alpha_{i/o}$ either stimulate or inactivate adenylate cyclases.

$G\alpha_{q/11}$ activates phospholipase C and the $G\alpha_{12/13}$ family stimulates the Na^+/H^+-exchanger pathway (Elefsinioti et al. 2004). The α-subunits possess an intrinsic GTPase activity domain necessary for the hydrolysis of GTP in the G–protein cycle (Fig. 5). At least 21 distinct α-subunits, five β-subunits and twelve γ-subunits have been identified so far (Cabrera-Vera et al. 2003, Hepler and Gilman 1992) accounting for a vast array of different combinations.

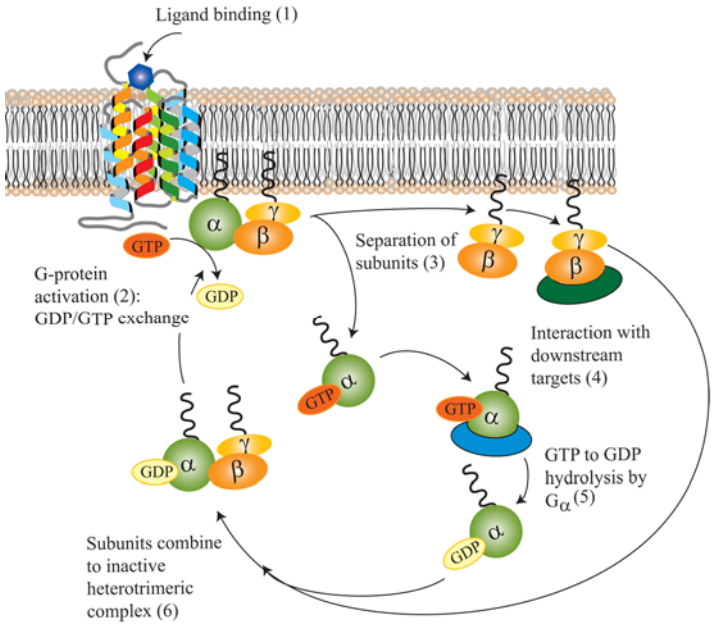


Fig. 5: Schematic representation of GPCR activation and initiation of downstream signalling.

In the basic G-protein cycle agonist activation leads to a conformational rearrangement within the receptor which might especially involve TMS 3 and TMS 6 (Ballesteros and Palczewski 2001, Gether et al. 1997). Subsequent interaction of the receptors with cytoplasmic G-proteins involves the second and third IL as well as the cytoplasmic ends of the adjacent TMSs (Kobilka 2007, Kristiansen 2004, Mirzadegan et al. 2003). The activated receptors serve as guanine nucleotide exchange factors and initiate GDP exchange to GTP at the respective α-subunit. This promotes the dissociation of the heterotrimeric G-protein from the receptors and its disintegration into the separately acting α-subunit and the βγ-dimer which further stimulate their downstream effectors. Subsequent auto-hydrolysis of GTP to GDP mediated by the α-subunit increases the affinity to the βγ-complex and leads to the reassociation of the heterotrimer and the termination of the G-protein cycle (Fig. 5).

In contrast to the activation of GPCRs by the G-protein heterotrimer as demonstrated above, G-protein independent pathways have also been elucidated (Azzi et al. 2003, Lefkowitz and Shenoy 2005, Luttrell and Lefkowitz 2002). These involve PDZ scaffolding proteins as shown for instance for the β1-adrenergic receptor (Pak et al. 2002) or β-arrestin transducers as

monitored for the β2-adrenergic and the endothelin receptors which may stimulate cell proliferation through β-arrestin-1-Src-mediated pathways (Kristiansen 2004). Other interaction partners for GPCRs are molecular chaperones (e.g. Nina A or calnexin) mediating proper folding of the GPCR or its transport to the plasma membrane as well as activity modifying proteins such as RAMPs (reviewed in Brady and Limbird 2002).

1.3.3. Biological membranes and their composition: Necessary lipids for MPs, especially for GPCRs

Biological membranes are essential for any living organism as they constitute specialized permeability barriers and compartimentalize cells in different functional units. Specific lipids or hydrophobic environments can play important roles in the functional folding or stabilization of MPs (Hunte 2005, Lee 2004, Opekarova and Tanner 2003).

Many crystallized MP complexes show defined protein-lipid contacts and folding and function of bacterial MPs can also depend on the presence of specific lipids, as reported for the lactose permease in *E. coli* (Xie et al. 2006). Most observed lipid effects on MPs are still difficult to interpret. They could either take place on the molecular and supermolecular level between lipids and proteins like hydrogen bonding, hydrophobic effects and charge interactions or on the physical level such as altered membrane properties like changes in fluidity, hydrophobic thickness, lateral pressure and curvature. Lipids lead to the positioning of MPs in correct orientations within the membrane, to changes in their secondary structure and to structural stabilization by direct lipid-MP interactions but can also promote aggregation and misfolding if their composition is unfavourable for the embedded and affected proteins (Lee 2004). The specific structure of lipids especially the charge of their headgroups as well as the length of their acyl chain was shown to be for instance of high importance for the potassium channel KscA which essentially needs anionic lipids for its activity (Heginbotham et al. 1998) as well as for the Ca^{2+}-ATPase (Starling et al. 1996). Acyl chain length effects of fatty acids on activity states were investigated for an array of proteins in terms of hydrophobic mismatch and phospholipids which match biological membrane thickness (mostly C18) were highly favoured (Lee 2004).

The lipid compositions of host cells from the different expression systems vary considerably (Opekarova and Tanner 2003). Bacterial membranes are generally devoid of sterols, sphingolipids and polyunsaturated side chains. Phosphatidylethanolamine (PE) is one of the most abundant glycerolphospholipids in bacteria followed by phosphatidylserine (PS), phosphatidylglycerol (PG), cardiolipin and phosphatidylinositol (PI). In contrast, one of the major lipid classes in eukaryotic plasma membranes is phosphatidylcholine (PC). Sterols, that completely lack in bacteria, and sphingolipids are further components of eukaryotic membranes in which PS constitutes the predominant anionic lipid. Of major concern for the selection of an

expression system or cellular background should therefore be the lipid composition of the host cell membranes in addition to specific codon usages and prevalent glycosylation patterns (Junge et al. 2008).

Cholesterol is an essential compound of mammalian cells which is engaged in the organization of membranes, mostly by formation of lipid rafts or modulation of membrane fluidity or permeability (Burger et al. 2000) and it is often discussed as indispensable lipid for the functional expression of MPs, especially for GPCRs (Opekarova and Tanner 2003). Its molar ratio to phospholipids might even be as high as 1:1 supporting its importance for plasma membranes (Yeagle 1985). The interaction mechanism of cholesterol with GPCRs in order to modulate their function is individual to each receptor and can hardly be predicted. It might either act via direct specific lipid/protein interaction, alteration of physical properties of the membrane in which the receptor is localised or by binding to proposed 'nonannular' sites in the immediate proximity of the receptor (Paila and Chattopadhyay 2009). Cholesterol or its analogue cholesteryl hemisuccinate (CHS) promoted for instance proper function and thermal stability of the rat neurotensin receptor (Tucker and Grisshammer 1996), the oxytocin and brain cholecystokinin receptor (Gimpl et al. 1995, Gimpl et al. 1997, Harikumar et al. 2005) the human adenosine A2a receptor (Weiss and Grisshammer 2002) and the human β2-adrenergic receptor (AR) (Yao and Kobilka 2005) and proved essential in stabilizing the crystal-packing interface of the AR dimer and its thermal stability (Cherezov et al. 2007, Hanson et al. 2008). Several amino acid sequences or whole stretches in MPs are associated with an ability to form interaction interfaces with sterols such as the CRAC motif with its consensus pattern L/V–$(x)_{1-5}$-Y-$(x)_{1-5}$-R/K (Li and Papadopoulos 1998), the sterol-sensing domain (SSD) which consists of ~180 aa in five consecutive TMS (Kuwabara and Labouesse 2002) or the cholesterol consensus motif (CCM) found in the β2-AR and 21 % of other human GPCRs. The CCM displays the consensus sequence [4.39-4.43 (R,K)]-[4.50 (W,Y)]-[4.46 (I,L,V)]-[2.41 (F,Y)] after the Ballesteros-Weinstein numbering scheme. A revised CCM (rCCM) in which the aromatic amino acids in the second TMS are absent should interact with cholesterol in a similar fashion increasing the number of human class A GPCRs containing this motif to over 44 % (Hanson et al. 2008).

1.3.4. GPCR dimerization / oligomerization: An evaluation of current models

Emerging evidence of GPCRs forming oligomeric structures which consist of at least two receptor subunits (dimers or oligomers) has grown, and more and more empirical data support these findings (George et al. 2002). Dimerization is a prominent mechanism for receptor activation of members of the glutamate family (Ji et al. 1998). For instance, only the simultaneous expression of two isoforms of the GABA receptor resulted in a functional receptor unit at the cell surface (Kaupmann et al. 1998, Kuner et al. 1999, White et al. 1998).

The role of dimerization in the rhodopsin-like family of GPCRs is just emerging. Data gained from atomic force microscopy and cryo-electron microscopy of discs from mouse rod outer segments suggest dimerization of rhodopsin (Liang et al. 2003) and of squid rhodopsin which clearly showed higher organisation of rhodopsin in membranes (Davies et al. 2001). A variety of functional implications for homo- as well as heterodimerization have been discussed so far which more and more gain in importance in pharmaceutical research. Homo-oligomerization is thought to already occur at the level of protein synthesis in the ER or during maturation of the GPCR therefore possibly influencing protein folding prior to cell surface delivery (Bulenger et al. 2005). A distinct role in protein folding has been determined for many GPCRs (reviewed in George et al. 2002, Milligan 2007). Mutant or truncated receptors act as dominant negatives and inhibit the transport of the native receptors to the cell membrane. Another function of homo-oligomerization has been seen in altered signal transduction that might result in signal amplification as exemplified with rhodopsin (Chabre and Le Maire 2005, Jastrzebska et al. 2006). Proposed functional consequences of hetero-oligomerization are altered G-protein selectivity as demonstrated for the dopamine D1 and D2 receptor dimer (Lee et al. 2004) in which adenylate cyclase signalling switched to phospholipase C stimulation or signal alteration upon allosteric molulation (Springael et al. 2007) which often results in an increased agonist potency transmitted by one of the protomers (Hilairet et al. 2003).

Currently, two mechanistic models of interaction between the single protomers of an oligomeric complex exist (George et al. 2002, Milligan 2007). In ‘contact’ dimers each protomer folds independently and interacts with its partner involving TMS contacts. In the ‘domain-swapped’ model the functional unit of each protomer is generated by its own first five TMS in combination with the last two TMS of the other protomer. This model is thought to be less likely. But investigations of the histamine H1 receptor suggest that both models might co-exist (Bakker et al. 2004).

On the structural level, GPCR oligomers might be generated by disulfide bridge formation, by interactions between ILs as well as ECLs or by hydrophobic interactions between TM domains (George et al. 2002, Milligan 2007). In the rhodopsin-like family the model of robust hydrophobic interaction is favoured as SDS-resistant oligomers are often observed (Lemmon et al. 1992, Hebert and Bouvier 1998). A universal mechanism can however not be deduced so far. Involvements of almost all TMS (1, 2, 4, 5, 6) including parts of extracellular domains have been analysed for a variety of different GPCRs (Milligan 2007). The role of agonist-induced formation or disruption of oligomers in the rhodopsin-like GPCRs is also a matter of debate and far from consistency (Angers et al. 2002, Milligan 2007). The concept of bivalent ligands which possess two distinct receptor recognition sites, hence able to bridge between two receptor monomers, has been suggested for ET receptors (Harada et al. 2002) and opioid receptors (Xie et al. 2005) and comes more and more into focus in drug design.

Pharmaceuticals based on those bivalent structures are already used in the treatment of migraines (George et al. 2002, Milligan 2007).

GPCR oligomerization clearly provides a new platform for the treatment of diseases. Nevertheless, until now these applications are still restricted to trial and error since the exact mechanisms are not yet elucidated. It therefore remains important to investigate the ligand-induced structural rearrangements of the individual GPCR monomers which contributes to the understanding of the complex GPCR physiology.

1.3.5. Expression systems for the functional and structural characterization of GPCRs

High-level recombinant expression of active GPCRs is still a matter of trial and error and mostly restricted to individual targets whose crystallization success bases on excessive fundamental research. Vapour diffusion as well as lipidic mesophase techniques (Caffrey and Cherezov 2009) have been employed for X-ray crystallization of GPCRs. Five crystal structures comprising bovine rhodopsin and ligand-free opsin obtained from either highly abundant natural sources or heterologously expressed *in vivo* (Okada et al. 2004, Palczewski et al. 2000, Park et al. 2008, Scheerer et al. 2008, Standfuss et al. 2007), squid rhodopsin (Murakami and Kouyama 2008, Shimamura et al. 2008) as well as various mutation-stabilized and engineered versions of the human β2-adrenergic receptor in which T4 lysozyme replaces the third IL (Cherezov et al. 2007, Hanson et al. 2008, Rasmussen et al. 2007), mutation-stabilized turkey β1-adrenergic receptor (Warne et al. 2008) and the human adenosine A2a receptor in complex with a high affinity antagonist (Jaakola et al. 2008) are recent path-breaking achievements ranging from 2.2 to 3.7 Å resolution.

Major challenges to successful crystallization are (I) the choice and the stable set-up of a suitable and efficient expression technique yielding large amounts of receptor and (II) generating a homogenous, thermostable, purified active receptor population in detergent-solubilized state by overcoming inherent problems of thermodynamic instability (Serrano-Vega et al. 2008) and proteolytic degradation especially of their ECL regions (Rosenbaum et al. 2009).

Functional studies on receptors are commonly performed in mammalian cells and production levels of 1.7 mg/L culture of the hamster β2-adrenergic receptor could be achieved (Chelikani et al. 2006). The structural genomics network MePNet recently screened 101 GPCRs using a combination of mammalian host cells and Semiliki Forest virus at high success rates of 70 active GPCRs displaying expression levels compatible with structural investigations (Hassaine et al. 2006).

Insect cells such as Sf9 or Hi5 (Standfuss et al. 2007) are suitable systems for the functional expression of GPCRs. They are relatively easy to scale up and promote the correct

folding, PTMs identical to mammalian cells and the supply of several Gα subunits (McCusker et al. 2007). The list of GPCRs expressed at high amounts for structural studies comprises for instance the human A2a adenosine, human α2a and α2b-adrenergic, histamine H1, β1- and β2-adrenergic and cannabinoid receptor CB1 (Akermoun et al. 2005) as well as many others (e.g. Massotte et al. 1999). Tremendous drawbacks are the time-consuming and laborious set-up and the necessity of high-titer viral stocks to infect the cultures (Lundstrom 2006, McCusker et al. 2007).

Yeast expression systems combine prokaryotic as well as eukaryotic characteristics. Single cells, fast growth rates, inexpensive media, high cell-densities and the well developed variety of genetic tools make yeasts to an attractive eukaryotic host for mammalian GPCRs if controlled by inducible promotors such as the GAL or AOX1 promotor to overcome their toxicity to yeast cells (Bill 2001, Macauley-Patrick et al. 2005, McCusker et al. 2007, Romanos et al. 1992). Some eukaryotic specific PTMs such as proteolytic processing and lipidation can be performed in yeasts (Eckart and Bussineau 1996). However, significant differences exist in membrane composition, in particular due to the absence of cholesterol, which might influence the functionality of mammalian MPs (Opekarova and Tanner 2003) as well as the success of crystallisation trials (Long et al. 2005). PTMs are often not reliably performed thus increasing the inhomogeneity of the receptor (McCusker et al. 2007). Yeasts are nevertheless one of the preferred hosts for the production of GPCRs (Reiländer and Weiss 1998). In a concerted approach to express 100 different GPCRs, more than 90 % of the targets were successfully produced in *P. pastoris* (Lundstrom 2006). A systematic optimization screen was successful and 8 of the 20 tested GPCRs were finally obtained in amounts of more than 0.35 mg/L culture (20 pmol per mg total membrane) (Andre et al. 2006). Oxygen supply and defined pH values were further found to be valuable for the expression of functional GPCRs (Sarramegna et al. 2002). Supplementation of lipid derivatives like CHS during solubilization or purification has proven effective for the stabilization of the active conformation of the human adenosine A2a receptor resulting in 1 mg/mL functional purified receptor (O'Malley et al. 2007).

Based on its long tradition as genetic model system the prokaryote *E. coli* can be considered as the oldest and still most popular host for the overproduction of MPs (Sorensen and Mortensen 2005). Major drawbacks for the expression of active GPCRs in *E. coli* cells are obvious, as they provide neither PTMs nor native G- or other accessory proteins for the functional expression and their lipid composition greatly differs from eukaryotic cells (e.g. no cholesterol). Despite those limitations a number of active GPCRs have been expressed in this prokaryotic host by implementing strategies such as fusions of *E. coli* signal peptide or maltose binding protein (MBP) to the termini of the target GPCR. It has been reported that GPCR expression without tags or fusion proteins resulted in only low or no expression yield (McCusker et al. 2007). Those strategies were successfully applied for instance to the human adenosine receptor A2a (Weiss and Grisshammer 2002), rat neurotensin receptor (White et al.

2004), human 5-HT1a receptor (Bertin et al. 1992), human β2-adrenergic receptor (Hampe et al. 2000) and the human cannabinoid CB2 receptor (Yeliseev et al. 2005).

The expression of MPs in IBs in *E. coli* display attractive advantages as they are resistant to proteolytic degradation, reduce the toxicity of the target protein expression and can be harvested at high amounts. *In vitro* refolding has therefore also been exploited for GPCRs. However, refolding procedures for α-helical MPs are neither straightforward nor generally applicable techniques. So far, only the human leukotriene B4 receptor (Baneres et al. 2003), the human serotonin 5-HT4a receptor (Baneres et al. 2005) as well as the OR5 olfactory receptor (Kiefer et al. 1996) have been refolded from IBs.

Recent crystallization success of GPCRs bases on the combination of different techniques adapted to the respective target and are designed to stabilize the active conformation as well as the thermal and proteolytic stability of the receptors (Rosenbaum et al. 2009). The binding of stabilizing ligands was a successful tool in recent submitted GPCR structures. Enhancing thermostability upon mutation of single amino acids was an important parameter towards crystallization of recombinant bovine rhodopsin and human adenosine A2a receptor, respectively (Magnani et al. 2008, Standfuss et al. 2007). Alteration of receptors by point mutation also enhanced the thermostability of the β1-adrenergic receptor and of the neurotensin 1 receptor compared with its highest stability in ligand bound native form (Serrano-Vega et al. 2008, Shibata et al. 2009). Addition of lipids during solubilization and purification and high salt concentrations as well as low pH might also increase receptor stability (Rosenbaum et al. 2009, Vogel et al. 2001). Engineering of the receptors by removal of glycosylation sites in line with truncation of disordered and flexible regions such as the C-terminus were applied. Introduction of well-folded fusion protein domains from T4 lysozyme into the unstable third IL or complex formation with an antibody Fab fragment which enhanced the formation of crystal-lattice contacts and stabilized the region of TM5/6 were further successfully used for the determination of the crystal structures of human β2-adrenergic receptor and the human adenosine A2a receptor (Cherezov et al. 2007, Hanson et al. 2008, Jaakola et al. 2008, Rasmussen et al. 2007). However, the approach to mutate and to engineer GPCRs always involves the risk of changing their native pharmacological behaviour. Reliable active states for drug design strategies will only be obtained in their natural form, favorably in complex with adequate G-proteins (Rosenbaum et al. 2009).

1.4. Structural and functional investigations on CF expressed MPs and GPCRs

To date not only diverse prokaryotic but also eukaryotic MPs have been successfully studied in CF systems of different sources and functional investigations as well as structural determinations are more and more brought into focus (Table 2). Members of a wide array of MP classes such as pores, channels, transporters, enzymes and GPCRs have already been targets for

either extensive MP library screens (Schwarz et al. 2010) or CF expression followed by subsequent functional and structural investigations (Junge et al. 2010b).

So far, only two CF expressed MPs have been reported to crystallize. The small multidrug resistance protein EmrE was P-CF produced as selenomethionine-labelled variant and employed for crystallographic phasing (Chen et al. 2007). Conditions for the crystallization of the human β-barrel VDAC1 have been extensively evaluated and first crystals of D-CF expressed MP could be achieved (Deniaud et al. 2010, Nguyen et al. 2010), however still displaying a limited diffraction (Deniaud et al. 2010).

Studies of around 20 GPCRs synthesised in CF systems have been published to date (Table 2) (Ishihara et al. 2005, Junge et al. 2010a, Kaiser et al. 2008, Kamonchanok et al. 2008, Klammt et al. 2005, 2007b, 2007c, Schneider et al. 2010). They were either expressed in P-CF or D-CF mode and their respective yields have been found to be enhanced considerably by addition expression tags like the T7-tag (14 aa) or thioredoxin (Trx) fused to the N-terminus. Expression screens without any further functional or structural investigations of the respective receptors have already highlighted the CF potential in synthesizing twelve rhodopsin-like GPCRs without extensive optimization in yields ranging from 10 µg up to >500 µg/mL RM.

Table 2: Cell-free expressed G-protein coupled receptors (adapted from Junge et al. 2010a)

GPCR	Origin	GPCR class	Size (kDa)	CF-mode	Extract source	Quality control	Funtional Assay
β2AR-GαS	Human	A	94	D-CF	*E. coli*	Activity	Filter binding
AChM2	Human	A	52	D-CF	*E. coli*	-	-
CRF	Rat	B	47	D-CF	*E. coli*	Reconstitution	-
ETA	Human	A	51	P-CF	*E. coli*	Activity	SPR
ETB	Human	A	52	D-CF	*E.coli*	Activity	SPR, co-elution, TIRFS
HRH1	Human	A	55	P-CF	*E. coli*	Activity	Filter binding
MTNR1A	Human	A	39	P-CF	*E. coli*	-	-
MTNR1B	Human	A	40	D-CF	*E.coli*	-	-
NPY2R	Human	A	43	P-CF	*E. coli*	-	-
NPY4R	Human	A	45	D-CF	*E. coli*	-	-
NPY5R	Human	A	50	P-CF	*E. coli*	-	-
NTR	Rat	A	59	D-CF	*E. coli*	-	-
OR17-4	Human	A	36	D-CF	wheat	Activity	SPR
OR23	Mouse	A	34	D-CF	wheat	-	-
S51	Mouse	A	18	P-CF	wheat	-	-
SSTR1	Human	A	43	P-CF	*E. coli*	-	-
SSTR2	Human	A	42	P-CF	*E. coli*	-	-
V1BR	Human	A	47	P-CF	*E. coli*	-	-
V2R	Human	A	43	D-CF	*E.coli*	Reconstitution	
V2R	Porcine	A	42	D-CF	*E. coli*	Reconstitution	

-, not analysed

1.5. The endothelin system

1.5.1. Evolution of the endothelin core system in the animal kingdom: Gene phylogeny indicates its vertebrate specific origin

The endothelin (ET) ligands and their respective receptors are known to play essential roles as regulators of the cardiovascular system (La and Reid 1995, Yanagisawa et al. 1988) as well as in the development of the neural crest and its derivatives (Pla and Larue 2003). Both systems represent hot spots of diversity especially in vertebrates and therefore participated in their evolutionary success (Braasch et al. 2009). Bioinformatic analyses in combination with phylogenetic reconstructions throughout the whole animal kindom suggest that the ET ligands as well as their receptors can be classified as a vertebrate specific innovation having emerged before the divergence of gnathostomes and lampreys. However, the ET system might also be even more diverse and older than anticipated since the genomes of lower vertebrates are far less analysed (Braasch et al. 2009, Hyndman et al. 2009).

So far, three ET receptor gene subfamilies designated as endothelin A (ETA), endothelin B (ETB) and endothelin C (ETC) extended by lineage-specific duplications in teleosts (ETA3/4, ETB1/2) and *Xenopus laevis* (ETA1/2, ETC1/2) as well as four ET ligand subtypes ET-1, -2, -3 and -4 have been identified. It is assumed that the ET core system has been created by two rounds of whole-genome duplications in an early gnathostome resulting in four ligands and receptors that were secondarily rearranged by gene losses and co-evolution events. The fourth ligand exclusively persisted in teleost fish and has completely been lost in tetrapods while the ETC receptor has only been lost in therian mammals possibly due to chromosomal deletion (Braasch et al. 2009, Hyndman et al. 2009).

1.5.2. The human ET system: Receptors, ligands and binding kinetics

Summarizing gene phylogeny studies, the human ET core system can be concentrated to the two class A GPCR subtypes ETA (Arai et al. 1990) and ETB (Sakurai et al. 1990) characterized by an extracellular N-terminus including a signal sequence and a cytoplasmic C-terminal part. They consist of seven TMSs each composed of around 20-27 hydrophobic amino acids. Both native receptors, that are approximately 50 kDa in mass, are primarily located in vascular smooth muscles and in the endothelium (Davenport and Battistini 2002). Downstream signalling of ETA is primarily mediated by the activation of G-proteins of the $G\alpha_{q/11}$ family but also of $G\alpha_S$, $G\alpha_{i2}$ and $G_{i/0}$. ETB further couples to $G\alpha_{q/13}$ (Douglas and Ohlstein 1997, Simonson 2001, Takigawa et al. 1995) stimulating phospholipase Cβ, an increase of the intracellular Ca^{2+}concentration and immediate early genes (Douglas and Ohlstein 1997).

Despite 59 % amino acid identity, the signalling function and targeting mechanisms of the activated ETA and ETB receptors are quite different (Cramer et al. 2001, Sakurai et al. 1992).

Vasoconstriction and cardiac inotropy is a result of ETA stimulation whereas vasodilatation can be attributed to ETB (Haynes and Webb 1998).

Identified natural agonists of the ET receptors are the three 21 aa isopeptides ET-1, ET-2 and ET-3 (Inoue et al. 1989, Yanagisawa et al. 1988). Their synthesis involves two enzymatic cleavage steps forming the mature peptides out of biologically inactive precursors (Davenport and Maguire 2006).

ET-2 and ET-3 differ in their mature forms in two and six amino acids from ET-1, respectively (Fig. 6). Major features of these three peptides reside in their highly hydrophobic, α-helical C-terminus and their bicyclic structure created by two disulfide bridges linking cysteine residues 1 and 15 and 3 and 11 (Janes et al. 1994, Kedzierski and Yanagisawa 2001).

The principal natural agonist in the human cardiovascular system is ET-1 that carries out a long-lasting vasoconstriction in human blood vessels. ET-1 is controlled by a dual secretory pathway. Besides being constitutively released from vascular endothelial cells thereby maintaining the endogenous vascular tonus by constriction of the adjacent smooth muscle cells, it can be induced by external stimuli being subsequently released by endothelial Weibel-Palade bodies in order to specifically increase its vasoconstrictory effect (Russell et al. 1998).

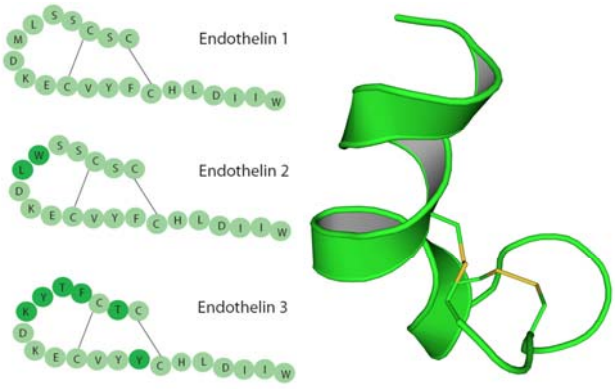


Fig. 6: Schematic representation of the three native ET-peptides with structure of human ET-1 (right). The changes in the amino acid sequence compared to ET-1 are highlighted in dark green. Disulfide bridges between involved cysteines are indicated. The crystal structure of ET-1 was modified after PDB 1EDN (Janes et al. 1994) in Pymol and shows the two disulfide bridges stabilizing α-helical secondary structure elements.

Due to its low concentrations in the plasma of approximately 1 pM (Battistini et al. 1993) ET-1 can rather be classified as locally acting than circulating hormone.

ET-2 is also attributed to cardiovascular tissues while mature ET-3 has been detected in plasma as well as brain and heart tissues (Davenport and Battistini 2002). Although all three native peptide ligands display picomolar to low nanomolar binding to both receptors *in vivo* (Davenport et al. 1995, Maguire and Davenport 1995, Saeki et al.1991) the receptors differ in their relative affinities to the respective ligands. Whereas ETB binds all three peptides with similar affinities, ETA is characterized by its relative insensitivity to ET-3 stimulation if compared with the other two ligands (Davenport and Battistini 2002). The C-terminal tryptophan 21 (Doherty et al. 1991) as well as the disulfide bridges (Saeki et al. 1991) have further been classified as essential binding residues of the ET-1 for high affinity binding to its receptors, especially with respect to ETA. Whereas

high affinity binding of 4-Ala-ET-1 to ETB is not disturbed by mutation of all four cysteines to alanines, ETA displays a significant reduction in its sensitivity to the mutated ligand of approximately 2000-fold (Saeki et al. 1991).

Evidently, the complex structure of the ET peptides in combination with their hydrophobicity render these ligands a very challenging tool for *in vitro* studies. However, structural studies by liquid-state NMR or X-ray crystallography require detergent-solubilized receptors. High background in ligand binding experiments might therefore be considered due to unspecific interaction of the hydrophobic ligands with hydrophobic micelles or liposomes. Furthermore, their bicyclic structure has to be sustained outside the cellular environment and their reactive cysteines have to be kept in an oxidized state to prevent aggregation as well as unspecific intermolecular disulfide bridges with the receptor cysteines. So far, most studies of the ET system have been conducted *in vivo* with the receptors surrounded by their natural environment therefore avoiding the aforementioned difficulties (**see 5.3.1.** and **5.3.3.**).

1.5.3. Physiology and pathophysiology of the human ET system

The human ET system displays a very diverse expression pattern (Fig. 7) clearly related to the wide array of physiological and pathophysiological functions (Yanagisawa et al. 1988).

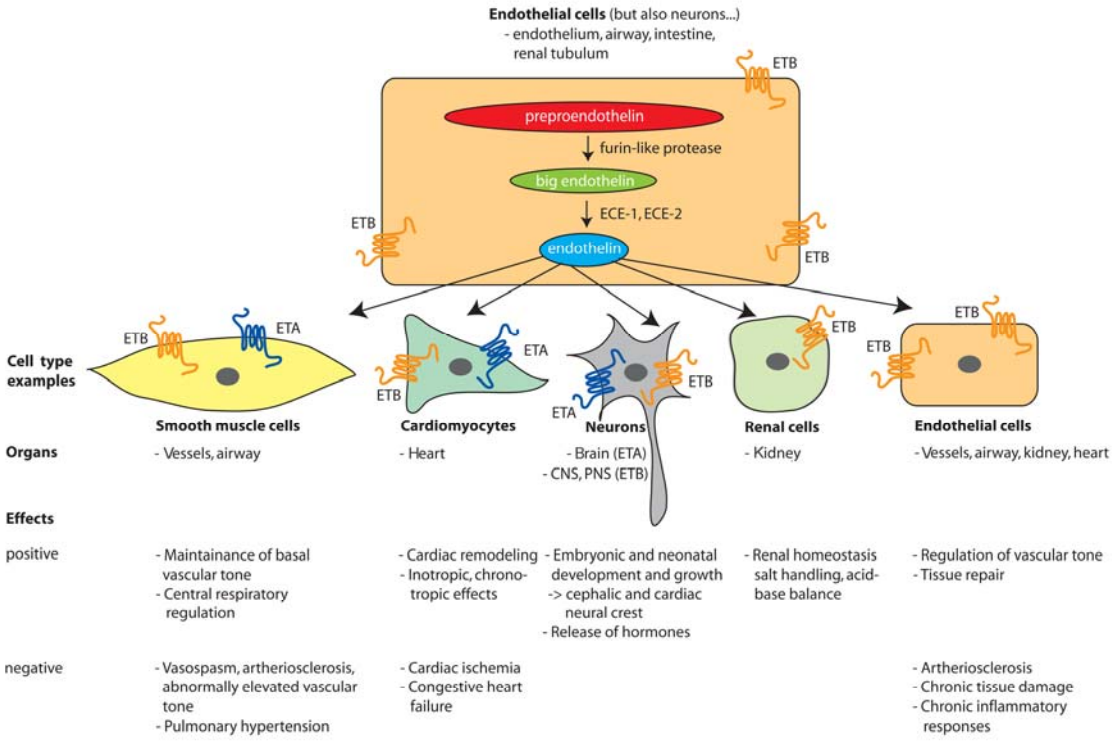


Fig. 7: Exemplary overview of the human ET systems' expression pattern. Illustrated are the expression, processing and release of the ET ligands by endothelial cells and their influence on the ETA ETB receptors on a selection of different cell types and tissues. Examples of respective physiological as well as pathophysiological responses are listed below.

The system generally acts as a major modulator in cardiovascular regulation and shows a striking diversity in biological responses affecting vasoconstriction, neurotransmission, embryonic development involved in the correct formation of neural crest-derived structures (Kedzierski and Yanagisawa 2001), renal function and hormone production. It is even involved in cancer by its mitogenic effects promoting aberrant cell proliferation, apoptotic escape and metastatic dissemination (Nelson et al. 2003). It is therefore not surprising that its components' expression has been found associated to a variety of different tissues. These include blood vessels, the cardiovascular system, renal cells, glial cells and neurons in the central as well as peripheral nervous system, pituitary, adrenal, immune, hepatic, genitourinary and endocrine tissues (reviewed in Watts 2009).

ET receptors are present on a variety of different cell types. Both receptors exist on smooth muscle cells on which ETA represents the major subtype with >85 %, whereas ETB is the predominant receptor subtype in kidney tissue and human cortex (Davenport and Maguire 2006, Kedzierski and Yanagisawa 2001).

On vascular smooth muscle cells the human ETA exhibits vasoconstriction upon its activation. ETB, being the main receptor subtype in kidney tissue, is supposed to act as prevalent clearing receptor for ET-1 in a variety of organs including lung, kidney and heart. This conception of ETB's clearing function bases on an empirically perceived significant rise in ET-1 levels if ETB is blocked by adequate antagonists (Davenport and Maguire 2006). Supporting its supposed role as ETA counter-regulatory receptor, activation of ETB stimulates endothelial cells to release muscular relaxing factors such as nitric oxide or prostacyclin resulting in a ETB-mediated transient vasodilatation (Berti et al. 1993, De Nucci et al. 1988, Verhaar et al. 1998). ET antagonism could be a possible clinical treatment for many civilisatory disorders including chronic heart failure, different types of pulmonary hypertension, diabetes, pain, renal failure, artherosclerosis and cancer (Watts 2009). The ET-1/ETA interaction constitutes the primary target in fighting those diseases whereas ETB antagonism only plays an inferior role in most of the pathophysiologies (Davenport and Maguire 2006, Kedzierski and Yanagisawa 2001). A variety of different subtype specific agonists and antagonists have been evaluated in clinical development by conventional drug design, bosentan (Tracleer) being the first combined ETA/ETB antagonist approved in human clinical treatment for pulmonary artery hypertension (Davenport and Battistini 2002, Davenport and Maguire 2006).

ETA antagonists show for instance beneficial effects by decrease of blood pressure in the treatment of hypertension (Miyauchi et al. 1993), in cardiac ischemia and congestive heart failure (Grover et al. 1993, Sakai et al. 1996) and attenuated the detrimental symptoms in renal failure (Chan et al. 1994, Gellai et al. 1995). Blockage of ETA receptors by specific antagonists further significantly reduced cell proliferation in prostate, cervical and ovary cancer cells, whereas ETB antagonism did not show any remarkable effect (Nelson et al. 2003) which underlines ETA's high pharmacological relevance (Kedzierski and Yanagisawa 2001).

1.5.4. Lipid composition of mammalian ET receptor membranes: Requirement of essential lipids?

Since ET receptors have been classified as vertebrate specific innovation (**see 1.5.1.**) essential lipids as well as lipid composition of the surrounding natural membranes are characterized by their eukaryotic origin.

For some of the mammalian ET receptor cell types their lipid composition has been elucidated. In human cultures of endothelial cells PC represents the most abundant lipid class accounting for approximately 60 % of total phospholipid content. Cholesterol constitutes around 40-45 µg/mg of cell protein. Predominant fatty acids were found to be mostly of the saturated and monounsaturated type with oleate, stearate and palmitate representing the predominant molecular species (Cansell et al. 1997).

PC and PE constitute the predominant phospholipids in rat liver cells, heart cells and neuronal cell bodies from cultured dorsal root ganglia (Calderon et al. 1995, Christie 1985). Latter are further characterized by a relatively high content of cholesterol and esters thereof at approximately 20 %.

As discussed for the GPCR family of receptors above they are often characterized by a special need of cholesterol (**see 1.3.3.**). Regarding the ET receptors no specific requirement of cholesterol or any other sterol derivative in terms of direct interaction has been investigated so far. However, an enrichment of ET receptors in caveolae or caveolae-like domains characterized by a high cholesterol content and the presence of the structural cholesterol-binding protein caveolin along with an intrinsic caveolin-binding motif might already point to an interplay of the ET receptors with this lipid (Burger et al. 2000, Couet et al. 1997). An analysis of the amino acid sequence of both ETA and ETB receptors further revealed a stretch of L-$(x)_3$-Y-$(x)_2$-K in the first IL that might act as a putative CRAC motif (**see 1.3.3.**). The rCCM motif (**see 1.3.3.**) was additionally found in the ETB receptor implying that cholesterol might also exhibit pharmacological relevance for the human ET receptors.

1.5.5. Homo- and heterodimerization of ET receptors

The traditional assumption of GPCRs solely acting as monomers in signalling processes has nowadays been corrected (**see 1.3.4.**) and corresponding investigations have also been performed with both ET receptors.

In 1997 Ozaki and coworkers suggested a cross-talk between ETA and ETB receptors in human Girardi heart cell cultures stably expressing ETB and subsequently transfected with human ETA cDNA. They could demonstrate that ETA stimulation by ET-1 resulted in a significantly lowered affinity of ETB for its selective agonist BQ-3020 and for its selective antagonist BQ-788 than in cells solely expressing ETB. They assumed a characteristic change in the ETB receptor upon stimulation of ETA. This interaction between both receptors influenced

downstream effects such as intracellular Ca^{2+} and cAMP levels (Ozaki et al. 1997). In the rat anterior pituitary gland, a tissue expressing both ETA and ETB receptors, Harada and colleagues suggested by quantitative autoradiography the formation of a heterodimer, which is mediated by the bivalent ligand ET-1 bridging between both receptors. In their ligand-dependent model of ET receptor interaction based on the message-address concept initially drawn by Portoghese (1989), the bivalent ET-1 binds to the address-recognition domain via its N-terminal address domain whereas the C-terminal message sequence recognizes the message subdomain of ETB (Harada et al. 2002).

Both ETA and ETB receptors have further been investigated for their ability to form homo– as well as heterodimers in cell cultures of HEK293 cells by fluorescence resonance energy transfer (FRET) and co-immunoprecipitation. Independently, stable FRET efficiencies between 12 % and 27 % indicated robust, constitutive homo- as well as heterodimerization of ETA/ETA, ETB/ETB and ETA/ETB. Furthermore, co-immunoprecipitation of the respective receptors was not altered by preincubation with agonists or antagonists indicating that the ligands directly neither promote dimer formation nor dissociation (Evans and Walker 2008, Gregan et al. 2004a, 2004b). This finding is in clear contrast to the supposed model of Harada and coworkers and differs from receptor tyrosine kinases which need ligands for their dimerization (Ottensmeyer et al. 2000). It is further supposed that homodimerization might already be promoted during biosynthesis in the ER and that heterodimer formation is a reversible process and depends on receptor ETB sequestration whose onset is retarded in heterodimers (Gregan et al. 2004a, 2004b).

Possible physiological implications of ET receptor interaction were found in sustained Ca^{2+}-signalling controlled by heterodimers as the increase in intracellular Ca^{2+} concentration could only be reversed by antagonizing both receptor subtypes whereas single antagonists did not show the ability to convert the sustained to a transient signal (Evans and Walker 2008). These investigations underline the necessity to understand ligand binding and downstream signalling of the ET receptors as a very complex and diverse process which might require not only the investigation of single, monomeric receptors and their kinetics but should also include solving receptor interaction interfaces.

1.6. CF characterization of the human ETB receptor

Optimized CF expression of the ETB receptor in P-CF mode resulted in high preparative yields of 3 mg/mL RM that could be obtained as well after D-CF expression in 1 % B78 and 1.5 % B58. Further detergent evaluations revealed that B35 and digitonin reduced the final yield of soluble receptor to less than 500 µg/mL of RM suggesting long-chain Brij derivatives as favourable detergents for the production of ETB. P-CF produced ETB precipitate resolubilized completely in 1 % LMPG and its subsequent reconstitution into *E. coli* total liposomes resulted

in homogenously distributed particles. Purification of the receptor either out of P-CF or D-CF mode was performed by immobilized metal affinity chromatography (IMAC) yielding 1.8 mg of purified receptor per mL RM (Klammt et al. 2007b, 2007c).

The quality of the detergent-solubilized ET receptor was analysed by a variety of complementary approaches. Size exclusion chromatography (SEC) and negative staining electron microscopy (EM) concomitant with single particle analysis of D-CF expressed ETB in B78 revealed monodisperse receptor preparations displaying dimensions comparable to the rhodopsin dimers suggesting also dimeric character of the receptor. In contrast, P-CF produced ETB resolubilized in LMPG resulted in high aggregation (Klammt et al. 2007c). Further evaluation of dimer formation capacity for the ETB receptor indicated Brij derivatives and digitonin as prominently able to produce SDS-resistant dimers (Klammt et al. 2007b) which seems to be a characteristic feature for many *in vivo* expressed GPCRs (**see 1.3.4.**). It has therefore been assumed that dimerization of ETB in different hydrophobic environments might account for native folding. Summarizing, the ETB receptor quality was found to be highest if produced in D-CF mode preferably in B78. Most of the binding studies were therefore performed using the aforementioned conditions.

Ligand binding studies on detergent-solubilized full-length ETB receptor was analysed by total internal reflection fluorescence spectroscopy with a f-4-Ala-ET-1 mutant as well as surface plasmon resonance (SPR) measurements with biotinylated ET-1 (b-ET-1). Equilibrium dissociation constants (K_D) of 29 nM and 6.2 nM respectively pointed at a considerably reduced receptor affinity if compared with K_D values of 50-300 pM determined *in vivo* in native lipid membranes (Hara et al. 1998). The reduction in binding affinity was speculated to result from potentially different receptor kinetics in an artificial hydrophobic micellar environment or the lack of G-proteins stabilizing the receptor (Klammt et al. 2007b, 2007c).

Further ligand binding studies were performed by co-elution of a Cy3-labelled ET-1 variant with different solubilized ETB receptor preparations in combination with SEC. D-CF expressed ETB in B78 revealed the highest degree of ligand binding of approximately 50 % comparable to values obtained by conventional insect cells expression. P-CF ETB resolubilized in LMPG showed no potency in binding of the ligand (Klammt et al. 2007c).

In order to confine the ligand binding and homodimerization interfaces, different truncated derivatives of the full-length receptor were designed and D-CF expressed in B78 achieving high final yields. By use of affinity pull-down experiments of either individually or co-expressed full-length strep-tagged ETB and single poly$(His)_{10}$-tagged truncations, the TMS 1 was found to be essential for the homodimerization of ETB. SPR measurements on truncations as well as affinity pull-down experiments of ETB truncations in B78 by b-ET-1 further suggested that this segment might also be essential for ligand binding (Klammt et al. 2007c).

However, SPR measurements with ET-1 might be delicate to interpret since it was demonstrated in the present study that by using SPR ET-1 also bound to related GPCRs with almost the same

affinity (**see 4.8.2.** and **5.3.3.**).

1.7. Aim of this project

CF expression approaches overcome major limitations of conventional *in vivo* expression systems and are thus a competing alternative for the large-scale production of MPs. Instead of using classical approaches for the synthesis of MPs, CF expression systems with focus on the prokaryotic S30 *E. coli* extract, were therefore to be used for the production of the human ET receptors. Based on previous studies on ETB, accomplished by Dr. Christian Klammt, CF synthesis was to be extended to and optimized for the ETA receptor as basis for the characterization of the whole human ET receptom.

Individual preparative scale production protocols along with the evaluation of rational purification and quality control strategies were to be developed as essential prerequisite for functional analyses of ETA and -to some extent- of ETB in detergent-solubilized and in co- and post-translationally liposome-reconstituted form. The intrinsic versatility of the CF expression system should be used to modulate the quality of the target protein at different stages of the production process. Parameters, compounds and processes able to modify ETA sample properties after solubilization should be identified and analysed by evaluation of homogeneity and protein stability. As complementary approach for analysing the functional folding of CF produced GPCRs these quality control strategies should finally allow the set-up of optimized production protocols for the generation of high quality ETA receptor samples which could serve as future guidelines for the CF preparation of GPCRs.

The implementation and development of straightforward biochemical or biophysical assays for CF synthesized GPCRs to readily assess their ligand binding competence in different *in vitro* environments represented a further major aim of the present study. Ligand binding experiments with liposome-reconstituted receptor were intended to compare CF produced ETA binding kinetics to *in vivo* expressed membrane-embedded receptor. In particular, the new L-CF mode should be approached as promising alternative to the conventional *in vitro* reconstitution of MPs in lipid environments.

Studies of homo– and hetero-oligomerization between ETA and ETB should further be initiated.

2. Materials

2.1. Equipment

CF extract and T7 RNA polymerase preparation:

Cell disruptor Constant Systems	IUL Instruments GmbH (Germany)
Fermenter, 10-liter	B. Braun Biotech GmbH (Germany)
French pressure cell disruptor	SLM Aminco Instruments Inc. (USA)

DNA template preparation and analysis:

Agarose gel electrophoresis system	Peqlab Biotechnologie GmbH (Germany)
Midi DNA preparation kit	Macherey-Nagel GmbH+Co. KG (Germany)
QIAquick gel extraction kit	Qiagen GmbH (Germany)
QIAquick PCR purification kit	Qiagen GmbH (Germany)
Thermocycler	Biometra AG (Germany), Eppendorf (Germany)

General equipment:

Autoclave	Tecnomara AG (Switzerland), Gettinge AB (Sweden)
Centrifuge Sorvall Evolution RC	Sorvall Instruments (Germany)
Centrifuge Sorvall RC 5B, C	Sorvall Instruments (Germany)
Cooled table top centrifuge Micro 22R	Hettich GmbH +Co.KG (Germany)
Incubating shakers	Infors AG (Germany), New Brunswick Scientific Inc. (USA)
MS2 Minishaker	IKA Werke GmbH+Co. KG (Germany)
NanoDrop 1000	Peqlab Biotechnologie GmbH (Germany)

Protein expression and analysis:

Dialysis tubes type 27/30, MWCO 12-14 kDa	Spectrum Laboratories Inc. (USA)
Immobilon-P PVDF membrane	Millipore GmbH (Germany)
Lumi Imager F1	Roche Diagnostics GmbH (Germany)
Mini-Protean Electrophoresis system	Bio-Rad GmbH (Germany)
Slide-A-lyzer dialysis cassette, MWCO 10 kDa	Thermo Fisher Scientific GmbH (Germany)
Waterbath shaker	New Brunswick Scientific Inc. (USA)

Protein processing, quality and functional analysis:

Äkta purifier FPLC system	GE Healthcare (Germany)
BAF 060 freeze-fracturing unit	Bal-Tec Inc. (Principality of Lichtenstein)
Bandelin Sonorex Super RK 510	SCHALLTEC GmbH (Germany)
Biobeads SM-2	Bio-Rad GmbH (Germany)

Cobra II Auto gamma	PerkinElmer (Germany)
Columns (Superdex 200 3.2/30, Superdex 200 10/300, Q-sepharose)	GE Healthcare (Germany)
Freeze-fracture EM	Bal-Tec Inc. (Principality of Lichtenstein)
HPLC unit	Jasco Labortechnik GmbH (Germany)
Mini Dawn Treos (3 angles)	Wyatt Technology Europe GmbH (Germany)
Mini-Extruder	Avanti Polar Lipids Inc. (USA)
MultiScreen HTS GF/B filter	Millipore GmbH (Germany)
MultiScreen HTS vacuum filtration	Millipore GmbH (Germany)
Optilab rex (RI detector)	Wyatt Technology Europe GmbH (Germany)
Spectrofluorometer, FP-6500	Jasco Labortechnik GmbH (Germany)
Spectropolarimeter, J-180	Jasco Labortechnik GmbH (Germany)
Sonifier Labsonic U	B. Braun Biotech GmbH (Germany)
Ultracentrifuges (L-70, Optima TLX, Optima LE-80K)	Beckman Coulter GmbH (Germany)
Ultra-Clear Centrifuge Tubes	Beckman Coulter GmbH (Germany)

2.2. Software

ASTRA 5.3.4.13	Processing and evaluation software for multi angle light scattering (MALS) data
Chromas lite	Processing software for DNA sequencing chromatogram files
Graph Pad Prism 5	Software for biostatistics, graph fittings and scientific graphing
UNICORN 5.11	Software package for control, supervision of chromatograpy systems and processing of chromatograms

2.3. Reagents and chemicals

Antibodies:

Anti-biotin goat pAb Peroxidase conjugate	Calbiochem, MERCK chemicals KG aA (Germany)
Anti-mouse IgG HRP conjugate from goat	Sigma-Aldrich Chemie GmbH (Germany)
Anti-penta His IgG from mouse	Qiagen GmbH (Germany)
T7 tag antibody HRP conjugate	Novagen, MERCK chemicals KG (Germany)

Detergents:

Sigma-Aldrich Chemie GmbH (Germany):

B35, Brij 35, polyoxyethylene-(23)-lauryl-ether

B58, Brij 58, polyoxyethylene-(20)-cetyl-ether

B78, Brij 78, polyoxyethylene-(20)-stearylether

B98, Brij 98, polyoxyethylene-(20)-oleyl-ether

Carl Roth GmbH+Co.KG (Germany):

CHAPS, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propansulfonat

SDS, sodium dodecylsulfate

Anatrace, Affymetrix Inc. (USA):

DDM, n-dodecyl-β-D-maltoside (also Applichem GmbH, Germany)

DPC, Fos-12, n-dodecylphosphocholine

Fos-16, n-hexadecylphosphocholine

HDM, n-hexadecyl-β-D-maltopyranoside

LMPC, 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac(1-choline)]

LMPG, 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)]

LPPG, 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)]

TDM, n-tetradecyl-β-D-maltopyranoside

General reagents and chemicals:

1,4-Dithiotreitol (DTT)	Carl Roth GmbH+Co.KG (Germany)
2-Mercaptoethanol	Carl Roth GmbH+Co.KG (Germany)
Acetyl phosphate lithium potassium salt	Sigma-Aldrich Chemie GmbH (Germany)
Adenosine 5'-triphosphate (ATP)	Roche Diagnostics GmbH (Germany)
Amino acids (unlabelled)	Sigma-Aldrich Chemie GmbH (Germany)
Antifoam Y-30 emulsion	Sigma-Aldrich Chemie GmbH (Germany)
Bactotryptone	Carl Roth GmbH+Co.KG (Germany)
Bovine serum albumin Fraction V	Sigma-Aldrich Chemie GmbH (Germany)
Complete Protease Inhibitor Cocktail	Roche Diagnostics GmbH (Germany)
Cholesteryl hemisuccinate tris salt	Sigma-Aldrich Chemie GmbH (Germany)
Cytidine 5' triphosphate di-sodium salt	Fluka, Sigma-Aldrich Chemie GmbH (Germany)
Dimethylsulfoxide	Carl Roth GmbH+Co.KG (Germany)
Ethidiumbromide	Carl Roth GmbH+Co.KG (Germany)
Folinic acid calcium salt	Sigma-Aldrich Chemie GmbH (Germany)
Gene ruler 100bp, 1kb DNA ladder	Fermentas GmbH (Germany)
Glucose monohydrate	Carl Roth GmbH+Co.KG (Germany)
Guanosine 5' triphosphate di-sodium salt	Fluka, Sigma-Aldrich Chemie GmbH (Germany)
HEPES, Imidazole, IPTG, K_2HPO_4, KCl, KH_2PO_4	Carl Roth GmbH+Co.KG (Germany)
Magnesium acetate tetrahydrate	Sigma-Aldrich Chemie GmbH (Germany)

NaCl	Carl Roth GmbH+Co.KG (Germany)
NaN_3	Fluka, Sigma-Aldrich Chemie GmbH (Germany)
Ni-NTA Agarose	Qiagen GmbH (Germany)
Nonidet P-40, P-40 alternative	MERCK chemicals (Germany)
PEG8000	Sigma-Aldrich Chemie GmbH (Germany)
Peptone, tryptic digest	Carl Roth GmbH+Co.KG (Germany)
Phenylmethylsufonyl fluoride (PMSF)	Sigma-Aldrich Chemie GmbH (Germany)
Phosphoenol pyruvic acid (PEP), monopotassium salt	Sigma-Aldrich Chemie GmbH (Germany)
PIERCE Monomeric Avidin Agarose	Thermo Fisher Scientific (Germany)
Potassium acetate	Sigma-Aldrich Chemie GmbH (Germany)
Pyruvate kinase	Roche Diagnostics GmbH (Germany)
Restriction enzymes	New England Biolabs GmbH (Germany)
RiboLock RNase Inhibitor	Fermentas GmbH (Germany)
Spermidine	Fluka, Sigma-Aldrich Chemie GmbH (Germany)
T4 DNA-ligase	New England Biolabs GmbH (Germany)
TCEP	PIERCE, Thermo Fisher Scientific (Germany)
Tris-(hydroxymethyl)-aminomethane	Carl Roth GmbH+Co.KG (Germany)
tRNA *E. coli* MRE 600	Roche Diagnostics GmbH (Germany)
Turbo-Pfu DNA polymerase	Stratagene AG (Germany)
Uridine 5' triphosphate tri-sodium salt	Fluka, Sigma-Aldrich Chemie GmbH (Germany)
VentDNA Polymerase	New England Biolabs GmbH (Germany)
Yeast extract	Carl Roth GmbH+Co.KG (Germany)

Lipids:

Avanti Polar Lipids Inc. (USA):

DMPC, 1,2-dimyristoyl-*sn*-phosphatidylcholine

DOPE, 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine

DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine

E. coli total lipid extract

E. coli polar lipid extract (Chloroform)

POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt)

Sigma-Aldrich Chemie GmbH (Germany):

L-α-Phosphatidylcholine from soybean, Type IV-S

Cholesterol

Plasmid vectors with T7 regulatory elements:

pET21a(+), pET28a(+)	Novagen, MERCK chemicals KG (Germany)
pIVEX	Roche Diagnostics GmbH (Germany)

2.4. Labelled chemicals and ligands

4-Ala-endothelin-1 (ET-1)	Dr. Michael Beyermann, FMP (Berlin)
L-[^{35}S]- methionine, 2 mCi	Hartmann Analytic GmbH (Germany)
Biotin [Lys9]-ET-1	Dr. Michael Beyermann, FMP (Berlin)
Cy3-ET-1	Dr. Michael Beyermann, FMP (Berlin)
ET-1 (Human, Porcine), [^{125}I]-ET-1, [^{125}I]Tyr^{13}-; 5, 10, 50 µCi	PerkinElmer (Germany)
ET-1, ET-3	Dr. Michael Beyermann, FMP (Berlin)
Fluorescein-4-Ala-ET-1 (f-4-Ala-ET1)	Dr. Michael Beyermann, FMP (Berlin)

The ligands, their aa sequences and labels are illustrated in the Appendix (**see 7.3.**).

2.5. Microbial strains

Strain	Relevant genotype	Reference
E. coli DH5α	[F$^-$,*end*A1, *hsd*R17 ($r_k^-m_k^-$), *sup*E44, *thi*-1, *rec*A1, *gyr*A (Nal^r), *rel*A1, Δ(*lac*ZYA-*arg*F)U169, Φ80*lac*ZΔM15]	(Hanahan et al. 1983)
E. coli BL21 (DE3)	*hsd*S, *gal* [λcI, *ts*857, *cnd*1, *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1]	(Studier and Moffat 1986)
E. coli K-12 strain A19	[*rna19 gdh A2 his95 relA1 spoT1 metB1*]	*E. coli* Genetic stock center, New Haven, CT

2.6. Oligonucleotides

All primers were ordered from MWG Biotech AG or Biospring if not stated otherwise. Sequences as well as melting temperatures are provided in the Appendix (**see 7.1.**).

2.7. Common buffers, media and reagents

All solutions are prepared with Milli Q water and kept at –20 °C if not stated otherwise. Sterilization by filtering is performed using 0.22 µm filters.

2.7.1. General buffers and media for the preparation of cell-free extract, T7 RNA polymerase and bacterial growth

Ampicillin stock, 1000-fold: 150 mg/mL Na^+-ampicillin salt in 50 % EtOH.

DB salt: 5.22 mM $NH_4H_2PO_4$, 0.05 mM $MgSO_4$ x $7H_2O$, 0.2 mM KCl, 44 % glycerol (w/v) in H_2O. Mix thoroughly on a stirring device for 20 minutes (min) and sterilize by autoclaving. Store at room temperature (RT).

Dialysis buffer for T7RNAP: 10 mM K_2HPO_4, pH 8.0, 10 mM NaCl, 0.5 mM EDTA, 1 mM DTT.

IPTG stock: 1 M in H_2O.

Kanamycin stock, 1000-fold: 75 mg/mL kanamycin sulfate in H_2O.

LB medium (1 liter): 10 g bactotryptone, 5 g yeast extract, 10 g NaCl. Sterilize by autoclaving.

M9 T7-TG medium (1 liter): Combine 900 mL TG medium and 100 mL 10x M9 stalts; add 0.2 % glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, stock solutions of latter mentioned solutions have to be sterilized by filtering.

10 x M9 salts (1 liter): 382 mM Na_2HPO_4, 220 mM KH_2PO_4, 186 mM NH_4Cl, 855 mM NaCl. Dissolve in H_2O, sterilize by autoclaving and store at 4°C.

Protease inhibitor cocktail: 1.197 mg/mL AEBSF, 0.238 mg/mL leupeptin, 0.154 mg/mL bestatin, 0.098 mg/mL aprotinin, 0.089 mg/mL E-64, 0.034 mg/mL pepstatin.

Q sepharose buffer A for T7RNAP: 30 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 5 % glycerol. Store at 4°C.

Q sepharose buffer B for T7RNAP: 30 mM Tris, pH 8.0,1 M NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 5 % glycerol. Store at 4°C.

Resuspension buffer for T7RNAP: 30 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM EDTA, 10 mM β-mercaptoethanol, 5 % glycerol, protease inhibitor cocktail

SOC medium (1 liter): 20 g bactotryptone, 5 g yeast extract, 0.5 g NaCl. Adjust to pH 7.0 with NaOH and sterilize by autoclaving. Add final concentrations of 25 mM KCl, 10 mM $MgCl_2$ and 20 mM glucose solution. All latter mentioned solutions have to be sterilized by filtering before addition. Store at 4 °C.

S30 A buffer: 10 mM Tris-acetate, pH 8.2, 14 mM $Mg(OAc)_2$, 60 mM KCl, 6 mM β-mercaptoethanol. Sterilize by filtering and store at 4°C.

S30 B buffer: 10 mM Tris-acetate, pH 8.2, 14 mM $Mg(OAc)_2$, 60 mM KCl, 1 mM DTT and 0.1 mM PMSF. It is recommended to prepare a 50-fold stock solution S30 A/B buffer out of Tris-acetate, $Mg(OAc)_2$ and KCl. Sterilize by filtering and store at 4°C. Add β-mercaptoethanol, DTT and PMSF freshly to the 1-fold buffers.

S30 C buffer: 10 mM Tris-acetate, pH 8.2, 14 mM $Mg(OAc)_2$, 60 mM KOAc, 0.5 mM DTT. Store at 4°C. It is recommended to prepare a 50-fold stock without DTT. Add DTT freshly to the 1-fold buffer.

TG medium (1 liter): 10 g bactotryptone, 0.2 % glycerol (v/v). Dissolve in H_2O and sterilize by autoclaving. Store at RT.

WGE buffer 40 mM HEPES-KOH pH, 7.6, 5 mM $Mg(OAc)_2$, 100 mM KOAc, 4 mM DTT, 0.3 mM of each of the 20 amino acids. Dissolve in H_2O and sterilize by filtering.

2 x YTPG medium (10 liters of aqueous solution): 22 mM KH_2PO_4, 40 mM K_2HPO_4, 100 mM glucose, 160 g bactotryptone, 100 g yeast extract, 50 g NaCl. Sterilize glucose separately by filtering. Sterilize phosphate buffer components separately by autoclaving. Dissolve remaining media components in distilled water and sterilize by autoclaving. The components should all be freshly prepared. Store at RT.

2.7.2. Reagents and buffers for cell-free expression and mRNA preparation

Acetyl phosphate lithium potassium salt, AcP: 1 M stock solution, pH 7.0, adjusted with 10 M KOH.

Amino acid stock: 20 amino acids containing stock solution at a final concentration of 8 mM each and dissolve in H_2O. Adjust pH to 8.0 with 10 M KOH. Suspensions are pipetted to the cell-free reaction set-up.

Complete® protease inhibitors cocktail with EDTA: 50-fold stock solution in H_2O.

Creatine kinase: 10 mg/mL stock in H_2O.

DTT: 500 mM stock in H_2O.

Folinic acid calcium salt: 10 mg/mL stock in H_2O. Completely dissolve by incubation at 37°C.

HEPES: 2.4 M stock. Adjust to pH 8.0 with 10 M KOH.

Lithium chloride, LiCl: 14 M stock solution in distilled RNase free water. Sterilize by filtering. Store at RT.

Magnesium acetate tetrahydrate, $Mg(OAc)_2$: 1 M solution in H_2O.

NTP mix: 75-fold stock containing 90 mM ATP, 60 mM each GTP, CTP and UTP, pH 7.0 adjusted with NaOH.

Phospho(enol)pyruvic acid monopotassium salt, PEP: 1 M stock. Adjust to pH 7.0 with 10 M KOH.

Polyethylenglycol 8000, PEG8000: 40 % (w/v) solution dissolved in H_2O by shaking over night (o. n.) at 37°C.

Potassium acetate, KOAc: 4 M in H_2O.

Pyruvat kinase (Roche Diagnostics): Stock of 10 mg/mL.Commercial, no separate preparation necessary.

RiboLock™ RNase inhibitor (Fermentas): Stock of 40 U/µL. Commercial, no separate preparation necessary.

Sodium acetate, NaOAc: 3 M stock in distilled RNase free water, pH 5.5 adjusted with NaOH. Sterilize by filtering. Store at 4°C.

Sodium azide, NaN_3: 3 % and 10 % (w/v) stocks in H_2O.

Spermidine: 100 mM stock solution in H_2O.

Transcription buffer, 5-fold: 400 mM HEPES-KOH, pH 7.6, 90 mM $MgCl_2$. Sterilize by filtering.

Translation pre-mix, 20-fold: 500 mM HEPES-KOH, pH 7.6, 30 mM DTT, 5 mM spermidine, 8 mM GTP, 20 mM ATP. Sterilize by filtering.

tRNA *E. coli* MRE 600: 40 mg/mL stock in H_2O.

tRNA from brewer's yeast: 2 mg/mL stock in H_2O.

WGE-NTP mixture:100 mM stock of 25 mM each ATP, UTP, GTP and CTP in distilled RNAse free water, pH 7.0 adjusted with NaOH. Sterilize by filtering.

2.7.3. Buffers for DNA-Agarose gels, SDS-PAGE and immunodetection by Western Blotting

Agarose: e.g. 1 % (w/v) agarose boiled in 1-fold TAE buffer. Store at RT.

Ammoniumperoxid sulfate, APS: 10 % stock solution by dissolving 100 mg/mL ammoniumperoxid sulfate in H_2O. Store at 4°C.

Blotting buffer (Towbin): Dissolve 25 mM Tris, 192 mM Glycin, 3.5 mM (1 %) SDS, 15 % MeOH in H_2O. pH should self-adjust to pH 8.3. Store at 4 °C.

Coomassie brilliant blue-staining solution for SDS gels: 50 % (v/v) ethanol (96 %), 10 % (v/v) acetic acid (100 %) and 0.1 % (w/v) Coomassie Brilliant Blue G250. Dissolve in H_2O and store at RT in a dark bottle to avoid exposure to light.

DNA-loading dye, 6-fold: 40 % (w/v) sucrose, 0.25 % (w/v) bromphenol blue and 0.25 % xylene cyanol FF. Dissolve in H_2O and store at 4°C.

EtBr-stock solution: 10 mg/mL stock in H_2O.

ECL 1: 100 mM Tris-HCl, pH 7.5, 2.9 mM Luminol, 0.4 % p-coumaric acid in H_2O. Store at 4°C. Luminol and p-coumaric acid are dissolved in 100 % DMSO.

ECL 2: 100 mM Tris-HCl, pH 8.0, 0.06 % H_2O_2 in H_2O. Store at 4°C.

PBS wash buffer for western blots (10-fold): Dissolve 1.37 M NaCl, 0.03 M KCl, 80 mM Na_2HPO_4 and 15 mM KH_2PO_4 in H_2O. Add 0.05 % Tween 20 to the 1-fold dilution. Store at 4°C.

Rotiphorese Gel 30: 30 % (v/v) acrylamide, 0.8 % (v/v) bisacrylamide. Store at 4 °C.

Rotiphorese Gel 40: 40 % (v/v) acrylamide, 1 % (v/v) bisacrylamide. Store at 4 °C.

SDS gel buffers:

Running buffer: 25 mM Tris-HCl, pH 8.0, 0.1 % (w/v) SDS and 200 mM glycine. Store at RT.

Stacking gel buffer: 0.4 % (w/v) SDS, 0.5 M Tris-HCl, pH 6.8. Store at 4°C.

Separating gel buffer: 0.4 % (w/v) SDS, 1.5 M Tris-HCl, pH 8.9. Store at 4°C.

SDS-PAGE sample buffer, 5-fold: 25 % (w/v) glycerol, 25 % (v/v) β-mercaptoethanol, 7.5 % (w/v) SDS, 0.1 % (w/v) coomassie G250, 300 mM Tris-HCl, pH 6.8. Store at RT.

TAE buffer, 50-fold: Dissolve 2 M Tris , 5.7 % acetic acid (v/v) and 50 mM EDTA in H_2O. Store at RT.

Tricine gel buffers:

Anode buffer, 10-fold: 1 M Tris-HCl, pH 8.9. Store at RT.

Cathode buffer, 10-fold: 1 M Tris, 1 M tricine, 1 % SDS (w/v). The pH should self-adjust to 8.25. Store at RT.

Gel buffer, 3-fold: 3 M Tris-HCl, pH 8.45, 0.3 % SDS (w/v). Store at RT.

Table 3: Pipetting scheme for two 10 % Tricine separating and two 4 % stacking gels.

Compounds	**Separating gel (10 %)** [mL]	**Stacking gel (4 %)** [mL]
Rotiphorese Gel 30 (37.5:1)	4.5	0.5
Gel buffer, 3-fold	4.5	1.3
Glycerol (98 %)	1.1	-
H_2O	3.0	2.0
APS (10 %)	0.135	0.08
TEMED	0.015	0.005

2.7.4. Buffers for protein purification and protein analysis

Biotin Blocking buffer for MAM: 100 mM Na_2HPO_4, 150 mM NaCl, pH 7.0. Add 2 mM biotin as reversible competitor.

CD buffer: 10 mM sodium phosphate, pH 7.5. The required pH is adjusted by combination of 1 M Na_2HPO_4 and 1 M NaH_2PO_4.

Elution buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM biotin as reversible competitor. Add the appropriate detergent used for protein purification.

Filter binding / wash buffer for radioactive activity measurements on proteoliposomes: 20 mM potassium phosphate, pH 7.5, 150 mM NaCl, 0.1 % BSA. The pH is adjusted by combination of 1 M K_2HPO_4 and 1 M KH_2PO_4.

Fluorescence anisotropy buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % B35.

IMAC buffers for ETA and truncations:

Equilibration buffer: 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM imidazole.

Wash 1 buffer: 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 30 mM imidazole.

Wash 2 buffer: 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 80 mM imidazole.

Wash 3 buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl.

Elution buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 350 mM imidazole.

Add the appropriate detergent at a concentration of 5-10 times above CMC.

IMAC buffers for ETB and truncations:

See: IMAC buffers for ETA and truncations. Instead of Tris, 50 mM NaH_2PO_4 was used as buffer substance.

Inteine cleavage buffer: 50 mM Tris-HCl, pH 6.5, 0.5 M NaCl, 1 mM EDTA. Alternatively, 2 mM DTT were added to the buffer and the pH was adjusted to 7.0.

Liposome reconstitution buffer, 1-fold: 20 mM potassium phosphate, 150 mM NaCl. The pH is adjusted by combination of 1 M K_2HPO_4 and 1 M KH_2PO_4.

PBS buffer for MAM, 1-fold: 100 mM Na_2HPO_4, 150 mM NaCl. Adjust pH to 7.0.

Regeneration buffer for MAM: 100 mM glycine, adjust pH to 2.8 with HCl.

Resolubilization buffer for IMAC purification: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM TCEP, 1 % of the respective detergent.

Resolubilization buffer for reconstitution, 5-fold: 100 mM potassium phosphate, 0.75 M NaCl. The required pH (pH 7.0, pH 7.5) is adjusted by combination of 1 M K_2HPO_4 and 1 M KH_2PO_4.

SEC buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.04 % DDM or 0.1 % B35. Sterilize and degase by filtering. Store at 4 or 16 °C.

SEC buffer for inteines: 50 mM Tris-HCl, pH 7.0, 100 mM NaCl, 2 mM DTT, 1 mM EDTA and 0.08 % Fos-12. Store at 4 or 16 °C.

StrepII-tag affinity purification buffer:

Equilibration and wash buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % B35 / 0.1 % Fos-12.

Elution buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % B35 / 0.1% Fos-12, 2.5 mM desthiobiotin as a reversible binding specific competitor.

Tris-HCl: Prepare a 1 M stock solution of Tris and adjust the required pH at RT with HCl. For measurements at higher temperatures a ΔpKa/°C of -0.03°C was assumed.

Wash buffer for MAM: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl. Add the appropriate detergent used for protein purification.

3. Methods

3.1. Standard methods of molecular biology and microbiology

3.1.1. DNA techniques: Conventional polymerase chain reaction (PCR) and cloning

The coding region of the human ETA full-length receptor (Gene Bank accession number: AY275462) was amplified by conventional PCR (Mullis and Faloona 1987, Saiki et al. 1988) from cDNA obtained from the UMR cDNA Resource Center (www.cdna.org) by using VentDNA-polymerase (New England Biolabs, Frankfurt, Germany) and the oligonucleotide primers ETA_up: 5'-CGA AGA TCT ATG GAA ACC CTT TGC CTC AGG GCA TCC-3' and ETA_low: 5'-CCG CTC GAG CAT GCT GTC CTT ATG GCT GCT CCG-3'. Restriction sites for the enzymes *Bgl*II, an isoschizomere of *Bam*HI, and *Xho*I were introduced by suitable linkers. PCR fragments were purified, restriction digested and ligated with a *Bam*HI and *Xho*I digested derivative of the vector pET21a(+) (Merck, Darmstadt, Germany) (**see 7.2.**) encoding for a poly(His)$_{10}$-tag. Further coding regions of the human ETA resulting in truncations of the receptor were PCR-amplified using the corresponding oligonucleotide primers (**see 7.1.4.**), VentDNA-polymerase and the full-length ETA receptor construct in pET21a(+)poly(His)$_{10}$ as a template (see above). The PCR products were either cleaved with *Bgl*II and *Xho*I or *Bgl*II and *Nco*I in order to achieve cloning into pET21a(+)polyHis$_{10}$ or pET21a(+)strepII, another derivative of pET21a(+) encoding for a strepII-tag that was introduced by quick change mutagenesis. PCR products were purified, restricted and ligated either with a *Bam*HI and *Xho*I cleaved pET21a(+)poly(His)$_{10}$ or a *Bam*HI and *Nco*I cleaved pET21a(+)strepII, respectively.

The coding region of the human ETB receptor without its signal peptide (ETB-sign) was PCR-amplified using a full-length receptor containing plasmid as template (see PhD thesis Dr. Christian Klammt) and the oligonucleotide primers ETB1-bam_up: 5'-CGG GGA TCC GAG GAG AGA GGC TTC CCG CCT GAC AGG-3' and ETBlow: 5'-CGG CTC GAG AGA TGA GCT GTA TTT ATT ACT GGA ACG-3'. Restriction sites for the enzymes *Bam*HI and *Xho*I were introduced by appropriate linkers. PCR fragments were purified, restriction digested and ligated with a *Bam*HI and *Xho*I digested derivative of the vector pET21a(+)poly(His)$_{10}$.

The ETB intein fusion constructs pVSO 4.1 and pVSO 4.2 were kindly provided by Prof. Dr. Henning Mootz (Technical University Dortmund, Germany). Both constructs were restriction digested with *Bam*HI and *Xho*I and subsequently ligated into the equivalently cleaved vector pET21a(+)poly(His)$_{10}$. pVSO 4.1 harbors the coding sequence for TMS1 of ETB full-length receptor starting at amino acid position 93 and ending at position 130 fused to the split intein DnaE^{N} from *Nostoc punctiforme*. pVSO 4.2 contains the split intein DnaE^{C} from

N. punctiforme fused to the coding sequence for the following two TMS (TMS 2+3) starting at amino acid position 131 and ending at position 203. The intein fusion proteins were further termed ETB 4.1 and ETB 4.2 after their respective plasmids pVSO 4.1 and pVSO 4.2.
ETA and ETB as well as their truncations and intein fusions were expressed from these constructs with a N-terminal T7-tag and a C-terminal poly(His)$_{10}$-tag.

The ETB full-length construct pObe-ETB-TMV for the expression in the WGE harboring a poly(His)$_6$-tag at its C-terminus was a pleasant gift of Dr. Vladimir A. Shirokov (Institute of Protein Research, Russian Academy of Science, Pushchino, Moscow Region, Russia). It was cloned into a pUC18-modified vector containing a 5' UTR obelin leader sequence as well as a 3' UTR TMV sequence under the control of a T7 promotor. The pObe-ETB-TMV was expressed from this constructs with a C-terminal poly(His)$_6$-tag.

Standard PCR reactions were performed using plasmid DNA as template and a reaction volume of 100 µL if not stated otherwise. Analysis of the amplification reaction was carried out with volumes of 5-10 µL on 1 % agarose gels in 1-fold TAE buffer. PCR reaction samples were subsequently purified with Nucleospin PCR purification kits (Macherey-Nagel, Düren, Germany) and eluted either in water or in 10 mM Tris-HCl, pH 7.5 (Table 4).

Purification of plasmid DNA was achieved by modified alkaline/SDS lysis. Plasmid DNA resulting from molecular cloning procedures for sequencing–grade purity was commonly purified with NucleoSpin Plasmid (Macherey-Nagel, Düren, Germany) using a 3 mL o. n. culture. Plasmid DNA used as template for CF expression was isolated with NucleoBond AX100 commercial kits (Macherey-Nagel, Düren, Germany) using 150 mL o. n. culture according to manufacturer's recommendations.

Table 4: Composition of a standard PCR with program set-up. The annealing temperature was adjusted dependent on the melting temperature of the respective oligonucleotides. The elongation time was chosen dependent on the length of the target fragment.

Compounds [stock]	**Final concentration**	**Program**	
Template DNA	50 -300 ng		**Initial denaturation:**
		1 x	95°C, 7 min
5'-oligonucleotide [10 µM]	10 pmol	30 – 35 x	**Denaturation:** 95°C, 1 min
3'-oligonucleotide [10 µM]	10 pmol		**Annealing:** 42°C – 65°C, 30 s
dNTP mixture* [10 mM]	200 µM		**Elongation:** 72°C, 1 – 1min 30 s
Polymerase buffer, 10-fold	1-fold		**Final elongation:**
		1 x	72°C, 7 min
VentDNA polymerase	2 U	∞	4°C
$MgSO_4$ [100 mM]	2 – 6 mM		

*, dNTP mixture (10 mM): 2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 2.5 mM dTTP

DNA concentration in aqueous solution was spectrophotometrically measured at $\lambda = 260$ nm using a NanoDrop 1000 Spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany) assuming with Lambert-Beer's law that an absorption of 1 corresponds to a concentration of 50 µg/mL of double-stranded DNA.

3.1.2. Site-directed mutagenesis: Replacement of essential cysteines involved in disulfide bridge formation

To exchange the relevant codons of cysteines 158 and 239 involved in putative disulfide bridge formation in the ETA full-length receptor by bases coding for alanines site-directed mutagenesis by PCR was performed using the oligonucleotide primers ETA C/A 158_up 5'-GGC GTA TTT CTT GCC AGG CTG TTC CCC-3' and ETA C/A 158_low 5'-GGG GAA CAG CTT GGC AAG AAA TAC GCC-3' as well as ETA C/A 239_up 5'-CAG CAT AAA ACC GCT ATG GGC-3' and ETA C/A 239_low 5'-GCC ATT GAG CAT AGC GGT TTT ATG CTG-3' (**see 7.1.3.**). Therefore, either 25 or 50 ng of template plasmid DNA were combined with 1-fold PfuUltra buffer, 15 pmol of each primer, 200 µM dNTPs (Table 4), 2.5 U PfuUltra polymerase and water to give a final reaction volume of 50 µL.

The cycler program was chosen as given in Table 5. Directly after amplification 40 U of the restriction enzyme *Dpn*I per 50 µL reaction volume were added to the PCR sample and incubated at 37 °C for 2 h in order to completely digest the parental methylated DNA template. Subsequently, 10 µL of digested sample were transformed (**see 3.1.4.**) into chemically competent *E. coli* DH5α cells, grown at 37 °C in SOC medium without antibiotics for an additional hour and finally plated on agar plates containing the appropriate antibiotic. The plates were incubated o. n. at 37 °C and colonies were picked the following day for the cultivation of bacteria and subsequent DNA preparation. Clones were sequenced in order to verify the success of site-directed mutagenesis.

Table 5: Site-directed mutagenesis cycler program. The annealing temperature was set empirically at a minimum of 55 °C independent of the respective oligonucleotides melting temperatures. The elongation time was adapted to the length of the target plasmid DNA and the performance of PfuUltra polymerase of 1kb / 2min.

Site-directed mutagenensis cycler program	
	Initial denaturation:
1 x	95°C, 1 min
	Denaturation:
	95°C, 30 s
	Annealing:
20 x	55°C, 30 s
	Elongation:
	72°C, 14 min
	Final elongation:
1 x	72°C, 10 min
∞	4°C

3.1.3. Template design for CF expression using E. coli S30 extracts

Plasmid DNA that was used as template for the *E. coli* CF expression was provided under the control of a strong promoter recognized by the phage T7 RNA polymerase. Essential T7 regulatory sequences were therefore present to ensure optimal transcription efficiency such as the T7-promotor, ε-enhancer, ribosome binding site and a T7-terminator region (Fig. 8). Suitable vectors fulfilling the aforementioned conditions belong to the pET (Novagen) or pIVEX series (Roche Diagnostics) series. However, not only transcription but also translation efficiency had to be taken into account. Protein expression productivity in prokaryotic backgrounds strongly depends on the efficient initiation of translation which primarily depends itself on the first codons of a reading frame. Inappropriate codon structure can therefore cause failure of expression, in particular of eukaryotic MPs.

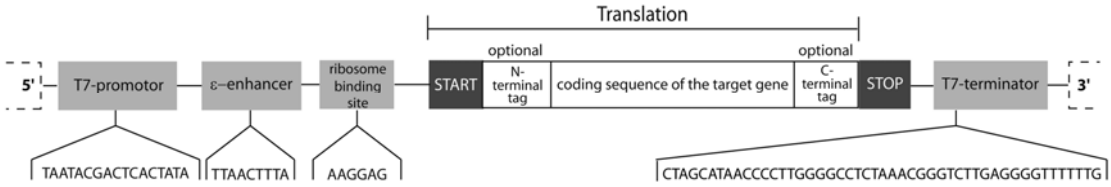


Fig. 8.: DNA template design for CF expression in *E. coli* extracts. T7 regulatory sequences are listed and highlighted in grey. Optional translated elements like N- as well as C-terminal tag are indicated.

Codon-optimized N-terminal tags like T7-tag or small fusion proteins such as Trx can help to avoid this problem and might even enhance expression (Junge et al. 2008, Murthy et al. 2004, Schwarz et al. 2007b).Therefore, all full-length constructs of the ET receptors as well as their respective truncations were cloned in a derivative of the standard expression vector pET21a(+) harbouring a N-terminal T7-tag and a poly(His)$_{10}$-tag strepII-tag at its C-terminus (Novagen, Darmstadt, Germany) for purification and immunodetection.

3.1.4. Basic working with E. coli: Transformation of plasmids, growth and storage

All heat resistant solutions as well as heat stable glassware were sterilized by autoclaving for 30 min at 120 °C and 2 bars. Other temperature sensitive reagents and solutions were sterilized by filtering through filters with a pore size of 0.22 µm.

For transformation of plasmid DNA into chemical competent DH5α cells (Inoue et al. 1990) 50-100 µL of cells initially stored at -80 °C were thawed on ice. Either 10 µL of a ligation sample or 50-200 ng of plasmid DNA were directly added to the cells and incubated on ice for 5 min followed by a heat shock of 42 °C for 45 s. Subsequently, cells were incubated on ice for additional 5 min. 750 µL of prewarmed SOC medium without antibiotics was added and cells were grown at 37 °C for 1 h. To concentrate bacteria, cells were centrifuged at 5,000 x g for 5-10 min at RT and resuspended in 1/5 of the supernatant. 100-200 µL of cell suspension was

finally plated on agar plates containing the appropriate antibiotic and incubated at 37 °C over night. Single colonies were picked and grown in LB-medium. DNA was isolated (**see 3.1.1.**), subjected to a test restriction and promising clones were sent to the sequencing service Seqlab Sequence Laboratories (Göttingen, Germany).

All used *E. coli* strains for standard techniques were grown in LB medium o. n. at 37 °C in shaking incubators at a speed of 170-190 rpm. Depending on the encoded antibiotic resistance of the used plasmid vector the appropriate antibiotic was added at a dilution of 1:1,000 with final concentrations of 100 µg/mL for ampicillin and 30 µg/mL for kanamycin.
An aliquot of bacterial suspension was plated on agar plates for single colony growth at 37 °C o. n.. Subsequently, a colony was picked, streaked on a separate agar plate and incubated again at 37 °C. Using inoculation loops, bacteria were finally transferred from these plates to 1 mL of DB salt and stored long-term at -80 °C.

3.2. Preparation of T7 RNA polymerase for cell-free expression

The T7 RNA polymerase (T7RNAP) is one of the most essential and most expensive compounds for the CF synthesis of proteins and can be purchased commercially (e.g. Roche Diagnostics GmbH, Penzberg, Germany). However, for efficient transcription high amounts combined with high concentrations of T7RNAP are needed and commercial polymerases are often too low in concentration.

The T7RNAP is characterized by a molecular mass of 98 kDa, a pI of 6.77 and is highly dependent on Mg^{2+} ions that are not only required as co-factors but also for the stabilization of RNA conformation and the interaction of the two subcomplexes in the 70S ribosome complex.
Since it has been shown that it is efficient to overexpress and purify this compound (Li et al. 1999), an individual protocol for the preparation of T7RNAP was set up (Schwarz et al. 2007a, Schneider et al. 2010). Overproduction of the T7RNAP in BL21 (DE3) star cells was achieved from the plasmid pAR1219 carrying ampicillin resistance (Davanloo et al. 1984). An overnight culture was prepared in LB medium including the adequate antibiotic at 37 °C. The following day, 1 L of M9 T7-TG medium containing ampicillin was inoculated 1:100 in a culture flask with the freshly prepared overnight culture and incubated at 37 °C with vigorous shaking to reach an OD_{600} of 0.6-0.8. Expression of the T7RNAP was subsequently induced by addition of 1 mM IPTG. After induction cells were further grown for additional 5 h at 37 °C and finally harvested by centrifugation at 4,500 x g and 4 °C for 15 min. At this step, cell pellets could either be stored at –80 °C for aproximately four weeks until further use or directly be subjected to cell disruption and purification procedures. The generated cell pellet was resuspended in 30 mL per L culture of resuspension buffer for T7RNAP. Cell disruption was achieved by 8 sonication cycles of 1 min each, interrupted by 30 s incubation on ice. The cell debris was then removed by centrifugation at 20,000 x g and 4 °C for 30 min. The supernatant was subsequently

kept on ice and adjusted dropwise to a final concentration of 2 % streptomycin sulfate to precipitate DNA. In order to gently mix after each addition of streptomycin sulfate the mixture was inverted six to eight times. DNA precipitate was removed from the turbid supernatant by a second centrifugation step at 20,000 x g and 4 °C for 30 min. The DNA-free supernatant was subjected to purification by anion exchange chromatography and further treated as mentioned in **3.5.2.**

Analysis of the catalytic activity and of the concentration units of the prepared T7RNAP was either achieved by CF synthesis of the reporter green fluorescent protein (GFP) or by *in vitro* transcription in comparison to a commercial polymerase. Plasmid DNA containing the target gene under the control of a T7 promotor was first linearized at the 3' end of the open reading frame with an appropriate restriction enzyme to generate a transcript of defined size by run-off transcription. The linear plasmid template was purified by phenol/chloroform extraction. Subsequently, the transcription reactions were set up containing 50 ng of template DNA, 0.1 U/µL RiboLock, 2 mM NTPs and 1-fold transcription buffer (40 mM Tris-HCl (pH 8.0), 12 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine; prepared as 10-fold stock) and T7RNAP in different concentrations. The mixture was incubated for 2 h at 37 °C and terminated by addition of 2 mM EDTA. Transcripts were analysed and quantified by eye on RNA-free agarose gels and equipment.

3.3. Cell-free expression of MPs

3.3.1. Preparation of an individual E. coli S30 extract

The extract preparation of the individual S30 extract of *E. coli* principally bases on the protocol developed by Zubay in the early 70s (Zubay 1973) but utilizes a modified run-off step. An additional heatshock that should induce the production of heatshock chaperones was occasionally introduced before cooling of the *E. coli* cells (see second day).

The CF extract constitutes the most essential basic compound for the CF expression of proteins and contains all indispensible components for the translational process (**see 1.2.2.**). It may furthermore harbor some residual *E.coli* lipids or membrane fragments of approximately. 50-100 µg/mL of S30 extract (**see 1.2.3.**). Other essential compounds for the complete expression process were supplemented individually.

In this work the RNAse deficient *E. coli* strain A19 was used for extract preparation (Schwarz et al. 2007a, Schneider et al. 2010).

The whole procedure takes three days and yields in between 70-100 mL of S30 extract out of 10 L 2 x YPTG medium. The first day concentrates on the preparations such as sterilization of media and buffers, supply of necessary working material and the inoculation of the pre-culture of A19.

For this purpose 100 mL of LB medium were prepared and sterilized by autoclaving. The medium was subsequently carefully inoculated with *E. coli* cells of strain A19 from a fresh plate without any supply of antibiotics. The pre-culture was then incubated o. n. at 37 °C with vigorous shaking. Preparation of 2 x YTPG medium was followed as described in **2.7.1.**. Yeast extract and bactotryptone were dissolved in 7 L of distilled water, filled into the fermenter and sterilized by autoclaving. Sterilized glucose and phosphate buffer were added the following day before initial inoculation. 50-fold stock solutions of S30 A/B buffer and S30 C buffer as well as 5 M NaCl were prepared, sterilized by filtering and stored at 4 °C until further use.

The second day started with the completion of the 2 x YTPG medium and its pre-heating to 37 °C under good aeration. Subsequently, the A19 pre-culture was inoculated to the oxygen enriched medium at a ratio of 1:100. Cells were then incubated at 37 °C under vigorous stirring at 500 rpm until an OD_{600} of 4.0-4.5 (mid-logarithmic phase) was reached.

An optional heat shock for the induction of chaperone formation was introduced at an OD_{600} of 3.5-4.0. Cells were heated up to 44 °C and grown at this temperature for 20 min, followed by cooling to 37 °C and further incubation for 20 min.

Cells were subsequently rapidly chilled below 12 °C in the fermenter by using internal and external cooling sources. The chilling should not exceed 45 min. The broth was then filled into centrifuge beakers that were constantly kept at 4 °C and cells were subsequently harvested by centrifugation at 5,000 x g and 4 °C for 15 min.

The pellet was resuspended in 300 mL of pre-cooled 1-fold S30 A buffer supplemented with β-mercaptoethanol and centrifuged for 15 min at 5,000 x g and 4 °C. This washing step was repeated twice with the same buffer but at an extended final centrifugation for 30 min. The resulting cell pellet was weighted and resuspended in 110 % (v/w) of pre-cooled 1-fold S30 B buffer supplemented with PMSF and DTT for cell lysis. The wet weight from a 10-liter fermentation was in between 50 and 70 g.

Cells were subsequently lysed either once by a pre-cooled French press cell disruptor (SLM Aminco Instruments Inc., USA) using a constant pressure of 20,000 psi or two to three times with a high-pressure homogenizer at 1.5-1.7 kbar (IUL Instruments GmbH, Germany). The disrupted cells were centrifuged at 30,000 x g for 30 min at 4 °C. The non-turbid upper part of the supernatant (~ 2/3 to 3/4 of the supernatant) was collected and subjected to a second centrifugation step at the aforementioned conditions. The supernatant was again judged by its turbidity. Only non-turbid extract was kept for following steps.

Subsequently, the modified run-off step was performed to remove endogenous mRNA. Therefore, the supernatant was adjusted to a final concentration of 400 mM NaCl and the extract was incubated in a waterbath at 42 °C for 45 min, resulting in a cloudy solution. The turbid extract was transferred to dialysis tubes with a MWCO of 12-14 kDa and was dialysed once against 5 L of cold S30 C buffer for at least 2 h at 4 °C. Then, the dialysis buffer was exchanged and the dialysis continued o. n. at 4 °C.

On the third day of preparation the dialysed extract was separated from precipitates by centrifugation at 30,000 x g for 30 min at 4 °C. The supernatant was immediately aliquoted to appropriate volumes and shock-frozen in liquid nitrogen. The frozen extract was stored at -80 °C for several months.

3.3.2. Continuous exchange cell-free configurations using E. coli extracts

CECF expression reactions in P-CF, D-CF as well as L-CF mode were basically performed as previously described in detail (Schwarz et al. 2007a, Schneider et al. 2010).

All reaction components for the CF expression of the ET receptors are listed in Table 9. A pipetting scheme for analytical scale reactions and salt optimization are given in Table 6. Analytical scale reactions for the optimization of expression conditions were carried out in 24-well microplates in Mini-CECF reactors. Appropriate dialysis membranes, type 27/32, having a MWCO of 12-14 kDa (Roth, Karlsruhe, Germany) were used to separate RM from FM. The RM volume was 55 µL with a RM to FM ratio of 1:15. Preparative scale expression was accomplished in Maxi-CECF reactors (Schneider et al. 2010) in RM volumes from 1 to 3 mL and with a RM to FM ratio of 1:17. CF reactions were incubated for 16 to 20 h with gentle shaking at 30 °C (Schwarz et al. 2007a). Optimal concentrations of Mg^{2+} and K^{+} ions for each S30 extract batch were analysed in analytical scale reactions in the P-CF mode. CF expressed protein was harvested as precipitate (P-CF) by centrifugation at 18,000 x g for 10 min at 4 °C. Successful P-CF synthesis resulted in a turbid RM after expression due to the precipitation of the target MPs. Those precipitates were subsequently washed and finally either resuspended in water or resolubilized in appropriate detergents equal to the volume of the initial RM for further analysis (**see 3.5.1.**). 3 µL or 1 µL of P-CF or D-CF overexpressed protein were analysed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) or western blotting, respectively (**see 3.4.1.**).

For soluble expression in the D-CF mode, detergents were added at the following final concentrations: Brij 35 (B35, 0.05 %, 0.1 %, 0.5 %), Brij 58 (B58, 1 %), Brij 78 (B78, 1 %), Brij 98 (B98, 1 %), digitonin (0.4 %), Triton X-100 (TX-100, 0.1 %), Fos-12 (0.1 %), n-hexadecyl-β-D-maltopyranoside (HDM, 0.05 %), n-tetradecyl-β-D-maltopyranoside (TDM, 0.01 %), DDM (0.1 %), CHAPS (2 %). Stock solutions of the relevant detergents were prepared in distilled water at a concentration of at least 10-fold to the working concentration and stored at 20 °C until further use. Stocks of Brij derivatives had to be completely solubilized at 60 °C for at least 1 h.

To express target proteins in the D-CF mode mastermixes were prepared as stated in Table 6 with minor changes. The appropriate detergents were added in desired amounts and their volumes were subtracted from the final water volume. D-CF expressed protein mainly stayed in the supernatant and was harvested by centrifugation at 18,000 x g for 10 min at 4 °C. Since

many soluble *E. coli* proteins also remain in the supernatant D-CF expressed protein displayed much less purity and could often only be identified by western blotting.

Table 6: Analytical scale pipetting scheme for P-CF mode expression. A pipetting scheme for two analytical scale reactions is given for RM volumes of 55 µL and 850 µL of FM.

	Concentration stock	final	Unit	Mastermix [µL]	FM [µL]	RM [µL]
NaN3	10	0.05	%	9.1		
PEG8000	40	2	%	90.5		
KOAc	4000	110	mM	49.8		
$Mg(OAc)_2$	1000	11.1	mM	20.1		
HEPES buffer	24	1	x	66.4		
Complete	50	1	x	36.2		
Folinic acid	10	0.1	mg/mL	18.1		
DTT	500	2	mM	7.2		
NTP mix	75	1	x	24.1		
PEP	1000	20	mM	36.2		
AcP	1000	20	mM	36.2		
AA-mix	8	0.6	mM	135.8	128	
RCWMDE-mix	16.7	1	mM	108.6		
Mastermix				638	599.2	38.8
S30 buffer	100	35	%		595	
Pyruvate kinase	10	0.04	mg/mL			0.44
tRNA *E.coli*	40	0.5	mg/mL			1.38
T7RNAP	3.8	0.05	mg/mL			1.45
RiboLock™	40	0.3	U/µL			0.83
Template DNA	200	0.03	µg/mL			16.5
S30 extract	100	35	%			38.5
MilliQ H_2O					378 12.1	
Total				1810	1700.2	110

[a], Value for reaction with 270 mM K^+ and 16 mM Mg^{2+} final concentration
[b], A, Carl Roth GmbH & Co. KG, Karlsruhe, Germany; B, Roche Diagnostics, Penzberg, Germany C, Sigma-Aldrich, Steinheim, Germany; D, MBI Fermentas, St. Leon-Rot, Germany

Alternatively, ET receptors were expressed in presence of preformed liposomes in the lipid based CF expression mode (L-CF). Liposomes composed of *E. coli* polar lipids/cholesterol (**see 3.9.2.**) or L-α-phosphatidylcholine from soybean, type IV-S, (aso-PC) were destabilized by detergents and subsequently added to the RM in final concentrations of 2 mg/mL for *E. coli* polar lipids/cholesterol and 4.5 mg/mL for soybean lipids. The proteoliposomes were harvested by centrifugation at 23,000 x g and 4 °C for 30 min. The pellet was washed once and finally resuspended in 1-fold Tris buffer or S30 C buffer equal to the RM volume and either analysed by ultracentrifugation (**see 3.9.2.**) or filter binding assays (**see 3.9.3.**).

3.3.3. Preparation of an individual S30 wheat germ extract

The isolation of wheat germ embryos as well as the subsequent preparation of the WGE was supervised by Dr. Vladimir A. Shirokov (Institute of Protein Research, Russian Academy of Science, Pushchino, Moscow Region, Russia) and accomplished according to initial protocols (Erickson and Blobel 1983, Madin et al. 2000, Sawasaki et al. 2007, Shirokov et al. 2007).

3.3.4. Preparation of mRNA templates for the cell-free translation in WGEs

To achieve efficient translation initiation in the WG system without eukaryotic 5'-cap structure appropriate 5' UTR as well as 3' UTR sequences were introduced to the coding sequence of the target gene (**see 3.1.1.**). The mRNA preparation and purification steps are of great importance for the subsequent translation reaction. If the quality of the prepared mRNA is not sufficient no or only minor translation will occur. Therefore, all steps were performed with RNase free material and solutions.

To increase the efficiency of the transcription reaction the ETB plasmid template was first linearized by the restriction endonuclease *Bsp*120I and subjected to DNA precipitation. The linear plasmid was subsequently used as template for the transcription reaction that was performed in volumes of 500 µL. Final concentrations of 50 µg/mL DNA, 1-fold transcription buffer, 20 mM WGE-NTP mixture, 2 mM spermidine and 40 mM DTT and RNase free water were combined and mixed thoroughly by vortexing. 0.5 U/µL RNase inbibitor and 40 U/µL T7RNAP were supplemented to the transcription reaction and the mixture was incubated for 3 h at 37 °C. Milky white precipitate due to pyrophosphate formed as by-product indicated successful transcription.

The mRNA was subsequently harvested by phenol/chloroform extraction. Therefore, the transcription reaction was terminated by the addition of 0.5 volumes of phenol/chloroform mixture (1:1, v/v), vortexed thoroughly and subjected to centrifugation at 18,000 x g and RT for 10 min. The aqueous phase was carefully harvested and transferred to a new tube. The yield of aqueous phase was increased by addition of 0.5 volumes of chloroform to the remainder of the previous step and repetition of the centrifugation. The mRNA was subsequently precipitated by addition of 0.5 volumes of saturated 14 M LiCl, mixed thoroughly and incubated on ice for at least 40 min. The mRNA was harvested by centrifugation at 18,000 x g and 4 °C for 20 min. The pellet was reconstituted in 200 µL of RNase free water followed by an addition of 0.1 volumes of 3 M NaOAc, pH 5.5 and 2.5 volumes of precooled 96 % ethanol. The mixture was mixed by vortexing and incubated o. n. at -20 °C. The sample was pelleted by centrifugation at 18,000 x g and 4 °C for 20 min. Washing steps at 18,000 x g and 4 °C for 5 min were repeated twice in 300 µL pre-cooled 75 % ethanol. The remaining pellet was dried at RT for at least 15 min and finally reconstituted in 30-40 µL of RNase free water. The concentration was determined at an absorption of 260 nm (**see 3.1.1.**).

3.3.5. Continuous exchange cell-free expression using WGE

The CF expression protocol set-up in WGE basically resembles expression of MPs in the *E. coli* extract (**see 3.3.2.**). Target proteins were expressed in analytical as well as preparative scale reactions. Dialysis membranes, type 27/32, with a MWCO of 12-14 kDa (Roth, Karlsruhe, Germany) were provided to separate RM from FM in the CECF reactions. For analytical scale and preparative scale reactions ratios of RM : FM of 1:14 or 1:17 were used, respectively. However, uncoupled transcription/translation was performed since magnesium optima for transcription in the wheat germ system are considerably higher (~15-18 mM Mg^{2+}) than those for translation (~ 3 mM Mg^{2+}) (Sawasaki and Endo 2007). Therefore, mRNA template was prepared beforehand (**see 3.3.4.**) which additionally renders the reaction more stable since the expression process is less susceptible to mRNA degradation.

For a 70 µL analytical scale translation reaction the final concentrations and the required volumes of the individual components are listed in Table 7.

Table 7: P-CF expression protocol in wheat germ extracts. Essential compounds as well as their stock and final concentrations in RM and FM are listed. A pipetting scheme for one analytical scale reaction is given for RM volumes of 70 µL and 1000 µL of FM.

	Concentration		
Compound [unit]	**Stock**	**Final concentration**	**Volume [µL]**
Feeding mix (FM): 1000 µL			
Translation pre-mix [x]	20	1	50
NaN_3 [%]	3	0.03	10
Glycerol [%]	40	2	50
Creatine phosphate [mM]	500	16	32
AA-mix [mM]	4	0.2	50
$Mg(OAc)_2$ [mM]	100	1.5	30
KOAc [M]	2.5	0.05	20
MilliQ H_2O			758
Reaction mix (RM): 70 µL			
mRNA template [mg/mL]	≥ 4	0.2	≤ 3.5
Wheat germ extract [%]	100	30	21
RiboLock™ [U/µL]	40	0.5	0.88
Creatine kinase [mg/mL]	10	0.1	0.7
NaN_3 [%]	3	0.03	0.7
tRNA (yeast) [mg/mL]	2	0.05	1.75
Translation pre-mix [x]	20	1	3.5
Glycerol [%]	40	2	3.5
MilliQ H_2O			adjust to 70 µL

All reaction compounds were thawed at RT and stored on ice except creatine kinase, T7RNAP, tRNA and the S30 WGE that were constantly kept on ice. The FM was prepared first, thoroughly mixed and incubated at 25 °C until further use. The RM was prepared on ice by pipetting in inverse order to Table 7. All components were well vortexed except creatine kinase, RNase inhibitor and the extract itself which were added after mixing. The required amount of WGE was added and the RM was subsequently incubated for 2 min at 25 °C before the reaction was completed by addition of mRNA. All the components were gently mixed and RM and FM were transferred to their respective compartments. The reactions were and incubated for 16-20 h at 25 °C with gentle shaking. The target proteins were harvested and subjected to SDS-PAGE or western blotting as described in **3.4.1.**.

3.4. Protein analysis

3.4.1. Electrophoresis and immunoblotting

Protein samples analysed by SDS-PAGE were supplemented 1:3 with SDS-PAGE sample buffer, 5-fold. Samples of full-length ETA and ETB receptors were separated on 12 % (w/v) Tris-Glycine-SDS gels (Laemmli et al. 1970) at 100 V for 15 min followed by 200 V for 45 min with constant current. For truncations and co-expression of ETA and ETB 16 % gels were used. Commonly, 2-3 µL of resuspended protein pellet or supernatant as well as 1 µL of RM were loaded on SDS-PAGE gels.

As Tris-Tricine-PAGE is better suited for the separation of proteins less than 30 kDa due to a higher resolution at lower molecular weights (Schägger and Von Jagow 1987) the smaller inteins and cleavage products were analysed by 10 % or 12 % Tris-Tricine-SDS gels (Table 3).

Gels were either stained with Coomassie blue or analysed by western blotting. For immunodetection of the poly$(His)_{10}$-tag, protein samples were transferred on an activated 0.45 µm Immobilon-P (polyvinylidene difluoride, PVDF) membrane (Millipore, Eschborn, Germany) and blotted in a wet western blot apparatus for 30-40 min at 340 mA (MiniProtean IV, Bio-Rad, München, Germany). The membrane was subsequently blocked for 1 h in 1-fold PBS supplemented with 8 % skim milk powder and 0.05 % Tween-20 followed by incubation with the first antibody at a dilution of 1:2,000 against the C-terminal His-tag (PentaHis Antibody, QIAGEN, Hilden, Germany) in 1-fold PBS / 0.05 % Tween 20 / 0.6 % skim milk powder. Incubation of the first antibody was performed at 4 °C o. n. with gentle shaking. As a second antibody an anti-mouse IgG HRP conjugate (Sigma-Aldrich, Germany) was added after two washing steps with 1-fold PBS and 0.05 % Tween-20 at a dilution of 1:5,000 for 1 h at RT (1-fold PBS / 0.05 % Tween 20 / 0.6 % skim milk powder). After extensive washing, the membranes were analysed by chemiluminescence in a Lumi-Imager F1™ (Roche Applied

Science, Mannheim, Germany) using each 5 mL ECL 1 and 2 solutions with an incubation time of 1 min, respectively.

To analyse the integrity of the receptors both tags had to be detected. Therefore, a T7-tag antibody directly conjugated to horseradish-peroxidase (Merck, Darmstadt, Germany) was used at a dilution of 1:10,000 in 1-fold PBS / 0.05 % Tween 20 / 0.6 % BSA and incubated with the membrane for 1 h at RT to detect the N-terminal T7-tag of the target proteins. Blocking as well as subsequent washing steps and chemiluminescence were performed as mentioned above.

3.4.2. Determination of protein concentration

Table 8: Molecular weights and molecular extinction coefficients necessary for the measurement of the protein concentration by NanoDrop. Listed are all parameters of the different ETA receptor constructs, truncations and of the ETB receptor minus signal sequence (ETB-Sign) with tags. All ligands, that were kindly provided by Dr. Michael Beyermann (FMP, Berlin), are included. Molecular weights and molar extinction coefficients were calculated on the ExPASy Proteomics Server using the Protparam Tool.

Protein/Peptide	Vector	Tag	Number of amino acids	Molecular weight [g/mol]	Molar ε_{280} [M^{-1} cm^{-1}], oxidized state
ETB-Sign cHx	pET21a	N-term T7 C-term H_{10}	441	49790.6	71485
ETA cHx	pET21a	N-term T7 C-term H_{10}	453	51716.5	66110
ETA strep	pET21a	N-term T7 C-term strepII	451	51426.2	77110
ETA197cHx	pET21a	N-term T7 C-term H_{10}	221	24740.5	25815
ETA197strep	pET21a	N-term T7 C-term strepII	219	24450.3	36815
ETA198strep	pET21a	N-term T7 C-term strepII	258	29988.1	40295
ETA107cHx	pET21a	N-term T7 C-term strepII	133	14990.0	11710
ETA107strep	pET21a	N-term T7 C-term strepII	131	14699.8	22710
ET-1	-	-	21	2490.6	7240
ET-3	-	-	21	2641.5	10220
b-ET-1	-	-	21	2716.1	7240
4-Ala-ET-1	-	-	21	2366.2	6990
f-4-Ala-ET-1	-	-	21	2698.5	6990

The concentration of purified proteins was routinely determined via Lambert-Beer's law by correlation of their respective absorptions at 280 nm using a NanoDrop 1000 Spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany) to the known extinction coefficients (ε) and molecular weight of the target protein (Table 8).

3.4.3. Acetone precipitation

Precipitation of protein samples with acetone was used to precipitate and simultaneously concentrate the respective target protein if initial concentrations were to low to be detected by conventional SDS-PAGE or immunoprecipitation.

Final concentrations of 90 % pre-cooled (-20 °C) acetone were added to the respective protein samples, mixed by vortexing and incubated for at least 20 min at -20 °C. Protein / acetone mixtures were then subjected to a cetrifugation step at 18,000 x g and 4 °C for 15 min. Discarding the supernatant had to follow very quickly on ice in order not to loose the precipitated protein. Pellets were subsequently dried at 50 °C for 20 min to completely remove residual acetone and were finally dissolved in desired volumes of water and SDS-PAGE sample buffer, 5-fold.

3.5. Protein purification and analysis of sample homogeneity

3.5.1. Resolubilization of MP precipitates

For resolubilization of P-CF produced precipitates, all tested detergents were supplied at a final concentration of 1 % in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl with addition of 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (PIERCE, Thermo Scientific, Bonn, Germany). P-CF produced MP precipitate was collected by centrifugation at 18,000 x g for 10 min followed by two washing steps in distilled water and resuspension buffer (20 mM Tris-HCl, pH 7.5 and 150 mM NaCl) or liposome buffer (20 mM potassium phosphate, 150 mM NaCl, pH 7.0). The precipitate was subsequently resuspended in resuspension buffer supplemented with 1 % detergent. The volume of resuspension was kept equal to the RM volume of the corresponding reaction. ETA solubilization was achieved by incubation for 2 h at 30 °C with gentle shaking. Residual precipitate was removed by centrifugation at 18,000 x g for 10 min and resuspended in a water volume equivalent to the supernatant for SDS-PAGE analysis. Routinely analysed detergents for resolubilization were LPPG, 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac(1-choline)] (LMPC) (Avanti Polar Lipids, Alabaster, AL), Fos-12, n-hexadecylphosphocholine (Fos-16) (Anatrace, Maumee, OH) and sodium dodecylsulfate (SDS) (Roth, Karlsruhe, Germany).

3.5.2. Anion exchange chromatography: Purification of individually prepared T7RNAP

The purification of the T7RNAP was performed at 16 °C and a flow rate of 2-4 mL/min using a self-packed fast flow Q-sepharose column with an approximate volume of 75 mL and a pressure limit set to 0.3 MPa on an Äkta purifier system (GE Healthcare Europe, München, Germany). The column was pre-equilibrated with 2 column volumes (CV) H_2O followed by 2 CV of

Q sepharose buffer A for T7RNAP. Subsequently, half of the lysate obtained from 1 L expression was loaded onto the column at a flow rate of 2 mL/min by an external pump. Before gradient set-up, buffer A was applied until a stable baseline was reached. Since the T7RNAP elutes at very low salt concentrations, the continuous gradient was set to moderate steepness from 50-500 mM NaCl in 90 min at a flow rate of 4 mL/min using Q-sepharose buffers A and B. The purification was followed by consecutive measurements of conductivity and absorption at 280 nm. To analyse most valuable fractions with high T7RNAP concentration and moderate to low contents in impurities a 12 % SDS-PAGE was performed prior to dialysis and concentration.

It is necessary to note that the T7RNAP does elute widely spread without being highly pure. Best fractions were pooled and dialyzed at 4 °C o. n. against dialysis buffer for T7RNAP. A final concentration of 10 % glycerol was added to the dialyzed protein solution followed by its concentration to 1-4 mg/mL of total protein. The T7RNAP preparation was finally adjusted to 50 % glycerol and stored in 500 µL aliquots for up to one year at -80 °C or at -20 °C for short term storage.

3.5.3. IMAC: Ni – and Co - NTA chromatography

IMAC was applied to purify the poly$(His)_{10}$- as well as poly $(His)_6$-tagged receptors and for the optional exchange of the first detergents used for solubilization against the second detergents used for analysis. Its principle is based on the reversible interaction between consecutive histidine side chains and immobilized metal ions (Porath et al. 1988).

For the purification of the ET receptors in batch mode either Ni^{2+}-NTA (QIAGEN, Hilden, Germany) or self-loaded Co^{2+}-NTA agarose beads were used. The advantage of the cobalt ions over nickel ions is their higher selectivity resulting in a higher purity of the eluted proteins since nickel-based IMAC resins tend to bind to host proteins' exposed hisitine side chains with a higher affinity than cobalt-based resins (Kasher et al. 1993). Therefore, proteins can already be eluted using less stringent conditions that might be beneficial for the stability of the target protein.

Resolubilized protein produced in the P-CF mode or RMs from D-CF reactions were centrifuged at 18,000 x g for 10 min prior to loading onto the IMAC agarose resins to remove residual precipitated proteins. The supernatant containing the detergent-solubilized MP was then diluted 1:5 or 1:10 in IMAC equilibration buffer in order to reduce the initial concentrations of detergent (e.g. starting from 1 % LPPG of resolubilized protein to 0.05 % LPPG) and reducing agents and was subsequently mixed with pre-equilibrated NTA-agarose beads (QIAGEN, Hilden, Germany) loaded with Ni^{2+} or Co^{2+} ions. 300-400 µL resin per 1 mL of diluted RM were employed. The mixture was incubated for 5-12 h at 4 °C on a shaker followed by the application to an empty gravity flow chromatography column. Chromatography was performed

on ice with ice-cold buffers. Bound protein was washed with 10 (CV) wash 1 buffer, followed by a second washing step with 10 CV of wash 2 buffer. The NaCl concentration was reduced to 150 mM by washing in 10 CV of wash 3 buffer to allow for direct application of the protein to ligand-binding studies. Immobilized protein was finally competitively eluted with 350 mM imidazole in IMAC elution buffer and collected in 4-5 fractions of 1 CV each with the first fraction having a volume of 0.5 CV. 10 µL aliquots of each fraction were analysed by 12 % SDS-PAGE.

3.5.4. Streptavidin affinity chromatography

ET full-length receptors in pET21a(+) as well as their truncations harboring a strepII-tag at the C-terminus were purified according to a slightly modified protocol based on the commercially available Strep-tag® Purification kit (IBA GmbH, Göttingen, Germany). As a modification, self-prepared buffers with the desired detergent were used.

The strepII epitope consists of eight consecutive amino acids W, S, H, P, Q, F, E and K and displays intrinsic affinity to streptavidin. The purification was performed by batch binding to pre-equilibrated streptavidin beads in a plastic gravity flow chromatography column. Prior to loading applied protein was treated as mentioned for IMAC purification (**see 3.5.3.**) to dispose residual precipitates. 500 µL resin were used per 1 mL of RM. Protein supernatant was diluted 1:5 in strepII-equilibration buffer in order to reduce the initial concentrations of the detergent and then incubated for 12 h or o. n. at 4 °C on a shaker. The protein was subsequently washed with 5 CV of strepII- wash buffer. The protein was finally competitively eluted in strepII-elution buffer containing 2.5 mM desthiobiotin in three to five fractions of 1 CV each. 10 µL aliquots of each fraction were analysed by SDS-PAGE.

3.5.5. Size exclusion chromatography: A basic method to study homogeneity and stability

SEC with prepacked columns (GE Healthcare Europe, München, Germany) was used in order to assess the homogeneity and the stability of the ET receptor samples produced. Protein preparations were centrifuged at 18,000 x g and 16 °C for 10 min prior to loading. Samples of 10 to 30 µg of purified solubilized or resolubilized receptor were separated on a pre-equilibrated (20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1 % B35 or 0.04 % DDM) analytical Superdex 200 column (3.2 mm/30 cm) (GE Healthcare Europe, München, Germany) at a flowrate of 50 µL/min on an Äkta Purifier station (GE Healthcare Europe, München, Germany). SEC was conducted at 16 °C and the absorption at 280 as well as at 260 nm was monitored during the run.

3.5.6. Multi angle light scattering: A more refined method for the calculation of protein mass and its oligomerization state in detergent

SEC is well suited as basic means to analyse protein homogeneity as well as its stability over time. However, it does not easily allow for the calculation of molecular masses of integral MPs since the amount of detergent associated with the protein in the protein / detergent micelle complex is usually not known. By using multi angle light scattering (MALS) it is possible to determine the absolute molecular mass as well as the oligomerization state of MPs by correlating molecular mass and concentration of the protein to the amount of scattered light. This technique does not take into account the shape of proteins nor does it need initial knowlegde of the amount of detergent bound to the protein (Slootboom et al. 2008).

Combined SEC-light scattering analysis was performed on a Superdex 200 10/300 column (GE Healthcare Europe, München, Germany) at a flow rate of 0.5 mL/min on a Jasco HPLC unit (Jasco Labor- und Datentechnik GmbH, Gross-Umstadt, Germany) connected to a light scattering detector measuring at three angles (mini Dawn Treos, Wyatt Technology Europe GmbH, Dernbach, Germany) and a refractive index detector (Optilab rex, Wyatt Technology Europe GmbH, Dernbach, Germany). The column was equilibrated for at least 16 h with SEC buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 0.005 % Fos-16 or 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % B35, filtered through 0.1 µm pore size VVLP filters (Millipore, Eschborn, Germany). 200 µL of protein samples (0.5-1 mg/mL) were separated on the column. Data analysis was accomplished using the ASTRA software package 5.3.4.13 (Wyatt Technology Europe GmbH, Dernbach, Germany).

3.6. Affinity chromatography: Immobilization as a means to study receptor-receptor interaction and ligand binding competence

3.6.1. Ligand affinity chromatography

Assessing the binding competence of a CF expressed receptor in proteomicelles is an essential tool for further structural studies by X-ray crystallography or liquid state NMR spectroscopy. Ligand-bound receptor tends to display a higher degree of homogeneity and stability, thereby increasing the possibility of one distinct receptor population and minimizing measurement disturbances.

The ligand-binding competent fraction of the CF-produced protein was isolated by binding to peptide ligands immobilized on monomeric avidin agarose beads (PIERCE, Thermo Scientific, Bonn, Germany). The matrix was prepared following the manufacturer's recommendations and subsequently incubated with 100 µg of biotinylated ET-1 or with 30 µg of biotinylated vasopressin per 100 µL (=1 CV) matrix. The peptide loading was conducted for 1 h at 4 °C in matrix buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 5 to 10 x CMC of the

selected detergent). Unbound ligand was removed by a washing step of 3 CV of matrix buffer. Residual bound ligand was estimated to approximately. 13 nmol per 100 µL matrix in case of b-ET-1. A volume of 200 µL of a 2 to 3 µM stock of MP was diluted 1:3 in matrix buffer and subsequently loaded onto the ligand affinity matrix. The loaded total amounts of MP were in between 0.33 and 0.67 nmol, resulting in an excess of bound ligand of 20 to 40-fold. The matrix was then incubated for 2 h at RT with gentle shaking. The flow-through of monomeric avidin agarose loaded with protein was collected and stored for western blot analysis. Unbound protein was removed by 10 washing steps (1 CV each) in matrix buffer. Elution of the ligand bound receptor was finally performed in one step of 0.5 CV followed by further 1 CV steps by releasing the immobilized peptide with 2 mM biotin dissolved in matrix buffer. Aliquots of 32.5 µL from fractions ≤1 CV and of 55 µL of fractions >1 CV were analysed by 12 % SDS-PAGE analysis followed by western blotting. The obtained percentages of ligand bound MP were normalized according to the initially loaded amounts of MP sample.

3.6.2. Analysis of homo- and heterodimerization by affinity chromatography pull-down

To investigate the homo- and heterodimerization potential of CF expressed ET receptors and their truncated derivatives, possessing either poly$(His)_{10}$-tag or strepII-tag, pull-down analysis was performed in batch binding on either co-expressed or separately expressed proteins by streptavidin or Co^{2+}-NTA chromatography. Plastic gravity flow columns were used for this purpose. The pull-down assay is based on the assumption that proteins that do not harbour the necessary tag can be co-eluted nonetheless if they are exhibiting interaction to a protein equipped with the relevant affinity tag.

Samples analysed by pull-down were expressed in analytical scale either in D-CF mode (0.5 % B35) or P-CF mode (resolubilized in 1 % LPMC or LPPG) and volumes of 100 µL co-expressed or 50 µL individually expressed receptor, that were combined to a final volume of 100 µL, were diluted 1:5 in strepII- equilibration buffer and subsequently applied to 70 µL of pre-equilibrated affinity resins. As negative controls (NCs) 50 µL of single receptors with the non-cognate tag were loaded on the resins to rule out any cross reactivity.

After an initial incubation for 2 h at RT with gentle shaking, pull-down samples were transferred to 4 °C and additionally incubated o. n.. For pull-down analyses using the strepII-tag, washing was performed in steps of 3 times 1 CV, followed by two times 2 CV with strepII-wash buffer. Elution was achieved by competition with 2.5 mM desthiobiotin in the elution buffer with volumes of once 0.5 CV, twice 1 CV and once 2 CV. For pull-down studies using the poly$(His)_{10}$ epitope, washing was performed with 10 CV wash 1 buffer, followed by10 CV wash 2 buffer. The NaCl concentration was reduced to 150 mM by washing in 10 CV of wash 3 buffer. Immobilized protein was finally competitively eluted with 350 mM imidazole in IMAC

elution buffer with 0.5 CV for the first fraction and 1 CV for the five subsequent fractions. All fractions were subjected to precipitation with acetone and analysed by immunoblotting or SDS-PAGE.

3.7. Spectroscopical and spectrometrical methods

3.7.1. Far-UV spectroscopy: Secondary structure analysis and thermostability

ETA samples to be measured by circular dicroism (CD) spectroscopy were purified by IMAC (**see 3.5.3.**) using suitable detergents. Peak elution fractions were pooled to give a concentration in between 0.5 to 0.7 mg/mL and they were subsequently dialysed at 4 °C against 10 mM sodium phosphate buffer, pH 7.5 (CD-buffer), containing the appropriate detergent. CD spectrometry and melting curves were measured with a Jasco J-180 spectropolarimeter (Jasco Labortechnik, Gross-Umstadt, Germany) in 10 mM CD-buffer. Experiments were carried out at standard sensitivity with a band width of 1 nm and a response of 1 s. The data pitch was set to 0.1 nm and a scanning speed of 100 nm/min was used. CD spectra were recorded from 190 to 260 nm at 20 °C in a cuvette of 1 mm cell length. The data represent an average of three accumulations and were baseline corrected by subtraction of a buffer spectrum recorded under identical conditions. Melting curves were measured with a band width of 1 nm, a response of 1 s and a data pitch set to 0.1 °C at a wavelength of 222 nm with a slope of 2 °C/min from 4 °C to 90 °C.

3.7.2. Ligand binding of detergent solubilized ETA monitored by fluorescence anisotropy

Fluorescence anisotropy measurements with detergent-solubilized ETA were performed using a Jasco spectrofluorometer FP-6500 (Jasco Labortechnik, Gross-Umstadt, Germany). ETA was expressed in the P-CF mode, resolubilized in 1 % LPPG and subsequently purified in 0.1 % B35. Residual imidazole was removed by over night dialysis against assay buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % B35). Protein samples purified by monomeric avidin matrix bound b-ET-1 ligand were measured without dialysis to retain the receptor-ligand complex. Ligand binding analysis was carried out with the fluorescein-labelled linear derivative 1,3,11,15-Ala-ET-1 (f-4-Ala-ET-1). Excitation and emission maxima of the f-4-Ala-ET-1 have been determined at 498 nm and 520 nm, respectively. The G-factor of the ligand was measured in assay buffer with a value of 1.50. For saturation binding, 30 nM of f-4-Ala-ET-1 was incubated with increasing concentrations of free (0-5 µM) or ligand-bound ETA-receptor (0-1 µM) for 1.5 h at RT in a final volume of 500 µL. Anisotropy was determined as mean value of seven repetitions per data point. The observed anisotropy signal was normalized by subtraction of the anisotropy values of 0.038 gained from free f-4-Ala-ET-1 in assay buffer.

Data were processed by non-linear regression curve fitting using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA).

3.7.3. Liquid-state NMR

CF expression of MPs offers a high potential for their structural analysis by NMR spectroscopy. The set-up is ideally suited for rapid screens of optimal expression and resolubilization conditions and highly concentrated protein samples can be obtained in less than 24 h. In order to study protein structure and dynamics by NMR spectroscopy, the backbone of a protein must first be assigned. However, the often large size of MPs results in slow rotational tumbling and concomitant broad linewidth. This problem is aggravated by the fact that MPs have to be solubilized in micelles, which contribute to the overall molecular weight. Furthermore, α-helical proteins tend to display narrower chemical shift dispersions, resulting in peak overlap, and the TM sections of these proteins consist predominantly of hydrophobic amino acids often leading to clustering of identical amino acids with very similar chemical shifts (Junge et al. 2010a).

The first TMS of the ETB receptor was analysed with respect to possible interaction and titration studies with unlabelled ET-1 as well as for comparison with split intein constructs (**see 3.11.**). Therefore, [^{15}N^{1}H]-TROSY HSQC of ETB-TMS1 were recorded in cooperation with Dr. Frank Löhr (Institute of Biophysical Chemistry, Goethe University, Frankfurt/Main) as reference spectra at 800 MHz and 303 K. The protein was uniformly ^{15}N- labelled by CF expression in the P-CF mode. For NMR measurements the same conditions were chosen as found for successful cleavage of ETB inteins. The pellet was washed two times in water and was finally resolubilized for 2 h at 32 °C in 2 % Fos-12, 50 mM Tris pH 7.0, 1 mM EDTA and 10 mM TCEP.

3.7.4. Mass spectrometry: Peptide mass fingerprinting (PMF)

Mass spectrometry of CF-expressed ETA was performed in cooperation with Dr. Julian Langer (group of Prof. Michel, Department of Molecular Membrane Biology, Max-Planck Institute of Biophysics, Frankfurt/Main).

Identification of SDS-PAGE separated ETA full-length receptor and its supposed dimer was performed on reduced and alkylated, trypsin- -digested samples prepared by standard mass spectrometry protocols (Proteoextract Digestion kit, CalBiochem).

Proteolytic digests were loaded (Proxeon easy-nLC) on reverse phase columns (trapping column: particle size 5 µm, C18, L=20mm; analytical column: particle size 5 µm, C18, L=10 cm; NanoSeparations, Nieuwkoop, The Netherlands) using a nano-HPLC, and eluted in gradients of water (0.1 % formic acid, buffer A) and acetonitrile (0.1 % formic acid, buffer B). Peptides eluting from the column were ionised online via a Bruker Apollo ESI-source with a

nanoSprayer emitter and analysed in a quadrupole time-of-flight mass spectrometer (Bruker micrOTOF-Q-II and Bruker maXis). Mass spectra were acquired over the mass range of 50-2200m/z, and sequence information was acquired by computer-controlled, data-dependent automated switching to MS/MS using collision energies based on mass and charge state of the candidate ions.

The data sets were processed by means of a standard proteomics script with the software Bruker DataAnalysis 4.0 Service Pack 1 Build 253 and exported as mascot generic files. Proteins were identified by matching the derived mass lists against the NCBInr database on a local Mascot server (Version 2.2.2, Matrix Science, UK). These data and the results from re-sequencing the open reading frames were analysed and validated using the BioTools 3.1 software package (build 2.22, Bruker Daltonics).

3.8. Electron microscopy: Assessing sample dispersity and reconstitution efficiency

3.8.1. Negative staining electron microscopy

The negative staining EM micrographs were provided by André Krüger from the group of Prof. Engel (Department of Structural Biology, M.E. Müller Institute for Microscopy, Biocenter, University of Basel, Switzerland).

Aliquots of 5 µL of different concentrations of purified ETA receptor preparations were adsorbed to glow-discharged 200 mesh carbon-film coated copper grids, washed with water and negatively stained with 2 % (w/v) uranyl acetate. Images were recorded using a Philips CM10 transmission electron microscope (Philips, Eindhoven, The Netherlands) operated at an acceleration voltage of 80 kV. Negatives were recorded and digitized on a 2000 x 2000 pixel charge coupled device camera (Veleta, Olympus Soft Imaging Solutions, Münster, Germany).

3.8.2. Freeze-fracture electron microscopy

The freeze-fracture EM micrographs were kindly performed by Friederike Joos (group of Prof. Kühlbrandt, Department of Structural Biology, MPI of Biophysics, Frankfurt/Main. Proteoliposomes in sample holders were frozen in ethane precooled to -180 °C under liquid nitrogen. The fracturing at -120 °C and replication at a shadowing angle of 45° with platinum/carbon was performed with a BAF 060 freeze-fracturing unit from Bal-Tec Inc. (Principality of Liechtenstein). The enlargement of the respective fracture images were achieved from a x 16k-x 25k primary magnification to a final magnification of x 116,800-x 194,500 and digitized in a 1024 x 1024 pixel format.

3.9. Analysing the functionality of cell-free produced ET receptors

3.9.1. Peptide labelling of the linear mutant 4-Ala-ET-1 with radioactive ^{125} iodine

For radioactive binding assays of the ET receptors with the mutant ET-1 ligand the peptide was labelled chemically with ^{125} iodine at its tryrosine residue 13 by a redox reaction mediated by the oxidizing agent chloramin T and the corresponding reducing agent sodium–metabisulfite (Hunter and Greenwood 1962).

17 µg of 4-Ala-ET-1 (Dr. Michael Beyermann, FMP, Berlin) in 1-fold PBS buffer and DMSO, pH 7.0 were gently mixed with 10 µL [^{125}I]NaI (1mCi) and 10 µL of chloramine T (1 mg/mL, in PBS) followed by an incubation of 5 min at RT. The reaction was terminated by the addition of 10 µL of sodium-metabisulfite solution (0.67 mg/mL, in PBS) and bound radioactivity was immediately separated by gel filtration on Sephadex G-10 spin columns with 1 mL bed volume, pre-equilibrated in PBS buffer. The whole reaction volume (approximately 60 µL) was subsequently loaded onto the column and the elution of the radioactively labelled ligand was performed in eight steps of 50 µL of PBS buffer, each followed by centrifugation at 500 x g for 10 s. 2 µL of each elution were measured on a gamma counter (Cobra II Auto gamma, PerkinElmer, Rodgau-Jügesheim, Germany) and fractions displaying highest counts were pooled. The radioactive ligand was finally adjusted to a total concentration of 10 µM by addition of cold ligand and stored at -20 °C until further use.

3.9.2. Following traditional tracks: Post-translational reconstitution of P-CF produced ETA and ETB receptors

Reconstitution of proteoliposomes was done with P-CF produced MP samples directly after resolubilization with 1 % LPPG in liposome reconstitution buffer at a concentration of approximately. 1.5 mg/mL. *E. coli* polar lipids and cholesterol at a ratio of 20:1 (w/w) were solubilized in 100 % chloroform, evaporated under vacuum to a thin film using a rotary evaporator and completely dried under vacuum over night. The film was resuspended in liposome reconstitution buffer to a concentration of 20 mg/mL, rotated and vortexed until a homogenous suspension was obtained. Finally, they were extruded to a size of 0.2 µm (Mini-Extruder, Avanti Polar Lipids Inc., USA). Solubilized protein samples were subsequently mixed at molar ratios of 1:350 or 1:1,000 with the prepared *E. coli* polar liposomes supplemented with 5 % cholesterol. The reconstitution mixture was incubated for 1 h at 30 °C. Detergent was removed by addition of 500 mg Biobeads SM-2 (Bio-Rad, München, Germany) pre-equilibrated with liposome buffer. After gentle agitation for 1 h at RT, Biobeads were refreshed and the mixture was further incubated for up to 48 h at 18 °C. Proteoliposomes were finally separated from empty liposomes in a discontinuous sucrose gradient of 10-20-30-40 % sucrose in liposome buffer by ultracentrifugation at 83,000 x g (SW28 swing bucket rotor, Beckman

Coulter, Krefeld, Germany) for 16 h at 4 °C. Proteoliposome layers were harvested and sucrose was removed by two subsequent washes with liposome buffer by centrifugation at 195,000 x g (rotor Type 70Ti, Beckman Coulter, Krefeld Germany) for 1 h at 4 °C. The pelleted proteoliposomes were resuspended in liposome buffer, shock frozen in liquid nitrogen and stored at -80 °C for functional analysis or freeze-fracture EM (**see 3.8.2.**).

Optionally, the P-CF expressed ETA receptor was purified in presence of 2 mM of the fluorinated surfactants F_6-TAC or F_8-TAC and subsequently reconstituted either into *E. coli* polar lipids/cholesterol or in liposomes of aso-PC at a molar protein-to-lipid ratio of 1:1,500.

3.9.3. Radioligand binding assay: Analysis of ET receptor proteoliposomes

Sucrose gradient purified and pelleted proteoliposomes of ETA were resuspended and concentrated in liposome reconstitution buffer. For competition studies, suitable aliquots of proteoliposomes (approximately. 6 µg protein/mL) were incubated in 96-well microplates with 500 pM [^{125}I]Tyr^{13} ET-1 (specific activity 2200 Ci/mmol) (PerkinElmer, Rodgau-Jügesheim, Germany) in the presence of unlabelled ET-1 (10^{-10}–10^{-4} M) in a final volume of 50 µL of binding buffer (20 mM potassium phosphate, 150 mM NaCl, 0.1 % (w/v) bovine serum albumine, pH 7.0) for 1 h at RT. Competition was stopped by addition of 100 µL ice-cold binding buffer to the reaction and samples were transferred to GF/B glass fibre filters (Millipore, Eschborn, Germany) that were pre-treated with 0.3 % polyethyleneimine (PEI) for 10 min and washed with 500 µL of binding buffer before use. Unbound ligand was removed by washing the filters 3 times with 150 µL of binding buffer. Dry filters were finally collected and the retained radioactivity was determined in a gamma counter (Cobra II Auto gamma, PerkinElmer, Rodgau-Jügesheim, Germany). Nonspecific binding was determined by preincubation of the proteoliposomes with 4 µM of unlabelled ET-1. Data were analysed by non-linear regression curve fitting using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA) and represent the mean value of three independent experiments.

3.9.4. Centrifugation-assisted gel filtration: Ligand binding competence of detergent solubilized ET-receptors

Saturation binding of individually labelled ^{125}I-4-Ala-ET-1 to soluble ET receptors was assayed by centrifugation-assisted gel filtration (Grisshammer et al. 1999).

Approximately 50 nM of D-CF expressed ETA receptor purified in 0.04 % DDM were incubated at RT for 1 h with increasing concentrations of labelled ligand ranging from 0 to 2 µM in a total volume of 25 µL in assay buffer (20 mM Tris-HCl, 150 mM NaCl, 0.04 % DDM and 0.01 % CHS). Non-specific binding was determined in presence of a 100-fold excess of unlabelled 4-Ala-ET-1. Separation of bound from free ligand was achieved by gel filtration on 1 mL columns (illustra MicroSpin G-50 columns, GE Healthcare, Europe, München,

Germany) of Sephadex G-50 matrix material. Columns were pre-equilibrated in assay buffer by three consecutive centrifugation steps of 400 µL buffer at 2,000 x g and 4 °C for 1 min each. Aliquots of 16 µL of the total reaction mixture were loaded onto the columns. Receptor and bound ligand were collected in the void volume by centrifugation at 740 x g and 4 °C for 3 min. The amount of bound radioactive ligand was analysed by gamma counting (Cobra II Auto gamma, PerkinElmer, Rodgau-Jügesheim, Germany). All data were analysed by non-linear regression curve fitting using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA).

3.9.5. Surface Plasmon Resonance measurements with detergent-solubilized ETB receptor

SPR measurements of the ETB receptor either CF-expressed in *E. coli* or in WGE were kindly carried out by the group of Dr. Michael Beyermann (FMP, Berlin) using a Biacore 2000 instrument (Biacore Intl. AB, Uppsala, Sweden). CM-5 chips (research grade, Biacore AB) were coated with ET-1 by amide coupling at different response units (RU) ranging from 700 to 1400 RU depending on the respective experiments. The control peptides urocortin and sauvagine were coupled to the chips via available cysteine residues. Subsequent interaction studies with ETB at dilutions of 2-120 µg/mL were performed in running buffer (20 mM Tris, 100mM NaCl, 0.005 % P-20 surfactant, pH 8.0) at 25 °C at a flow rate of 2 µL/min. Unspecific binding was subtracted using blank runs without ET-1 bound to the chip surface. Kinetic constants were calculated using BIAevaluation software 3.1 (Biacore).

3.10. Automated robotic screening of buffer conditions for X-ray crystallography

Under defined conditions proteins can be forced to crystallize. Undersaturated solutions of homogenous, stable, purified protein are supersaturated by the addition of protein precipitating agents like salts and polyethylene glycols. Precipitation and aggregation as a result of optimal conditions in this unstable supersaturated state might lead to the alignment of individual protein molecules in a repeating series of “unit cells” by adopting uniform orientations. Non-covalent interactions stabilize the crystal lattice. Crystallization of a protein serves as basis for the determination of its three-dimensional structure by X-ray diffraction. A variety of conditions and combinations have to be screened initially in order to find the optimal parameters for the crystallization of the respective target protein.

In cooperation with Dr. Yvonne Thielmann (group of Prof. Michel, Department of Molecular Membrane Biology, Max-Planck Institute of Biophysics, Frankfurt/Main) initial sitting drop crystallization of classical vapour diffusion as well as cubic phase series on the 96-well format robotic platform “CrystalMation” (Rigaku, Berlin, Germany) were performed on the ETA receptor. The advantage of automation on CrystalMation clearly resides in a very fast

experimental set-up combined with small sample sizes of 100 nL of concentrated protein solution overlaid with 100 nL buffer volumes.

ETA was therefore P-CF expressed, resolubilized in 1 % LPPG and exchanged by IMAC purification to 0.1 % B35 in 20 mM Tris and 150 mM NaCl. A protein purity of estimatedly >95 % was achieved. Gel filtration was not performed as second purification step since protein homogeneity was judged to be sufficient for initial trials. The purified protein was subsequently concentrated with or without addition of equal moles of ET-1 ligand to final volumes of 150 µL of around 5-8 mg/mL using an ultrafiltration device with a MWCO of 10 kDa (Millipore GmbH, Eschborn, Germany). Initial experiments designed for soluble proteins or MPs were performed in sitting drop with commercial as well as custom MPI screens available from the database of the CrystalTrak software (**see 7.5.**). Incubation within the robotic unit for the vapour diffusion plates was performed at 4 °C and 18 °C. Cubic phase plates were incubated externally at 22 °C. Imaging was carried out automatically twelve times at fixed intervals within a time window of three months.

3.11. Self-cleavage of ETB split intein constructs

The intein self-cleavage / fusion reactions of the ETB split intein constructs pVSO 4.1 and pVSO 4.2 (**see 3.1.1.**) were adapted to the CF reaction and modified from Hong et al. 2008. The intein constructs were separately expressed in P-CF mode, centrifuged at 18,000 x g and 4 °C for 10 min and resolubilized in 2 % LPPG, Fos-12, Triton X-100 or diC_6PC (solubilized in cleavage buffer) for 1 h 30 min at 30 °C. Resolubilization efficiency was analysed by western blotting of 1.5 µL of protein sample. The protein products further termed ETB 4.1 and ETB 4.2 were subsequently mixed 1:1 (v/v) in a final reaction volume of 30 µL. The mixture was incubated at different temperatures (16 °C, 23 °C and 40 °C) and samples were analysed for their self-cleavage efficiency after 1, 2, 4, 18 and 42 h. Therefore, 2.5 µL of collected samples were loaded on 12 % Tris-Tricine-SDS gels and subjected to western blotting (**see 3.4.1.**).

Alternatively, either ETB 4.1 or ETB 4.2 were CF expressed in presence of 125 µCi L-[^{35}S]- methionine (RM) and 40 µCi in the FM and cleavage was followed on 12 % Tris-Tricine-SDS autoradiographs after 45 min, 1, 3 and 65 h at 23 °C. Therefore, gels were dried for 1 h 30 min at 80 °C and finally incubated on phosphoimager plates from o. n. to 24 h.

4. Results

4.1. Cell-free expression optimization of the human endothelin A receptor

4.1.1. Basic parameters for the expression of ETA in the E. coli CECF system analysed in the P-CF mode

The coding sequence of the human ETA receptor was successfully cloned into the expression vector pET21a(+)poly(His)$_{10}$ (**see 3.1.1.**). Full-length protein synthesis resulted in the protein construct T7-ETA-poly(His)$_{10}$, further abbreviated ETA or ETAcHx, (453 aa, 51.72 kDa) (Fig. 9). The abbreviation cHx for poly(His)$_{10}$-tagged receptors simplifies their differentiation from the strep-tagged variants. The N-terminal T7-tag was chosen as expression enhancer as it already proved highly beneficial especially for the expression of GPCRs since almost no expression was observed without this tag for the porcine GPCR vasopressin 2 receptor (V2R) as well as for three other GPCRs (Klammt et al. 2005). Addition of the poly(His)$_{10}$-tag should ensure efficient IMAC purification. The strepII-tag, resulting upon expression of the protein construct T7-ETA-strep, abbreviated ETAstrep, was chosen for purification by streptavidin matrix and for homo- and heterodimerization pull-down experiments (Fig. 9). A list with all used ET constructs is available in **3.4.2.**.

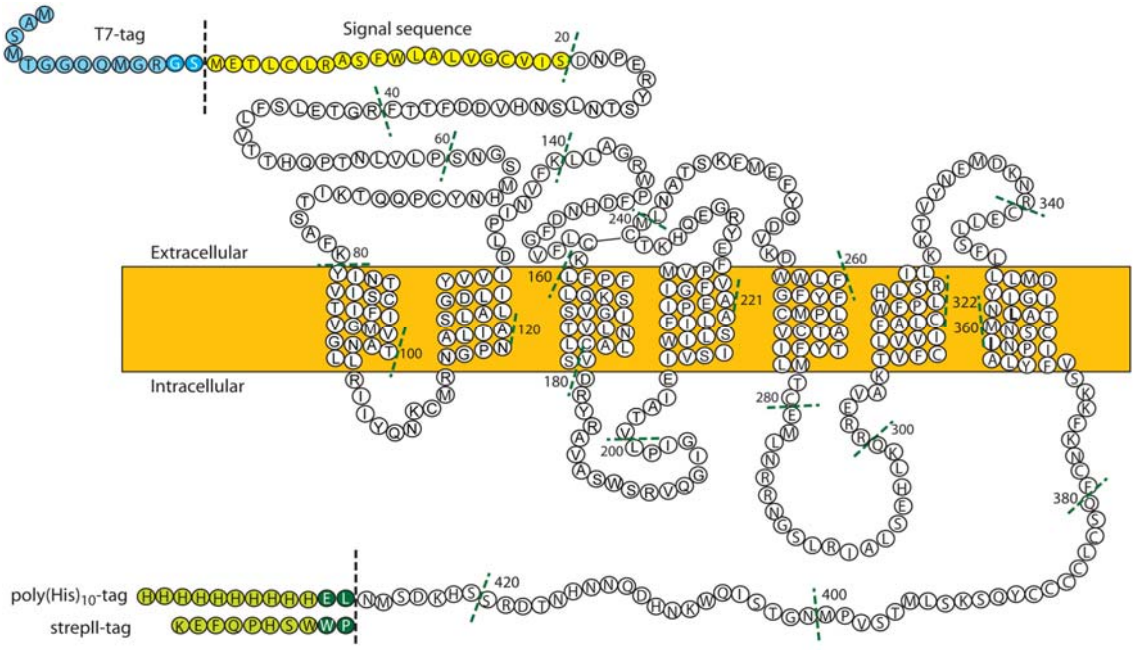


Fig. 9: Predicted secondary structure of the human ETA receptor. Highlighted in light blue and green are the N-terminal T7-tag as expression enhancing element and the C-terminal purification sequences poly(His)$_{10}$-tag or strepII-tag, respectively. Restriction site inserted amino acids are visualised by dark blue and green circles written in white. The internal signal sequence is depicted in yellow.

All three terminal extensions were furthermore used as immunotags for quantification and verification of the expression success. Expression of ETA was first performed by P-CF

synthesis using the optimized protocol for the standard control protein GFP. Production of ETA was subsequently analysed either by SDS-PAGE followed by Coomassie staining or immunodetection with poly(His)$_6$-tag or T7-tag antibodies.

Without addition of detergent, the ETA receptor precipitated quantitatively. After centrifugation of the RM at 18,000 x g and 4 °C for 10 min the whole synthesized protein was harvested in the pellet fraction of which 3 µL and 1.5 µL were loaded on SDS-PAGEs for either Coomassie or immunostaining, respectively (Fig. 10). ETAcHx or ETAstrep constructs were detected in 12 % SDS-gels at an apparent mass of approximately 40 kDa running faster than expected (Fig. 10 A) which might be explained by incomplete denaturation as already seen for the V2R as well as for many other MPs (Klammt et al. 2005). To investigate their full-length expression and to exclude the possibility of premature termination, ETA samples were subjected to immunodetection of both termini in parallel. Full-length synthesis could be verified from both ends for ETAcHx (Fig.10 B) as well as for ETAstrep (data not shown).

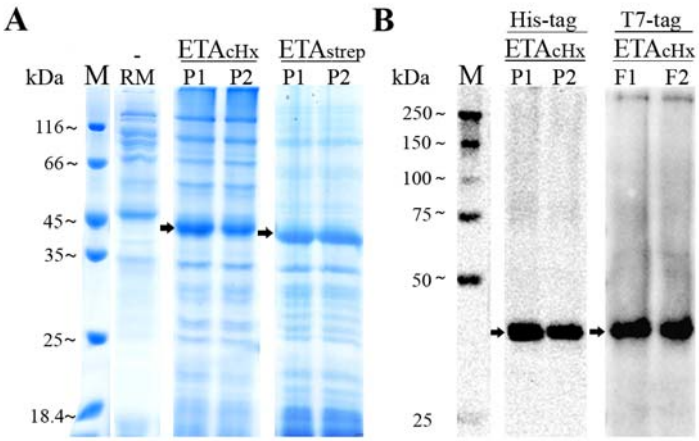


Fig. 10: Verification of ETA construct expression and full-length synthesis of ETAcHx. (A) Sample volumes of 3 µL of P-CF expressed ETAcHx or ETAstrep were loaded on 12 % SDS-Tris-glycine gels and subsequently stained with Coomassie Blue. (B) 1.5 µL of resuspended protein pellet or detergent-solubilized ETAcHx were separated by 12 % SDS-Tris-glycine PAGE and immunodetected by either His- or strep-tag specific antibodies. Arrows indicate ETA bands. M, marker proteins; P, pellet; F, soluble fraction; cHx, poly(His)$_{10}$-tag.

CECF reactions commonly display a very defined optimum for Mg^{2+} and K^+ ions which usually is in between 15-23 mM and 250-300 mM, respectively. This optimum is highly batch-dependent and individual to every target MP. Especially Mg^{2+} ensures highest yields if optimized in CECF systems.

Therefore, optimal concentrations of Mg^{2+} and K^+ ions for every S30 extract batch were analysed in analytical scale reactions in the P-CF mode. In case of ETA, optimal concentrations were found in between 16–22 mM Mg^{2+} and 270–300 mM K^+. Depending on the extract batch Mg^{2+} optima might be broader or more distinct (Fig. 11). The concentrations of other reaction components were optimized and finally kept constant for all further experiments with ETA as illustrated in Table 9.

Table 9: CF expression protocol for the optimized expression of ETA in the CECF configuration.

		Concentration			
Compound [unit]	**Stock**	**Final (RM)**	**Final (FM)**	**Supplier[b]**	**/Ref.**
NaN_3 [%]		10	0.05	0.05	A
PEG 8000 [%]		40	2	2	A
KOAc [mM]		4000	110[a]	110[a]	A
$Mg(OAc)_2$ [mM]		1000	11.1[a]	11.1[a]	A
HEPES buffer, pH 8.0 [M]		24	1	1	A
Complete [x]		50	1	1	B
Folinic acid [mg/mL]		10	0.1	0.1	C
DTT [mM]		500	2	2	A
NTP-mix [x]:		75	1	1	
ATP [mM]		360	4.8	4.8	B
UTP [mM]		240	3.2	3.2	C
GTP [mM]		240	3.2	3.2	C
CTP [mM]		240	3.2	3.2	C
PEP [mM]		1000	20	20	C
AcP [mM]		1000	20	20	C
AA-mix [mM]		4	0.6	1.2	C
RCWMDE-mix [mM]		16.7	1	1	C
S30 C buffer [%][c]		100	-	35	(Schwarz et al. 2007a)
Pyruvat kinase [mg/mL]		10	0.04	-	B
Plasmid DNA [mg/mL]		0.2	0.03	-	-
RiboLock™ [U/µL]		40	0.3	-	D
T7RNAP [mg/mL]		3.8	0.05	-	(Schwarz et al. 2007a)
tRNA *E. coli* [mg/mL]		40	0.5	-	B
S30 extract [%]		100	35	-	(Schwarz et al. 2007a)

[a], Value for reactions with 270 mM K^+ and 16 mM Mg^{2+} final concentration
[b], A, Carl Roth GmbH & Co. KG, Karlsruhe, Germany; B, Roche Diagnostics, Penzberg, Germany; C, Sigma-Aldrich, Steinheim, Germany; D, MBI Fermentas, St. Leon-Rot, Germany
[c], S30 C buffer (10 mM Tris-acetate, 14 mM $Mg(OAc)_2$, 0.6 KOAc, 0.5 mM DTT, pH 8.2), prepared according to (Schwarz et al. 2007a)

Using the optimized basic protocol with a good quality of the prepared S30 extract batch, expression rates in between 1-3 mg ETA per mL RM could be obtained in the P-CF mode.

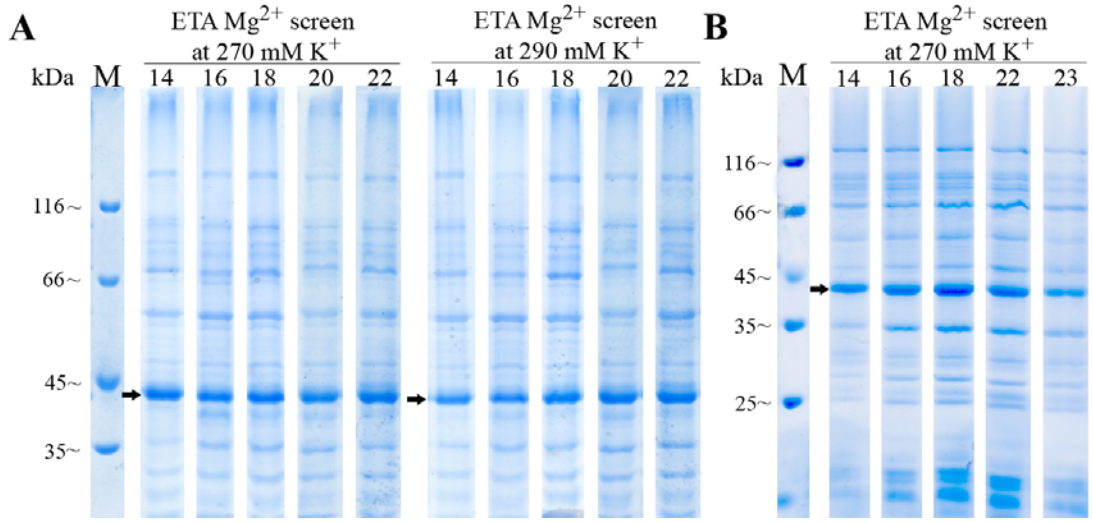


Fig. 11: Mg^{2+} and K^{+} optimization screen in the CECF configuration for two different S30 extract batches. Protein samples of 3 µL volume each of analytical scale P-CF reactions were loaded on 12 % SDS-Tris-glycine gels and stained with Coomassie Blue. Arrows point at ETA receptor bands. (A) Analysed Mg^{2+} concentrations in dependence of K^{+} ions kept in a concentration range of 270-290 mM. (B) Different extract batch, displaying a more distinct optimum for Mg^{2+} than (A). M, marker proteins.

4.1.2. Evaluation of detergents for co- and post-translational solubilization of ETA

The co-translational solubilization in the D-CF mode as well as its post-translational solubilization starting from P-CF mode were analysed for ETAcHx. Specific sets of detergent families which are either frequently used for MP solubilization out of membranes (e.g. DDM, Triton X-100, digitonin) or which already proved efficient especially for the expression of GPCRs (Brij derivatives, digitonin) (Klammt et al. 2005) were investigated in each mode (Fig. 12).

ETA precipitates produced in the P-CF mode in analytical reaction volumes were recovered by centrifugation at 18,000 x g and 4 °C for 10 min and subsequently washed twice, once with water, once in resuspension buffer (**see 3.5.1.**), to reduce the remaining impurities of *E. coli* extract proteins which co-precipitate with the target protein. ETA could completely be solubilized in 1 % final concentration of either the long-chain phosphoglycerol LPPG, the long-chain phosphocholine LMPC, SDS or Fos-16. Fos-12 in the same concentration was less effective and solubilized only estimated 10 % of ETA (Fig. 12 A). Even 5 % DDM did not resolubilize ETA at all. The harsh denaturing agent SDS was used as a standard for complete resolubilization. As already shown for ETB and V2R (Klammt et al. 2005, 2007b) the relatively mild long-chain phosphoglycerols like LMPG or LPPG appear to be highly efficient for complete solubilization of GPCRs including ETA.

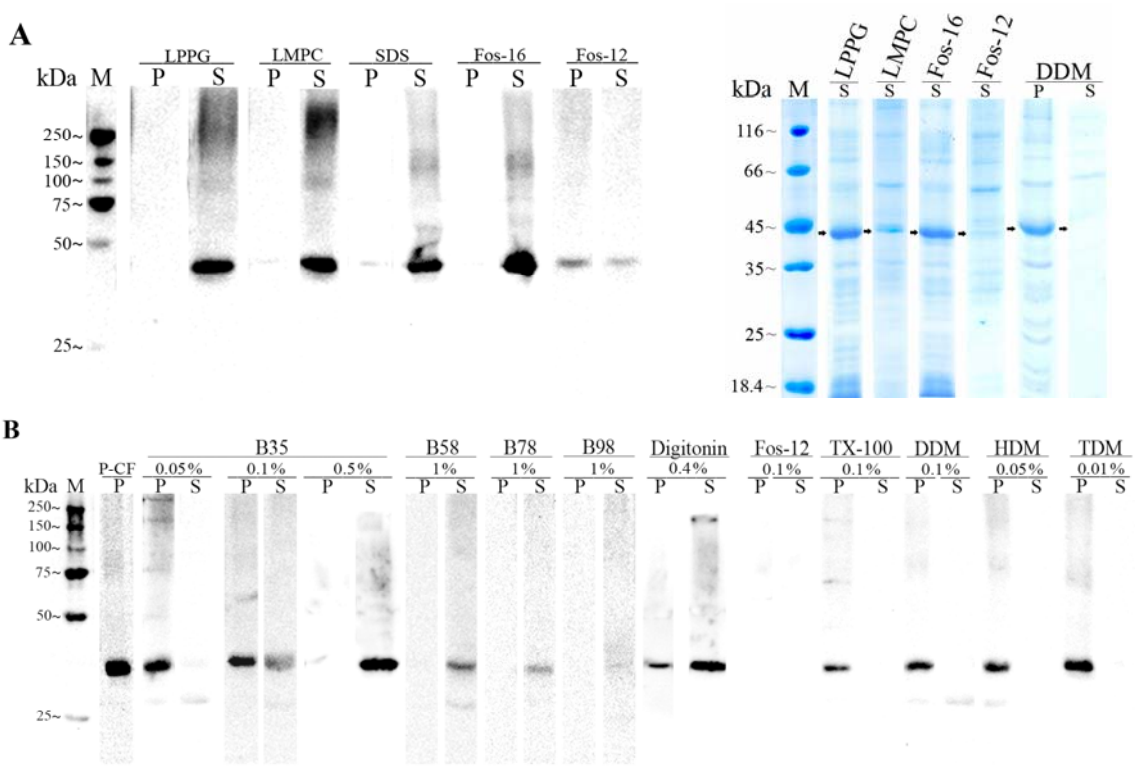


Fig. 12: Solubilization screen of CF expressed ETA. Sample volumes of 1-1.5 µL (Western Blot) and 3 µL (Coomassie) were loaded on 12 % SDS–Tris–glycine gels and subsequently immunodetected by anti-His-tag antibodies or stained by Coomassie Blue. (A) Resolubilization of P-CF produced ETA precipitates. All detergents were supplied in 1 % (w/v). (B) Detergent screen for the D-CF expression of ETA (partially adapted from the diploma thesis of Laura Luh under supervision of Friederike Junge, Institute of Biophysical Chemistry, Goethe University, Frankfurt/Main). The protein was synthesized in presence of various detergents at the indicated final concentrations (w/v). M, marker proteins; P, pellet; S, supernatant.

D-CF expression offers an alternative approach to the post-translational solubilization of MPs as it directly enables the production of soluble MP targets during protein synthesis. If protein quality of samples derived from post-translational solubilization, in which yields are usually higher, is not efficient, the operator can switch to the D-CF mode. For the evaluation of the adequate hydrophobic environment, different detergent families were tested in analytical scale reactions with respect to their effects on the overall CF production efficiencies and their solubilization capacity of ETA as well as with respect to their use in functional and structural approaches. Especially Brij derivatives and digitonin were promising as they were already used in the functional production of GPCRs as shown for the ETB receptor (Klammt et al. 2007b) and human olfactory receptors (Kaiser et al. 2008). All detergents were tested above their specific CMC.

In the D-CF screen highest yields of the ETA receptor of around 1 mg/mL RM were only achieved with polyoxyethylene derivatives (Brijs). B35 at final concentrations of 0.5 % corresponding to approximately 46 x CMC appeared to be most effective in the complete solubilization of the synthesized ETA. The related detergents B58 and B78 at final

concentrations of 1 % also resulted in complete ETA solubilization. However, these detergents appeared to reduce the expression efficiencies significantly (Fig. 12 B).

Table 10. Efficiencies of CF expression protocols for the production of solubilized ETA.

CF mode	1st Detergent	(%)	Solubilization[a] (%)	2nd Detergent (%)		Yield[b]	
P-CF	-		-	-		+++	(E)
	LPPG	1	100	-		+++	(S)
	LPPG	1	100	B35	0.1	+++	(P)
	LPPG	1	100	DDM	0.04	+/-	(P)
	LPPG	1	100	LPPG	0.05	+++	(P)
	LPPG	1	100	Fos-12	0.1	++	(P)
	LPPG	1	100	Fos-16	0.005	+++	(P)
	LPPG	1	100	F_6-TAC	0.35	+++	(P)
	LPPG	1	100	F_8-TAC	0.37	++	(P)
	LPPG	1	100	CHAPS	1	-	(P)
	LPPG	1	100	DHPC	1	-	(P)
	LPMC	1	100	-		+++	(S)
	SDS	1	100	-		+++	(S)
	Fos-16	1	100	-		+++	(S)
	Fos-12	1	30	-		++	(S)
D-CF	B35	0.5	100	-		+++	(E)
	B35	0.5	100	B35	0.1	++	(P)
	B35	0.5	100	DDM	0.04	+	(P)
	B58	1	100	-		+	(E)
	B78	1	100	-		+	(E)
	B98	1	-	-		-	(E)
	Digitonin	0.4	60	-		++	(E)
	Fos-12	0.1	-	-		-	(E)
	TX-100	0.1	-	-		+/-	(E)
	DDM	0.1	-	-		+	(E)
	HDM	0.05	-	-		+	(E)
	TDM	0.01	-	-		++	(E)

[a], solubilization efficiency in the 1st detergent judged by immunodetection.
[b], E, expression yield; S, yield after solubilization; P, yield after IMAC purification.

Thus, the number of polyoxyethylene moities as well as the chain length of the alkyl group seemed to be an important parameter for the efficiency of soluble ETA expression. The shortest of the investigated long chain Brij derivatives -possessing only a lauryl moiety ($C_{12}H_{20}$)- displayed best solubilization effects without any reduction in CF protein production yield. The steroid detergent digitonin at 0.4 % final concentration solubilized approximately 60 % of the

produced ETA. Increased digitonin concentrations however inhibited the expression system (Kaiser et al. 2008, Klammt et al. 2007b).

The alkyl glycoside detergents DDM, HDM and TDM as well as TX-100 were ineffective in ETA solubilization, although no inhibitory effect on the expression level could be observed. In contrast, addition of 0.1 % Fos-12 or 1 % B98 completely inhibited ETA production (Fig. 12 B). In summary, a list of detergents suitable for the production of soluble ETA in the D-CF mode (B35 > digitonin > B58, B78) as well as in the P-CF mode (LPPG, LMPC, SDS, Fos-16 > Fos-12) could be defined as basis for subsequent quality control approaches and their efficiencies are given in Table 10.

4.2. Quality control of cell-free expressed human ETA

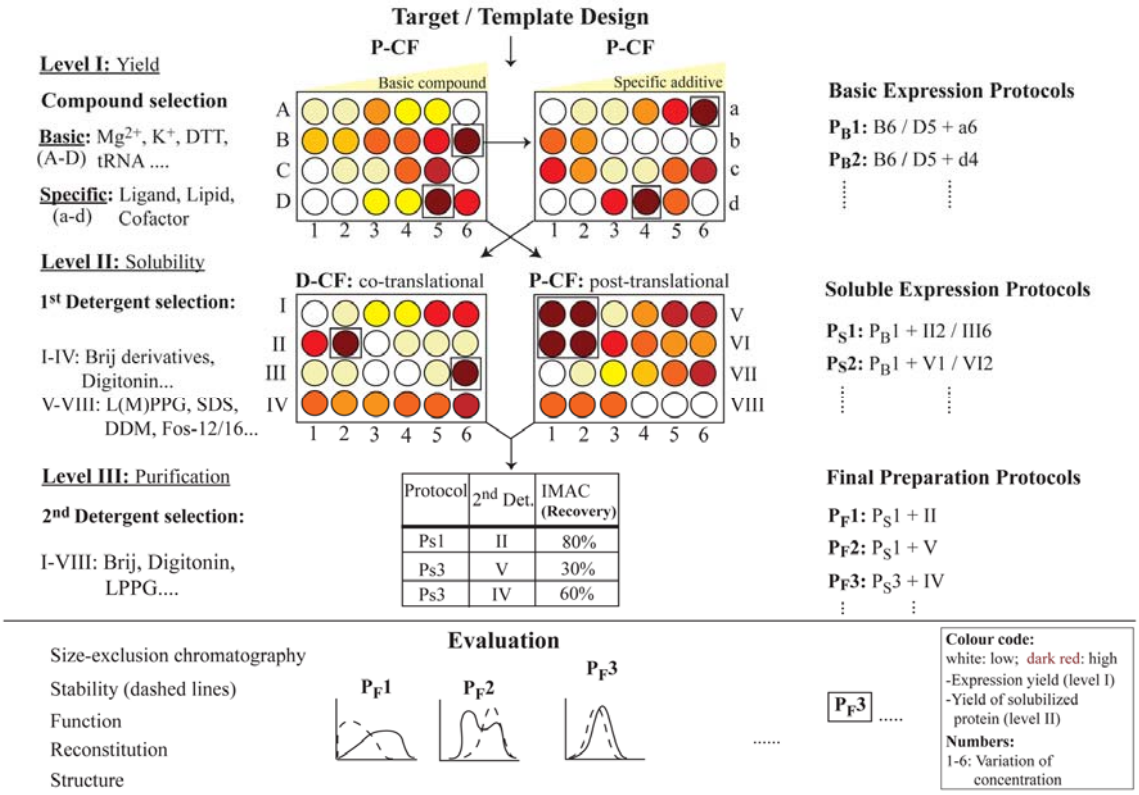


Fig. 13: Schematic flow-chart of protocol development for the CF expression of MPs. After successful template and target design, the expression screening can be organized into the three illustrated levels focussing on protein yield (level I), protein solubility (level II) and protein purity (level III). The obtained list of determined final preparation protocols provides the basis for subsequent quality evaluation screens implementing complementary techniques.

Integrity, homogeneity and monodispersity amongst other parameters of protein sample quality are clearly high-end aims if aiming at structural investigations. The strength of CF expression resides in the possibility to individualize expression protocols and to adapt them to the requirements of the target protein (Fig. 13). As for any other expression system, the effect of basic compounds, specific additives or of the provided detergent on the target after CF synthesis has further to be assessed by extensive quality analyses in order to achieve optimal conditions

for its production. An advantage of the CF system is the fast feed-back response possible from any level of the production process since the reaction volumes are small and the expression rates fast. A comprehensive strategy of sample quality analysis of ETA in different CF modes has therefore been developed and might finally serve as basic guideline for the production of related receptors in similar expression systems.

4.2.1. Evaluation of buffer conditions for the resolubilization and purification of ETA

Two different buffer substances Tris(hydroxymethyl)methylamine (Tris) and sodium phosphate at a near physiological pH of 8.0 were tested with respect to receptor homogeneity. Homogeneity was taken as a first hint to receptor quality which was assessed by SEC using a Superdex 200 3.2/30 column (Fig. 14). Sodium phosphate was analysed since it already generated stable and ligand binding competent ETB receptor (Klammt et al. 2007b, 2007c). Medium concentrations of Tris were chosen due to their common applicability in crystallization trials and their common use in functional assays for ETA membrane receptor preparations (De Leon and Garcia 1995, Tessier et al. 2005).

Compared to sodium phosphate, Tris buffer generated a less aggregated D-CF expressed ETA receptor in 0.5 % B35 purified to 0.1 % B35 with higher apparent homogeneity. The peak maximum of ETA purified in Tris buffer centred at about 1.34 mL corresponding to a molecular mass of approximately 280 kDa with a shoulder at around 1.51 mL (120 kDa). The sodium phosphate-buffer generated profile of ETA resulted in a much broader peak pattern, indicating a multitude of different oligomeric or aggregated states of ETA in those buffer conditions. Most pronounced states could be detected at 1.13 and 1.25 mL corresponding to approximately 780 kDa and 430 kDa, respectively with a shoulder at 1.51 mL (120 kDa) (Fig. 14.).

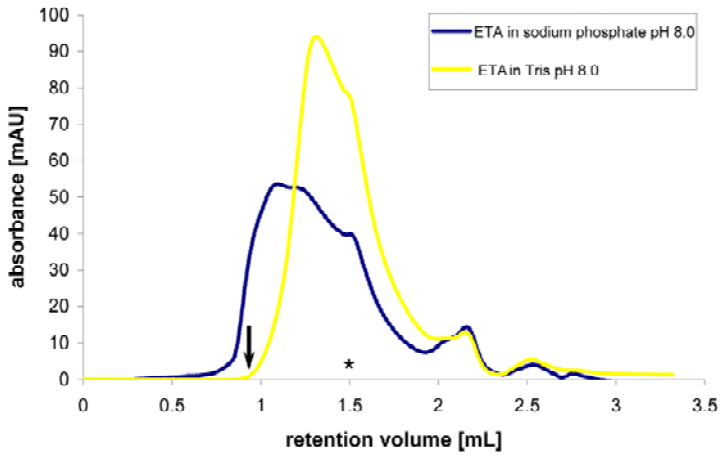


Fig. 14: Choice of optimal buffer substances for the purification of ETA. SEC elution profiles of IMAC purified ETA in 0.1 % B35 under different buffer conditions. Samples containing 10–15 µg of protein were analysed on a Superdex 200 3.2/30 column. Protein absorbance was recorded at 280 nm. The bold arrow indicates the void volume of the column in between 0.9 and 1.0 mL. The retention volume, indicated by an asterisk, corresponds to approximately 125 kDa (Fig. 62 A).

Tris buffer was therefore chosen as promising buffer and applied in subsequent functional and structural analyses. Lowering the pH to 6.5 of this buffer during purification decreased the final receptor yield to a minimum. Optimal purification of the ETA receptor was performed at pH 7.4 with decreasing salt concentrations from 500 mM for equilibration and washing steps to 150 mM sodium chloride in the elution fractions.

4.2.2. Characterization of resolubilized receptor sample quality by size exclusion chromatography

Analysing elution profiles of proteins on size exclusion columns is a first and general tool in order to detect aggregates or apparent heterogeneities in protein samples. P-CF produced ETA precipitates (Fig. 12 A) were directly applied to SEC analysis on a Superdex 200 3.2/30 column after resolubilization in 1 % of the indicated detergents (Fig. 15 A). To evaluate possible aggregation or oligomerization of native protein samples without any reducing and denaturing agents, 3-5 µg of freshly resolubilized samples of ETA in 1 % LPPG, LMPC as well as samples in SDS as a control were further subjected to gradient Blue Native-PAGE (BN-PAGE) analysis in cooperation with Dr. Sebastian Richers (Institute of Biophysical Chemistry, Goethe University, Frankfurt/Main) (Fig. 15 B).

Best elution profiles with highest yield and lowest degree of apparent receptor aggregation were achieved in LPPG and LMPC supplemented with 0.5 mM TCEP. A symmetric peak shape centred at 1.4 mL of retention volume corresponding to a molecular mass of approximately 250 kDa was obtained with the sample in 1 % LPPG (10^3 x CMC). Upon BN-PAGE separation, the resolubilized ETA receptor in LPPG behaved mostly monomeric at a molecular weight of around 50 kDa which is in accordance with its calculated mass of ~52 kDa. A faint band at estimated 100 kDa might possibly account for a minor dimerization potential in LPPG. Addition of 0.02 % CHS during the resolubilization process does however not seem to influence the running behaviour of ETA (Fig. 15 B). Pronounced bands at 440 and 880 kDa very likely represent impurities from the *E. coli* extract as they also occur in non related P-CF expressed proteins (data not shown). The resolubilization of ETA in 1 % LMPC (500 x CMC) generated a much broader peak presumably resulting from a mixture of lower and higher order ETA oligomers (Fig. 15 A).

ETA in LMPC showed monomeric as well as dimeric folding on BN-PAGE since two prominent bands at approximately 60 and 120 kDa could be detected. The slower migration of ETA in LMPC might result from a slightly different running behaviour in LMPC as compared to LPPG (Fig. 15 B).

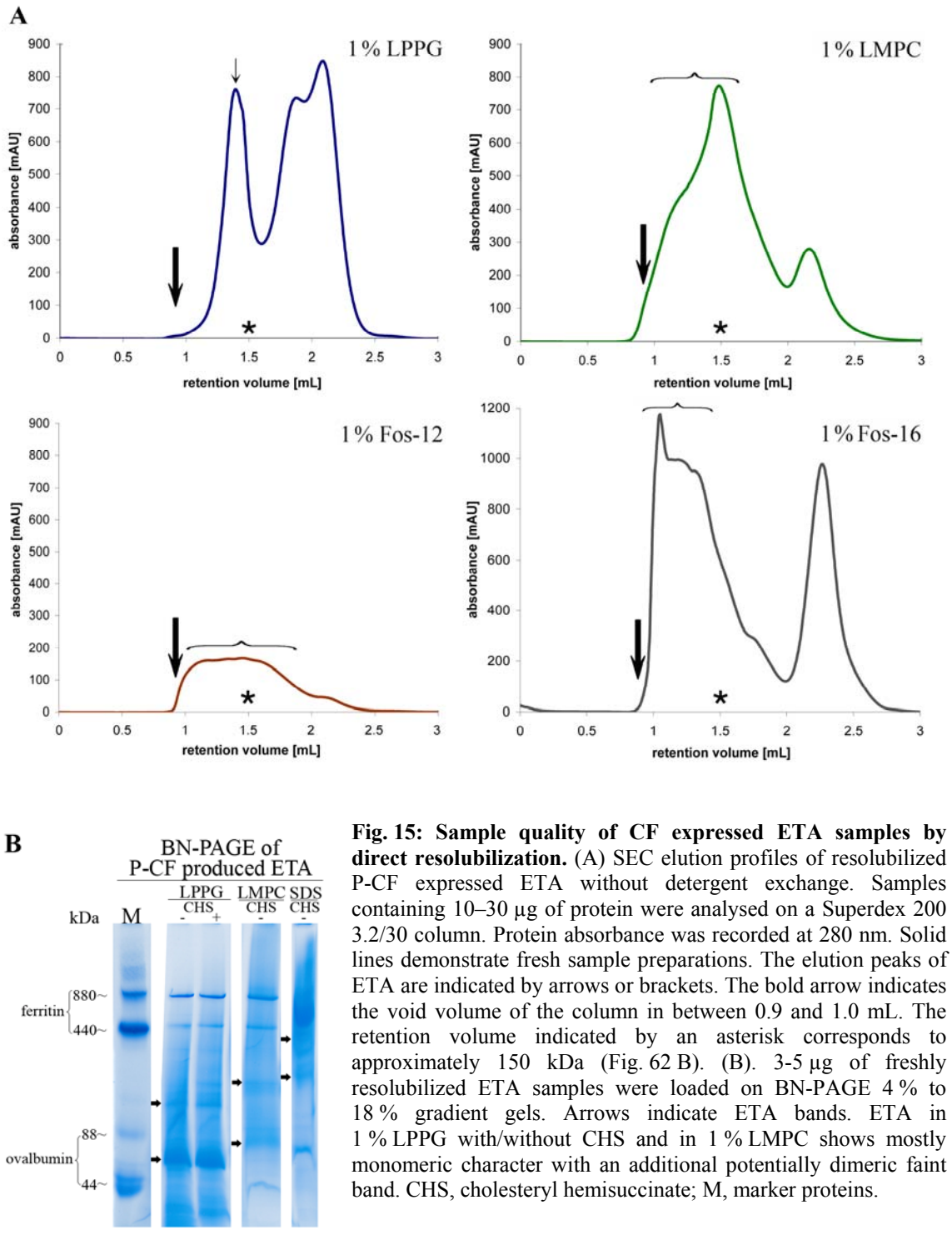


Fig. 15: Sample quality of CF expressed ETA samples by direct resolubilization. (A) SEC elution profiles of resolubilized P-CF expressed ETA without detergent exchange. Samples containing 10–30 µg of protein were analysed on a Superdex 200 3.2/30 column. Protein absorbance was recorded at 280 nm. Solid lines demonstrate fresh sample preparations. The elution peaks of ETA are indicated by arrows or brackets. The bold arrow indicates the void volume of the column in between 0.9 and 1.0 mL. The retention volume indicated by an asterisk corresponds to approximately 150 kDa (Fig. 62 B). (B). 3-5 µg of freshly resolubilized ETA samples were loaded on BN-PAGE 4 % to 18 % gradient gels. Arrows indicate ETA bands. ETA in 1 % LPPG with/without CHS and in 1 % LMPC shows mostly monomeric character with an additional potentially dimeric faint band. CHS, cholesteryl hemisuccinate; M, marker proteins.

SDS seemed to completely aggregate the ETA receptor as only bands at very high molecular weight occurred in this detergent (Fig. 15 B). After resolubilization in 1 % Fos-16 (2 x 10^3 x CMC; 1 mM TCEP) a prominent fraction of ETA eluted within the void volume of the column, indicating complexes of >800 kDa. ETA resolubilized in 1 % Fos-12 (20 x CMC) also showed a broad elution profile indicating a high degree of receptor heterogeneity (Fig. 15 A).

According to its SEC elution profile, 1 % LPPG was therefore chosen as most promising resolubilization detergent for P-CF produced ETA precipitates analysed in subsequent purification and detergent exchange steps (Fig. 15 A).

4.2.3. Purification of ETA by IMAC: Evaluation of chelating ions and secondary detergents

Purification of detergent solubilized receptors was approached by IMAC based on binding of the C-terminal poly(His)$_{10}$-tag at Co^{2+} loaded NTA-agarose beads (**see 3.5.3.**). First evaluations of IMAC purification demonstrated that the commonly used Ni^{2+} ions generated high initial yields of eluted ETA, however concomitant with significant impurities (Fig. 16).

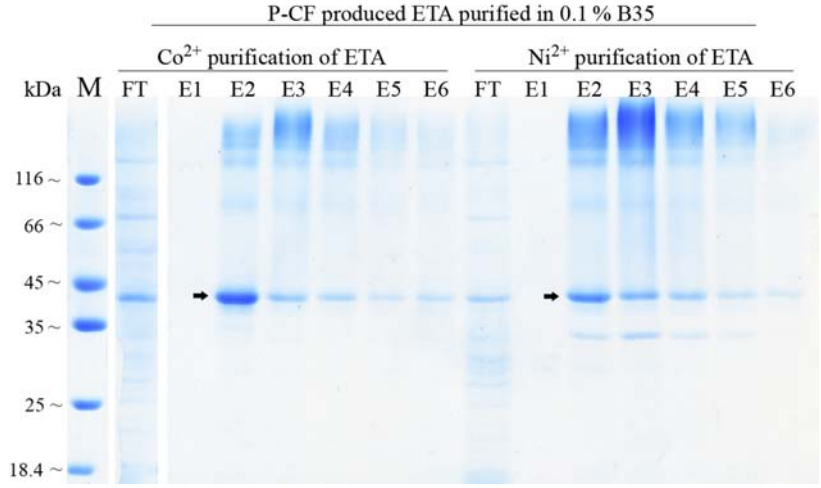


Fig. 16: Evaluation of chelating ions for the purification of ETA. Sample volumes of 10 µL fractions were separated on 12 % SDS-Tris-glycine gels, stained with Coomassie Blue. Chelated cobalt ions produced purer receptor than nickel ions, however with a more pronounced loss in the flow-through fraction. M, marker proteins; FT, flow-through; E, elution; B35, Brij 35.

Although final receptor yields were significantly lower, Co^{2+} ions were preferably employed since their use already resulted in a very high degree of final receptor purity of estimated 90-95 % in the first purification step (Table 11). Thereby, the need of gel filtration as a second purification step could be avoided.

Immobilization of MPs upon IMAC purification was used to modify the initially selected hydrophobic environment either by decreasing detergent concentrations or by exchanging the primary detergent with a second detergent having better properties for downstream processes. A selection of detergents frequently used in structural as well as in functional GPCR studies was evaluated with respect to their effects on ETA homogeneity and stability. LPPG and in particular Fos-12 as well as its derivative Fos-16 were chosen for their applicability in liquid-state NMR spectroscopy (Arora et al. 2001, Krueger-Koplin et al. 2004). B35, F_6-TAC and F_8-TAC as representatives of very mild detergents or surfactants (Klammt et al. 2005, Park et al. 2007) were analysed for their ability to retain or to increase the functionality of the ETA receptor. Their applied final concentrations are listed in Table 10.

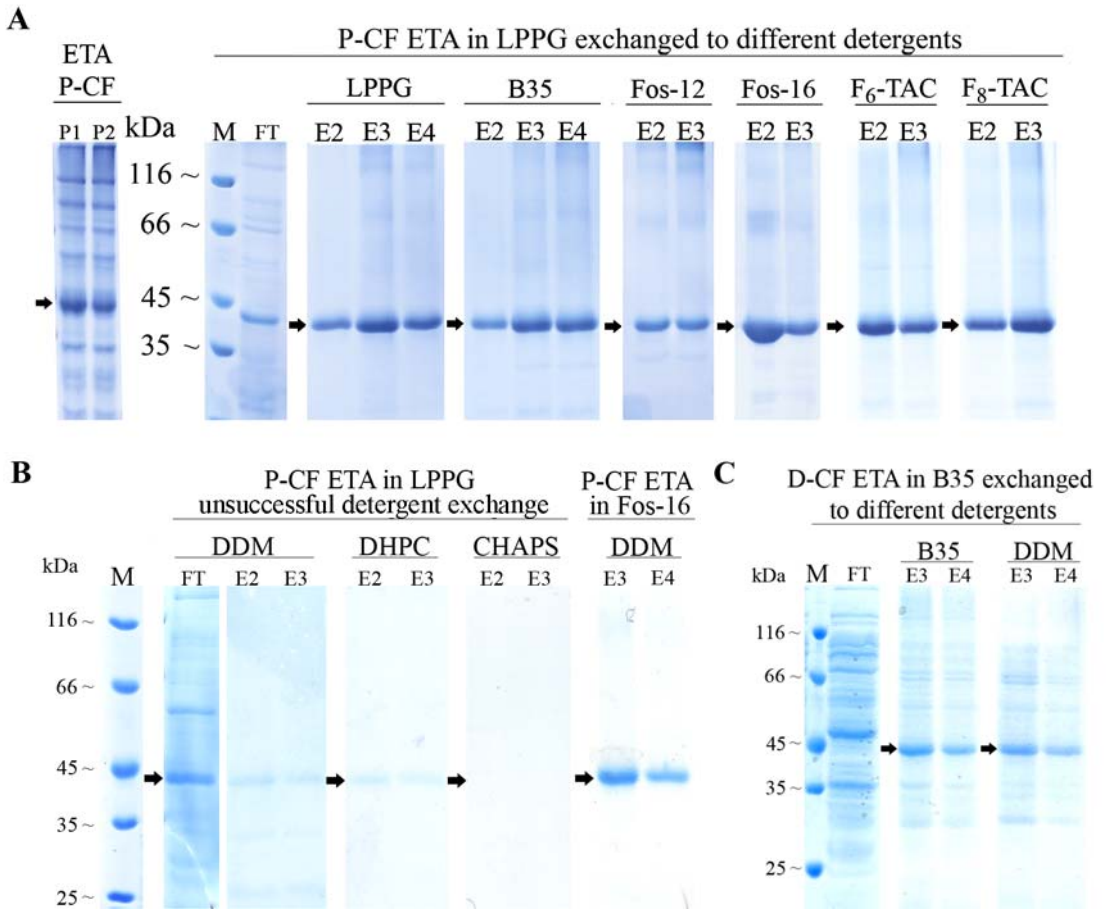


Fig. 17: Purification profiles of ETA in different detergents. Sample volumes of 10 µL were loaded on 12 % SDS-Tris-glycine gels and subsequently stained with Coomassie Blue. (A) Detergent exchange of resolubilized ETA from 1 % LPPG to the respective detergents was performed upon IMAC purification. ETA elution fractions are already apparently pure after the first purification step out of P-CF mode. (B) Unsuccessful detergent exchange from P-CF expressed ETA in 1 % LPPG. Quantitative detergent exchange to DDM can only be achieved from Fos-16 resolubilized ETA. (C) Detergent exchange or concentration reduction of D-CF expressed ETA in 0.5 % B35 to the respective detergents. M, marker proteins; P, pellet; FT, flow-through; E, elution.

P-CF produced ETA subsequently resolubilized in 1 % LPPG could be purified to a high degree in all tested detergents (Fig. 17 A) with highest average yields in 0.05 % LPPG. Exchange to B35, Fos-12 or Fos-16 resulted in comparably high amounts as seen with LPPG (Table 11).

Exchange to some detergents like DDM, CHAPS or 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC, diC_6PC) considerably reduced the ETA yield in the elution fractions since most of the protein was already lost during the low stringency washing steps (Table 11). For 2 % CHAPS and 1 % DHPC losses of up to 100 % were even detected. To achieve quantitative detergent exchange from P-CF synthesized protein to DDM, ETA had to be resolubilized in 1 % Fos-16 as its resolubilization in 1 % LPPG hardly allowed an exchange to DDM on column (Fig. 17 B). D-CF expressed ETA in 0.5 % B35 purified to 0.1 % B35 or 0.04 % DDM always displayed a significantly increased degree of impurities due to the presence of remaining soluble proteins of the *E. coli* extract (Fig. 17 C). Therefore, resolubilization out of P-CF mode can already be considered as efficient pre-purification step.

Table 11: Purification efficiencies of Co^{2+}-IMAC for ETA. Final estimated protein purity as well as final minimal and maximal yields in mg/mL determined by NanoDrop measurements at 280 nm (**see 3.4.2.**) are listed. Highlighted in bold are those conditions most promising for further studies.

CF mode	1st Detergent / 2nd Detergent	Average loss (FT / wash) (%)	Purity (%)	Yield[1] (mg/mL)
P-CF	-	-	50	1-3
	LPPG/LPPG	40	95	**0.9-1**
	LPPG/B35	60	95-98	**0.25-1.25**
	LPPG/Fos-12	80	95	0.25
	LPPG/Fos-16	54	95	**0.25-1**
	LPPG/F_6-TAC	60	95	0.25-0.6
	LPPG/F_8-TAC	70	95	0.25
	LPPG/CHAPS	100	-	0
	LPPG/DDM	95	-	0.025
	LPPG/DHPC	100	-	0
	Fos-16/DDM	80	95	0.2
D-CF	B35/B35	60	85	**0.5-0.6**
	B35/DDM	80	75-80	0.05-0.5
	B35/Fos-12	90	95	0.15

[1], mg ETA out of 1 mL RM after IMAC purification

Dependent on the detergent used for exchange upon IMAC purification, varying final receptor yields were achieved. Highest final amounts of purified ETA were approximately 1 mg/mL RM from the P-CF mode (LPPG/LPPG) and 0.5 mg/mL RM from the D-CF mode (B35/B35). Implementing the two expression modes in combination with variations in the 1st and 2nd detergent, a dozen conditions, some of them resulting in the preparative scale production of solubilized ETA, could be determined in total (Table 11). Subsequent quality controls as well as ligand binding analyses were performed without removing N-terminal and C-terminal peptide tags from the receptors.

4.2.4. Evaluation of receptor sample quality after IMAC purification by size exclusion chromatography

Those detergents which proved efficient for the purification of ETA (**see 4.2.3.**) and which are frequently used in structural as well as in functional GPCR studies were further evaluated with respect to their effects on ETA homogeneity and stability. Therefore, either LPPG resolubilized P-CF produced or D-CF expressed ETA in 0.5 % B35 was purified in the respective detergents by Co^{2+}-IMAC and the elution fractions were directly loaded on an analytical Superdex 200 3.2/30 column. In parallel, their stability was assessed by storing freshly purified proteins for one to two weeks at 4 °C before SEC analyses (Fig. 18, dashed lines).

The most homogenous elution profiles centred at approximately 1.35 mL (~300 kDa) could be achieved after exchange to 0.1 % B35 (~10 x CMC) as well as to 0.005 % Fos-16 (~10 x CMC) (Fig. 18 A, C solid lines). ETA was detected by immunoblotting against the C-terminal poly(His)$_{10}$-tag in both preparations after gel filtration (Fig. 18 B, D). For LPPG/B35 samples second order differentiation of the peak curve using the Unicorn software resulted in two main protein mass fractions, represented as minima, suggesting two oligomeric states in this receptor preparation (Fig. 18 A, inset, red curve). The same evaluation for LPPG/Fos-16 showed only one major protein mass fraction under the peak which might correspond to a distinct oligomeric or even monomeric state (Fig. 18 C, inset, red curve).

Furthermore, ETA showed a high degree of stability after storage in either of the two detergents at 4 °C. Only minor N-terminal degradation by the rightward shift of the peak tail and no aggregation of ETA in 0.1 % B35 might be expected after one week (Fig. 18 A, dashed lines). ETA exchanged from LPPG to 0.1 % Fos-12 (2 x CMC) also showed a reasonable profile eluting with its peak maximum at around 1.38 mL (~275 kDa) and a small shoulder at approximately 1.26 mL (~500 kDa) (Fig. 18 C). ETA in Fos-12 exhibited good stability with only little aggregation after one week, indicated by the small arrow in Fig. 18 C at approximately 1 mL of retention volume. Subjecting the Fos-12 curve to second order differentiation (Fig. 18 C, inset, red curve) one main minimum at ~1.38 mL and a small second minimum under the shoulder at 1.26 mL were calculated suggesting two multimeric ETA states in Fos-12. The immunodetection however resulted in only one clear band (Fig. 18 D).

Resolubilized ETA in 0.05 % LPPG (~50 x CMC) eluted at 1.41 mL (~230 kDa) retention volume with a shoulder at around 1.50 mL (~150 kDa) when loaded directly onto the column after purification (Fig. 18 A, solid line). ETA could furthermore be detected in the peak by immunoblotting (Fig. 18 D). When stored at 4 °C for two weeks, the profile shifted to a major peak at around 1.55 mL (~120 kDa) (Fig. 18 A, dashed line). Fractions of protein masses in the LPPG/LPPG sample by differentiation (Fig. 18 A, inset, red line) verified two major multimeric states for ETA in the fresh sample which disintegrated over time at 4° C to only one prominent state of lower apparent molecular weight (Fig. 18 A, dashed line).

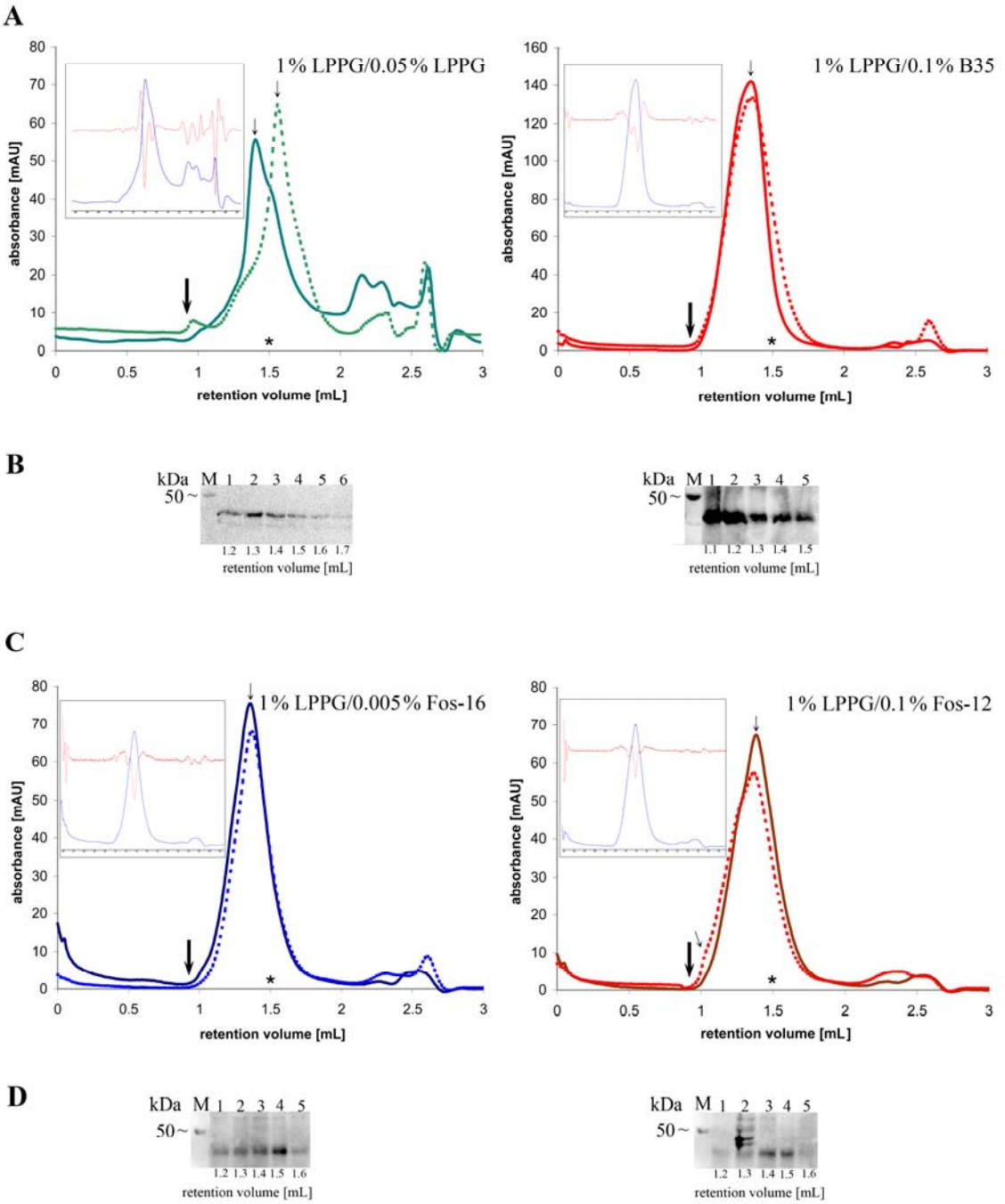


Fig. 18: Sample quality of P-CF expressed ETA samples exchanged from LPPG to different detergents upon IMAC purification. (A, C) SEC elution profiles of purified receptor samples solubilized in LPPG and B35 (A) and Fos-16 and Fos-12 (C). Grey boxes: The insets comprising blue and red curves represent initial smoothed curves (blue) subjected to second order differentiation (red) in the Unicorn 5.11 software. The analysed detergents are indicated (1st detergent/2nd detergent). Samples containing 10–30 µg of protein were analysed on a Superdex 200 3.2/30 column. Protein absorbance was recorded at 280 nm. Solid lines: fresh sample preparations; dashed lines: samples after storage at 4 °C for 1–2 weeks. The elution profiles of ETA are indicated by small arrows. The bold arrow indicates the void volume of the column in between 0.9 and 1.0 mL. The retention volume indicated by an asterisk corresponds to approximately 150 kDa (Fig. 62 B). (B, D) Elution fraction samples collected during SEC of the ETA preparations depicted above. 50 µL of 100 µL fractions were subjected to acetone precipitation, completely loaded on 12 % SDS-Tris-glycine gels and analysed by immunodetection with specific His-tag antibodies. M, marker proteins; 1-6, elutions fractions; 1.1-1.6, retention volume of the respective elution fractions [mL].

Samples of ETA D-CF produced in 0.5 % B35 (~50 x CMC) and exchanged to 0.1 % B35 (~10 x CMC) started to elute right after the determined void volume and showed a symmetric but considerably broader elution profile centred at approximately 1.21 mL corresponding to a theoretical size of ~640 kDa already hinting at potential higher average aggregation of ETA from D-CF mode expressed in 0.5 % B35 (Fig. 19 A, solid line). ETA could be verified as monomers without apparent aggregation in the SEC elution fractions by immunodetection against the C-terminal poly(His)$_{10}$-tag (Fig. 19 B). However, second order differentiation of the D-CF sample peak resulted in the detection of four different oligomeric states within the profile suggesting inhomogenous sample quality (Fig. 19 A, inset, red curve). The sample appeared to be stable when stored at 4 °C for one week (Fig. 19 A, dashed line).

As delineated above quantitative detergent exchange from P-CF synthesized protein to DDM was only possible from 1 % Fos-16 (**see 4.2.3.**).

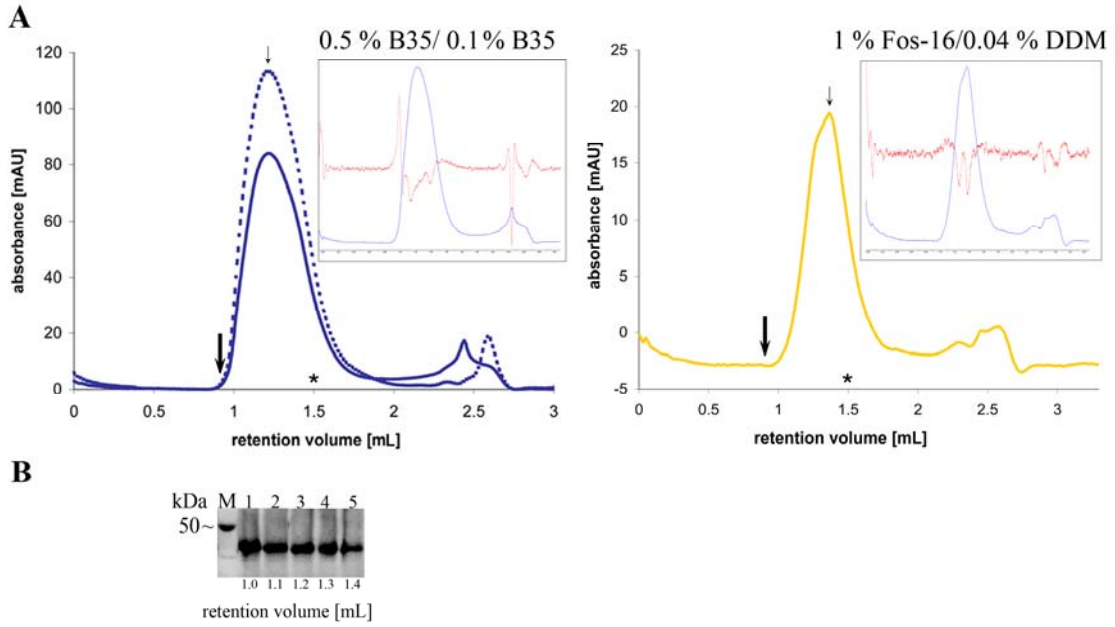


Fig. 19: Quality of D-CF (left) or P-CF (right) expressed ETA samples exchanged to different detergents upon purification. (A) SEC elution profiles of purified ETA solubilized in B35 and DDM. Grey boxes: The insets comprising blue and red curves represent initial smoothed curves (blue) subjected to second order differentiation (red) in the Unicorn 5.11 software. The analysed detergents are indicated (1st detergent/2nd detergent). Samples containing 5–20 µg of protein were analysed on a Superdex 200 3.2/30 column. Protein absorbance was recorded at 280 nm. Solid lines: fresh sample preparations; dashed lines: samples after storage at 4 °C for one week. The elution peaks of ETA are indicated by small arrows. The bold arrow indicates the void volume of the column in between 0.9 and 1.0 mL. The retention volume indicated by an asterisk corresponds to approximately 150 kDa (Fig. 62 B). (B) Elution fractions collected during SEC of the ETA preparations in 0.1 % B35. 50 µL of 100 µL fractions were subjected to acetone precipitation, completely loaded on 12 % SDS-Tris-glycine gels and analysed by immunodetection with anti-His-tag antibodies. M, marker proteins; 1-6, elutions fractions; 1.1-1.4, retention volume of the respective elution fractions [mL].

The ETA sample Fos-16/DDM showed a relatively symmetric elution profile centred at 1.36 mL (~295 kDa) with a shoulder oriented to higher molecular weights at 1.27 mL (~470 kDa) (Fig. 19 A, solid line). Due to the concomitant dilution effect its low absorbance of 19 mAU at 280 nm rendered the immunodetection of the protein in the elution fractions

impossible. Upon differentiation two major oligomeric states were calculated supporting the peak shape with shoulder (Fig. 19 A, inset, red curve).

4.2.5. Molecular mass determination of ETA/detergent complexes

MALS coupled to semi-preparative SEC on a Superdex 200 10/300 was employed in order to further analyse ETA sample qualities. The molecular mass of protein/detergent complexes as well as of the individual complex components can be analysed by this technique. SEC elution profiles of P-CF expressed ETA, resolubilized in 1 % LPPG and exchanged to 0.1 % B35 as well as to 0.005 % Fos-16 revealed the highest apparent degree of homogeneity (Fig. 18 A, C). These sample preparation conditions were therefore selected for analysis. P-CF produced ETA was changed to the respective detergents upon IMAC purification (Fig. 20 C, D) with a high degree of purity. From both preparations the second elution fractions were further subjected to MALS. Representative sections of the profiles depicted as red boxes were processed (Fig. 20 C, D).

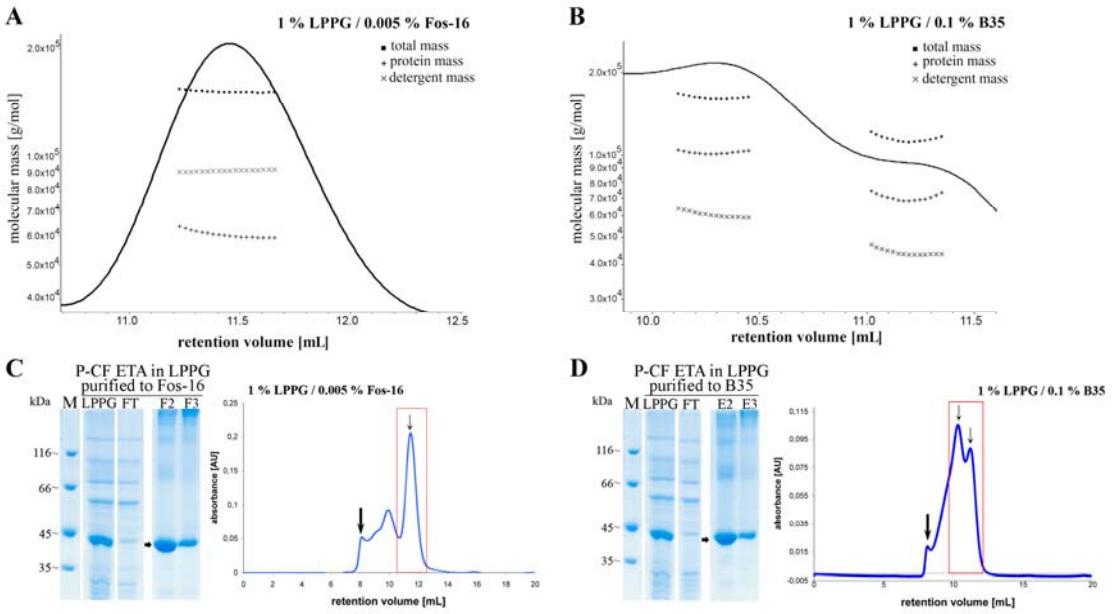


Fig. 20: Molecular mass analysis of P-CF produced ETA samples in different detergents by MALS. ETA precipitates were resolubilized in 1 % LPPG and exchanged to the indicated secondary detergents upon IMAC purification (C, D). MALS was coupled to SEC on a Superdex 200 10/300 column in HEPES buffered Fos-16 (A) or Tris buffered B35 (B). Relevant sections (thin arrows) used for the molecular mass calculation of the detergent-protein complexes are shown by red boxes in the gel filtration profiles (C, D). The void volume at 8 mL is represented by bold arrows.

Using the Astra software 5.3.4.13, the molecular mass of the whole ETA/detergent complex in Fos-16 micelles was calculated with a retention volume of 11.4 mL at approximately 151 kDa, resulting from a protein mass of ~60 kDa and a mass contribution of the Fos-16 detergent of ~91 kDa (aggregation number of ~220) which corresponds very well to the aggregation number

found in literature of ~180 in pure water. ETA therefore appeared to be monomeric under those conditions since its calculated molecular mass including the terminal tags is 52 kDa.

In B35 micelles, two major peak fractions had to be evaluated (Fig. 20 D). In the first peak at around 10.4 mL of elution volume, the mass of the ETA/detergent complex was determined at 163 kDa. The mass contributions of ETA were calculated at 102 kDa and of the B35 detergent at 61 kDa (aggregation number of ~50), respectively. The determined aggregation number is again comparable with the average aggregation number in water of 40 resulting in a pure micelle of 50 kDa. The second peak, eluting at approximately 11.3 mL, had a molecular mass of the ETA/detergent complex of 114 kDa, resulting from a protein mass of 70 kDa and a mass contribution of the B35 detergent of 44 kDa (aggregation number of ~36) (Fig. 20 B). However, the relative calculation errors were higher if compared with the ETA/Fos-16 sample. Considering the calculated molecular mass of the ETA monomer, it can be assumed that ETA in 0.1 % B35 shows monomeric as well as dimeric character.

The relative mass contributions of ETA and B35 to the ETA/detergent complex from D-CF expressed receptor in 0.5 % B35 could not be determined. On the Superdex 200 10/300 the whole receptor preparation generated a shark fin elution profile with a peak maximum at 8 mL corresponding to the determined void volume of the column (Fig. 21). Molecular weight determinations according to the calibration proteins resulted in a mass of 1 MDa for the D-CF expressed ETA receptor (Fig. 62 C). This observation is well in accordance with the broad elution profile of a comparable sample on the Superdex 200 3.2/30 (Fig. 19) hinting to unsatisfactory homogeneity and overall receptor quality of D-CF produced ETA exchanged to lower concentrations of B35.

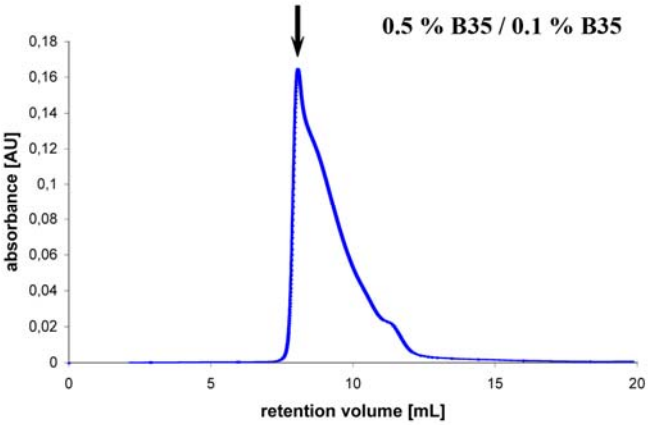


Fig. 21: Size exclusion profile of D-CF expressed ETA generated during MALS analysis. ETA is highly aggregated and almost completely elutes in the void volume indicated by a bold arrow.

4.2.6. Characterization of ETA receptor sample dispersity by negative staining electron microscopy

Negative staining EM was used as a complementary approach to support the proposed receptor quality in different detergents explored by SEC analysis. These experiments were kindly performed in cooperation with André Krüger (group of Prof. Andreas Engel, Department of Structural Biology at the M.E. Müller Institute for Microscopy, Biocenter, University of Basel,

Switzerland). 5 µL of P-CF expressed and subsequently purified ETA receptor at different concentrations in 0.05 % LPPG and 0.1 % B35, as well as D-CF expressed ETA exchanged to 0.1 % B35 upon IMAC, were analysed. Prior to the negative staining experiments the purity of the samples was explored by 12 % SDS-Tris-glycine PAGE.

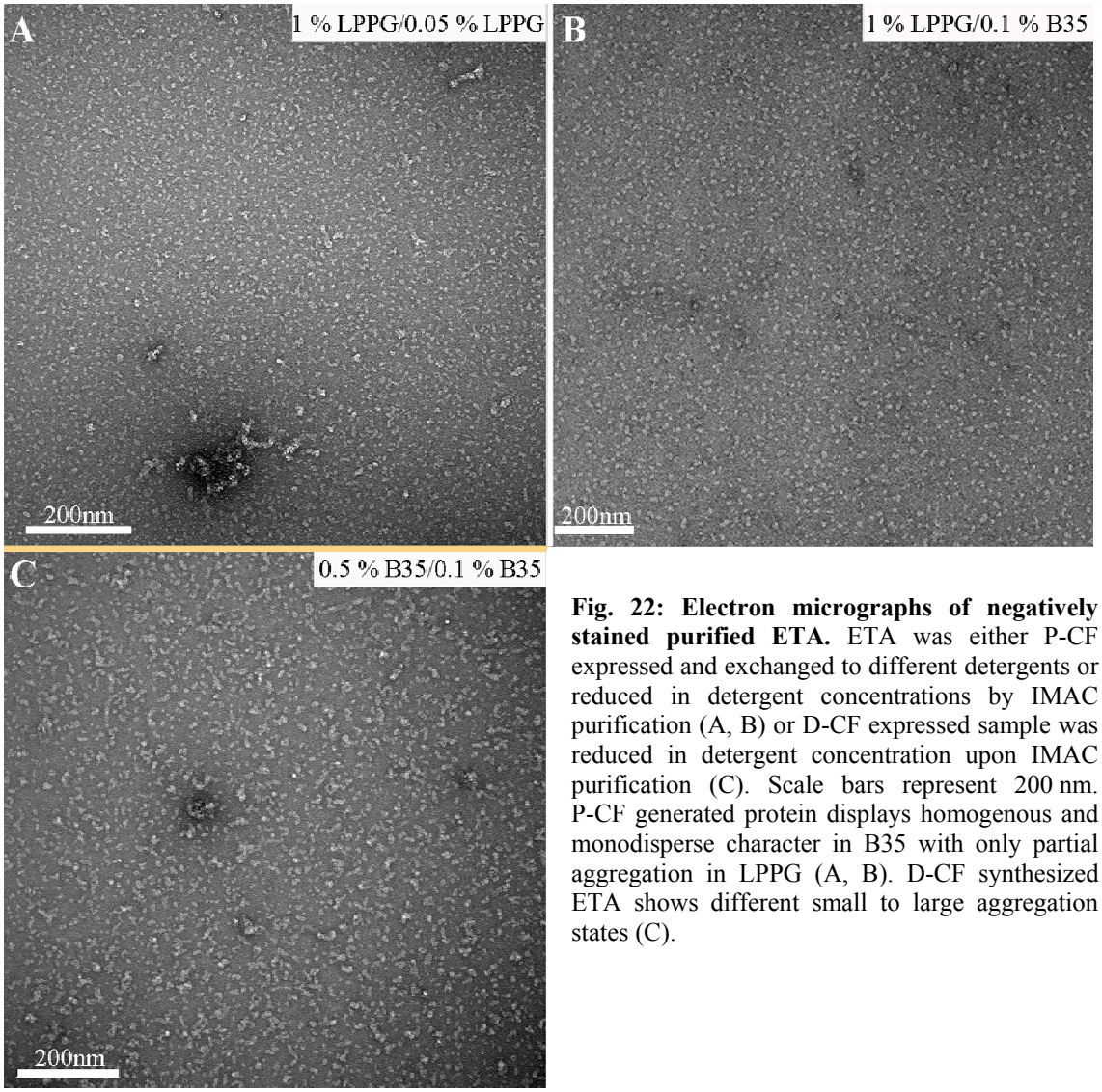


Fig. 22: Electron micrographs of negatively stained purified ETA. ETA was either P-CF expressed and exchanged to different detergents or reduced in detergent concentrations by IMAC purification (A, B) or D-CF expressed sample was reduced in detergent concentration upon IMAC purification (C). Scale bars represent 200 nm. P-CF generated protein displays homogenous and monodisperse character in B35 with only partial aggregation in LPPG (A, B). D-CF synthesized ETA shows different small to large aggregation states (C).

The electron micrographs of negatively stained purified ETA receptor showed different degrees of sample homogeneity depending on which expression mode and which final detergent was used. The negatively stained ETA particles from P-CF expressed protein purified to lower concentrations of LPPG or purified to B35 displayed equal sizes and therefore exhibited homogenous and monodisperse character. This indicated high sample quality from these preparation methods (Fig. 22 A, B). Upon reduction of the final concentration of LPPG to 0.05 % during IMAC purification only partial aggregation could be detected but the overall size distribution remained homogenous (Fig. 22 A). ETA expressed in 0.5 % B35 reduced to 0.1 % B35 resulted in differently sized particles with an apparently high degree of varying

aggregational states ranging from small to large structures assuming lower overall sample quality (Fig. 22 C). These findings are in accordance with the MALS and SEC results.

4.2.7. Characterization of secondary structure and its thermostability by far-UV CD spectroscopy

Far-UV CD spectroscopy with ETA was conducted to analyse the formation of its secondary structure elements in the best conditions supported by SEC, MALS and negative staining (**see 4.2.4.** to **4.2.6.**). CD spectra were thus recorded of P-CF produced ETA samples which were resolubilized in 1 % LPPG and exchanged to 0.1 % B35 and 0.005 % Fos-16, respectively. All spectra showed minima at 208 and 222 nm characteristic for α-helical proteins. The maximum at 190 nm which is typical for α-helical structure as well could not be detected due to high noise interference during the measurements under those buffer and detergent conditions (Fig. 23 D).

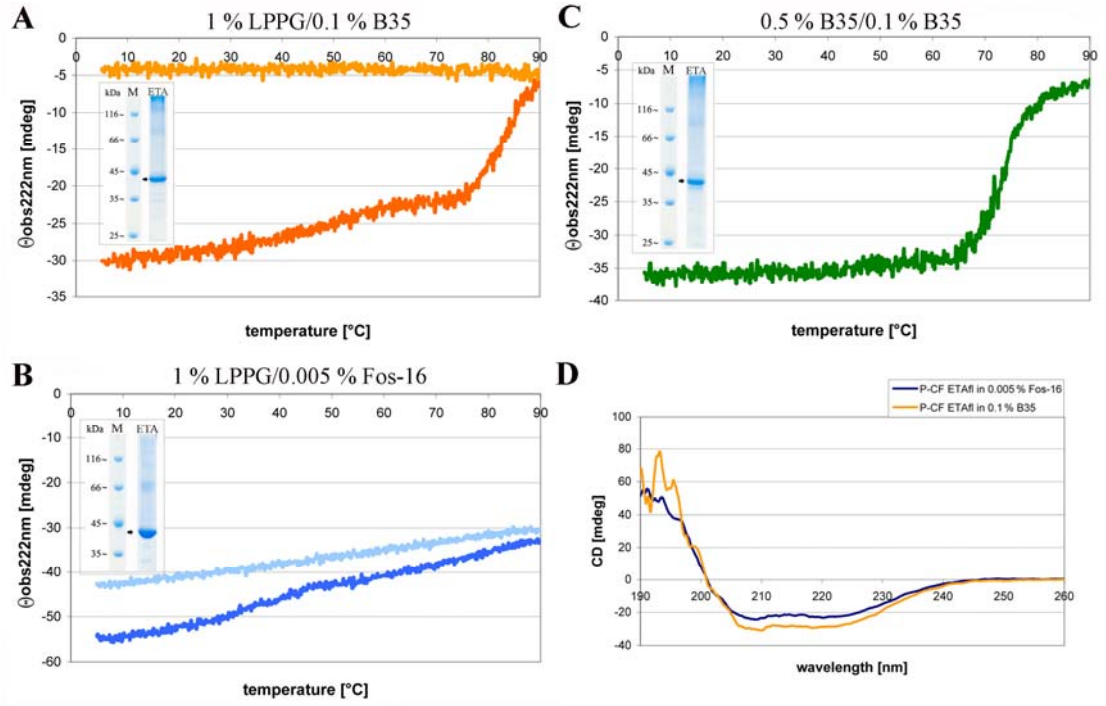


Fig. 23: Characterization of ETA by far-UV CD spectroscopy. ETA samples were expressed either in P-CF mode without detergent (A, B, D) or in D-CF mode in presence of 0.5 % B35 (C). They were IMAC purified and exchanged to the respective detergents at final concentrations of 12 µM (A), 14 µM (B) and 11.5 µM (C). The samples were analysed in 10 mM sodium phosphate buffer, pH 7.5, including the respective detergents and measured in a cuvette of 1 mm path length at 22 °C. Melting curves were performed at a fixed wavelength of 222 nm from 4 °C to 90 °C with reversed temperature screens back to 4 °C for A and B (light orange and light blue curves). T_m values of ~85 °C and ~72 °C could be determined for LPPG/B35 (A) and B35/B35 (C), respectively. LPPG/Fos-16 did not show any significant transition. (D) CD spectra of LPPG/B35 and Fos-16 respectively displayed the typical minima at 208 nm and 222 nm known for α-helical proteins. Insets: Coomassie stained SDS–PAGE of analysed samples.

The determined α-helical content could be processed to final values of 61 % in 0.005 % Fos-16 and 46 % in 0.1 % B35 according to the method of Chen (Chen et al. 1974). These

determinations correspond well to the predicted α-helical percentage of around 51 % performed by the secondary structure analysis servers PsiPred and APSSP2. Especially ETA in 0.1 % B35 of 46 % is comparable to CD data of other groups which claim an α-helical content of lower than 50 % for refolded ETA (Orry and Wallace 2000). Apparently, different detergents induce various degrees of α-helical folding in the ETA receptor as already seen for V2R and Tsx (Klammt et al. 2005).

The thermostability of the secondary structure elements of ETA was further analysed by far-UV CD spectroscopy as a change in ellipticity at 222 nm by thermal denaturation in a temperature range from 4 to 90 °C (Fig. 23 A, B, C). For ETA in LPPG/B35 (Fig. 23 A) two transitions could be detected. The minor one having its midpoint at ~40 °C might be caused by incomplete detergent exchange or a very small fraction of thermally instable elements as the ellipticity change only covers a range of 7 mdeg units. The major transition started at 60° C with an assumed endpoint to -5 mdeg at 90 °C. A T_m value for secondary structure melting could be determined at ~85 °C. Cooling down the denatured sample back to 4 °C did not show any refolding indicating complete denaturation at 90 °C, which was further supported by protein precipitates in the cuvette (Fig. 23 A). Denaturation of the B35/B35 treated ETA sample resulted in only one defined transition (Fig. 23 B). The melting curve showed a very clear shift from 60 °C towards a complete loss of secondary structure at 90 °C. Precipitation of ETA after heating was further observed. A T_m value of ~72°C was determined for this sample. Measurements with assay buffer containing only Brij detergent did not show any transition (data not shown). For both samples a highly stable secondary structure can be assumed in 0.1 % B35 micelles.

For ETA in LPPG/Fos-16 no significant changes in ellipticity and therefore no relevant decomposition of its secondary structure up to 90 °C could be detected. Only a minor fraction of structural elements might have started to melt at around 30 °C as the loss of ellipticity might indicate upon backward cooling (Fig. 23 C). This leads to the assumption that ETA in Fos-16 micelles seems to be heat resistant up to 90 °C. Heating this sample to higher temperatures would possibly allow a better understanding of its secondary structure melting behaviour.

4.2.8. Preliminary indication of ETA folding in P-CF precipitates by solid-state NMR

In the P-CF mode, no hydrophobic compounds are added into the reaction and translated hydrophobic MPs will consequently form a precipitate. This process apparently resembles the IB formation in *E. coli* cells. Increasing numbers of reports already indicate that even complex MPs can be functionally reconstituted from CF precipitates (Ishihara et al. 2005, Kamonchanok et al. 2008, Keller et al. 2008, Klammt et al. 2004). This might give evidence that some structural parts remain folded in the P-CF produced MP precipitates.

Since ETA was processed from P-CF precipitates for further functional and structural analyses, insights in its initial quality is of high relevance and might provide further strategies of quality improvement already during the production process. An indication of precipitate quality might be given by magic angle spinning solid-state NMR spectroscopy as in particular CαCβ chemical shifts are dependent on secondary structure elements (Wang and Jardetzky 2002, Wishart and Sykes 1994).

Solid-state experiments of ETA precipitates were performed in cooperation with Karsten Mörs (group of Prof. Glaubitz, Institute of Biophysical Chemistry, Goethe University, Frankfurt/Main) and preliminary data, results and discussion were kindly provided by him.

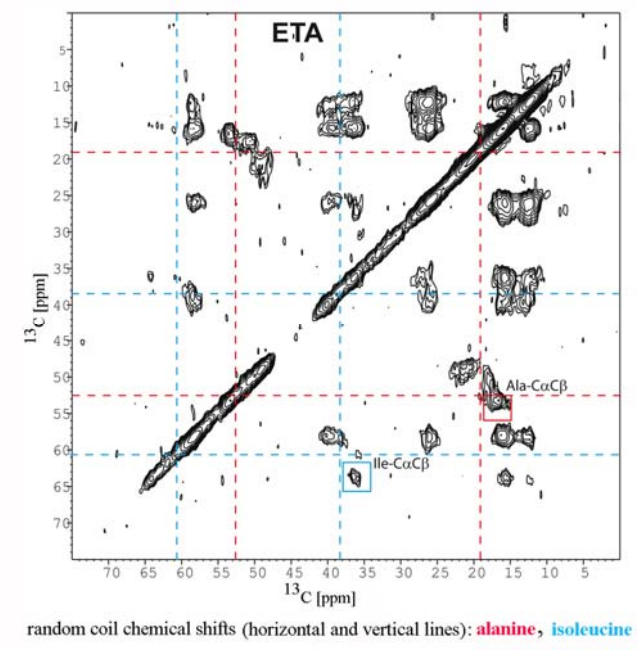


Fig. 24.: 2D ^{13}C-PDSD of ETA precipitates. The protein was expressed in the P-CF mode and selectively ^{15}N ^{13}C- alanine and -isoleucine-labelled. The spectrum was acquired on a Bruker Advance 600 spectrometer using a 4 mm MAS-DVT probe at 260 K and 10 KHz spinning rate. Random coil structures of the respective aa are indicated by the red and blue horizontal and vertical lines.

ETA was selectively ^{15}N ^{13}C- alanine and -isoleucine-labelled by CF expression in the P-CF mode. 3 mg of pellet was subsequently harvested, washed with 150 mM KCl, 5 mM EDTA, pH 7.3 and subjected to solid-state measurements at 260 K and 10 KHz spinning rate (Fig. 24). The sample was then loaded in a 4 mm MAS rotor. The 2D ^{13}C-PDSD experiment was recorded with 50 ms mixing time and 240 increments of 336 scans each. A SPINAL-64 pulse with a field strength of 125 KHz was applied during data acquisition.

Taking the lines for random coil chemical shifts as starting point in the lower half of the spectrum (blue and red dashed lines) the observed CαCβ cross-peaks of alanine and isoleucine displayed secondary chemical shifts in the ω_1-dimension to higher parts per million (ppm) and in the ω_2-dimension to lower ppm values which indicates α-helical structures, whereas shifts to lower (ω_1-dimension) and higher ppm values (ω_2-dimension) account for β-sheet content. Both structural elements were present in the ETA precipitate with potential α-helical folding at an estimated amount of 30-40 % as judged by the intensity of both alanine and isoleucine cross-peaks (Fig. 24). From this spectrum the assumption seems likely that partial α-helical folding already characterizes P-CF derived ETA precipitates.

4.3. Functional analyses of ETA by *in vitro* reconstitution into lipid bilayers

Due to the lack of published data and further information on the functional characteristics of *in vivo* expressed ETA receptor in detergent-solubilized form, *in vitro* reconstitution into proteoliposomes was implemented for the CF expressed ETA. As a wealth of binding studies have already been performed on membrane-inserted ETA receptor from *in vivo* material, evaluation of the ligand binding activity and thus the quality of CF produced receptor compared with *in vivo* produced receptor in lipid bilayers was approached.

In order to prove the quality of CF expressed ETA in liposomes and to finally confirm its ligand specific activity, different reconstitution conditions were tested and subjected to radioactive ligand binding experiments.

The specificity of the implemented radioactive assay was initially verified by a commercially purchased membrane preparation of Chem1 mammalian cells containing recombinant human ETA receptor (ChemiScreen™ GPCR Membrane Preparation, Millipore, Eschborn, Germany). Therefore, 5 µg of positive control membranes were incubated with increasing concentrations of ^{125}I-ET-1-labelled ligand. Unspecific binding was determined in presence of 4 µM unlabelled ET-1. High specificity of the assay and of the utilized ligands was shown and very low background binding could be detected upon pre-incubation of the membrane-incorporated ETA with unlabelled ligand (Fig. 25).

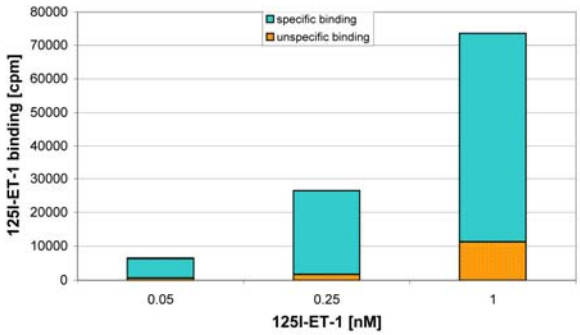


Fig. 25: Specificity of the radioactive binding test verified by commercially purchased membrane-inserted ETA.

4.3.1. Evaluation of reconstitution parameters: Lipids, detergents and protein-to-lipid ratio

A variety of ETA receptor preparations were initially tested with a selection of natural lipids or artificial lipid mixtures in liposome buffer (20 mM KPi, 150 mM NaCl, pH 7.0 or pH 7.5) to achieve reconstitution (**see 3.9.2.**). Proteoliposomes were harvested either with or without sucrose gradient ultracentrifugation (Fig. 26) and subsequently subjected to ligand binding assays (Table 12). Alternatively, they were stored at -80 °C until further use.

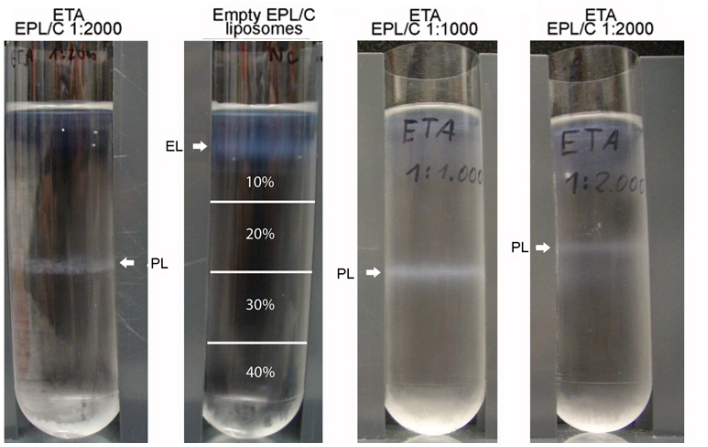


Fig. 26: Sucrose gradient analysis of ETA proteoliposomes. ETA was P-CF expressed, resolubilized in 1 % LPPG and subsequently reconstituted at different protein-to-lipid ratios (1:1,000, 1:2,000) and subjected to sucrose gradient ultracentrifugation. EPL/C, *E. coli* polar lipids plus cholesterol. Arrows indicate EL, empty liposomes and PL, proteoliposomes.

Best reconstitution with highest radioactive signal conservation of ET-1 specific binding was accomplished if empty liposomes were pre-destabilized with final concentrations of either 3.5 mM DDM or 4 mM Triton X-100 for 30 min at RT prior to the addition of the respective protein preparations. Destabilization by Triton X-100 especially proved valuable for the ETA incorporation purified in the fluorinated surfactant F_6TAC, while destabilization by DDM significantly decreased the observed specific binding (Fig. 27). Proteoliposome formation was performed by incubation of the liposome/protein mixture for 30 min at RT with an optional addition of 1 % glycerol. The removal of the detergents was conducted with sequential addition of Biobeads for 2 h at RT. Further incubation with Biobeads over night resulted in considerably higher apparent activity at 4 °C than at 16 °C or RT.

All parameters were evaluated by determination of the relative ligand binding activity of radioactively labelled ^{125}I-ET-1 to the proteoliposomes (Table 12). Successful reconstitution with a high incorporation rate was assumed if an adequate radioactive signal over background binding was achieved. For the measurements, different proteoliposome preparations were incubated with 0.5 nM to 1 nM of ^{125}I-ET-1 and background binding was determined by blocking the same sample with an excess of unlabelled ET-1 prior to ^{125}I-ET-1 addition (unspecific binding, background).

Separation of bound from unbound ligand was performed upon transfer of the samples to GF/B glass fibre filters and subsequent washing steps. Radioactivity retained on the filters was measured in a gamma counter (**see 3.9.3.**).

Table 12: Analysed reconstitution conditions for the ETA receptor by radioactive ET-1 ligand binding. The first detergent refers to the detergent used for either expression or resolubilization of ETA exchanged to the second detergent upon IMAC purification. %-ranges were determined by multiple independent experiments. 100 % correspond to the measured total radioactive signal for separate determinations.

Expression mode	1st detergent (%)	2nd detergent (%)	Lipids	P/L-ratio[a]	Destab. detergent	Activity[b] (%)
D-CF	B35 (0.5)	B35 (0.1)	EPL[c]/C[d]	1:1,200	DDM	0
P-CF	**LPPG (0.5)**	**-**	**EPL[c]/C[d]**	**1:350**	**DDM**	**40-60**
P-CF	LPPG (1)	-	EPL[c]/C[d]	1:700	DDM	10
P-CF	**LPPG (1)**	**-**	**EPL[c]/C[d]**	**1:1,000**	**DDM**	**30-40**
P-CF	LPPG (1)	-	EPL[c]/C[d]	1:1,000	Triton	20
P-CF	LPPG (1)	-	EPL[c]/C[d]	1:2,000	DDM	n.a.
P-CF	LPPG (1)	Fos-12 (0.1)	EPL[c]/C[d]	1:2,500	DDM	20
P-CF	**LPPG (1)**	**F_6-TAC (0.35)**	**EPL[c]/C[d]**	**1:1,500**	**Triton**	**50**
P-CF	LPPG (1)	F_6-TAC (0.35)	EPL[c]/C[d]	1:1,500	DDM	25
P-CF	LPPG (1)	-	PC/PE[e]/C[d]	1:1,000	DDM	50
P-CF	LPPG (1)	LPPG (0.05)	PC/PE/PS[e]/C[d]	1:2,900	DDM	0
P-CF	Fos-16 (1)	DDM (0.05)	EPL[c]/C[d]	1:1,500	DDM	30
P-CF	**LPPG (1)**	**-**	**aso-PC[f]**	**1:1,000**	**DDM**	**60**
P-CF	LPPG (1)	-	aso-PC[f]	1:1,000	Triton	30
P-CF	LPPG (1)	F_6-TAC (0.3)	aso-PC[f]	1:1,500	DDM	20

[a], P/L-ratio, protein-to-lipid ratio
[b], percentage of specific binding calculated from total measured signal and background binding determined by an excess of cold ET-1 ligand
[c], EPL, *E. coli* polar lipids; [d], C, cholesterol; [e], PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; [f], aso-PC, asolectin-phosphatidylcholine, type IV-S
n. a., not analysed
bold writing, most promising conditions chosen for saturation and competition radioligand assays

The initial screening of different reconstitution conditions by radioactive ligand binding allowed to determine the most promising combinations which could further be subjected to saturation as well as competition experiments (Fig. 27).

D-CF expressed ETA in 0.5 % B35 and exchanged to 0.1 % B35 did not show any remarkable hint of specific ligand binding activity in *E. coli* polar lipids supplemented with cholesterol. Therefore, D-CF expression of ETA in B35 was classified as inefficient for the quantitative reconstitution of active receptor right at the beginning (Fig. 27, first bar, left).
This decision also based on previous quality control by SEC and MALS which reported minor homogeneity for the B35 D-CF ETA preparation (**see 4.2.4.** and **4.2.5.**).

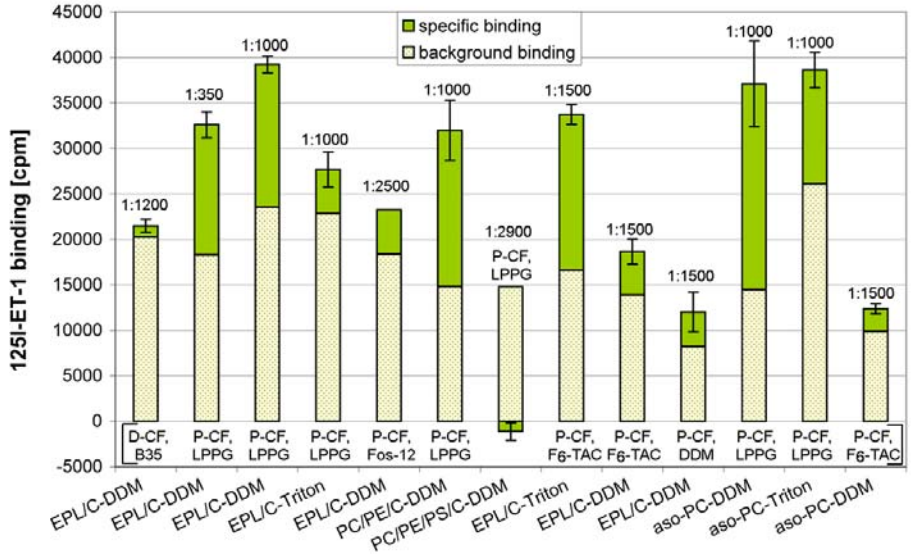


Fig. 27: Tested combinations for the *in vitro* reconstitution of ETA. Below the diagram the used lipids and the destabilizing detergents are listed. Above the bars the P/L-ratios are indicated. The expression mode and the final protein detergent are additionally given in brackets below the respective bars.

ETA was P-CF expressed and resolubilized in 1 % LPPG in most of the reconstitution trials. 1 % Fos-16 was only chosen for ETA sample preparation in DDM as IMAC purification to DDM was impossible using LPPG-resolubilized receptor (**see 4.2.3.**). High apparent binding was observed in *E. coli* polar lipids (EPL) and artificial PC/PE mixtures supplemented with cholesterol by using resolubilized ETA receptor directly without any further purification or detergent exchange step at protein-to-lipid ratios (P/L-ratios) of either 1:350 (40-60 % activity) or 1:1,000 (30-40 % activity) (Table 12). This leads to the assumption that dilution of initial protein concentration due to purification reduces the overall reconstitution efficiency, as ratios higher than 1:1,500 on average resulted in considerably decreased activity (Fig. 27). Very efficient also proved to be the application of the very mild fluorinated surfactant F_6-TAC which is supposed not to induce disintegration of liposomes (Park et al. 2007) and can be removed by Biobeads thus presumably allowing easier incorporation of MPs into liposomes. However, using F_6-TAC as protein detergent, the liposome-destabilizing detergent seemed to have a tremendous influence on total protein incorporation. While 50 % binding above background was detected with Triton X-100, DDM only generated low specific binding of ETA (Fig. 27).

A remarkable radioactive signal/background-ratio of 60 % was achieved using extracted eukaryotic PCs from soybean (aso-PC type IV-S) for the formation of proteoliposomes from LPPG resolubilized ETA receptor (Fig. 27).

To further determine the equilibrium dissociation constant K_D in saturation binding experiments as well as half maximal inhibitory concentration values (IC50) in homologous competition experiments of *in vitro* reconstituted receptor with radioactively labelled and unlabelled ET-1, the most promising conditions were chosen (highlighted in bold writing in Table 12).

In addition to the evaluation of reconstitution efficiency by activity measurements, one sample of ETA reconstituted into EPL enriched with 5 % cholesterol at a protein-to-lipid ratio

(P/L-ratio) of 1:700 could be further analysed by freeze-fracture EM in cooperation with Friederike Joos (MPI of Biophysics, Frankfurt/Main) to determine the incorporation efficiency of ETA (Fig. 28).

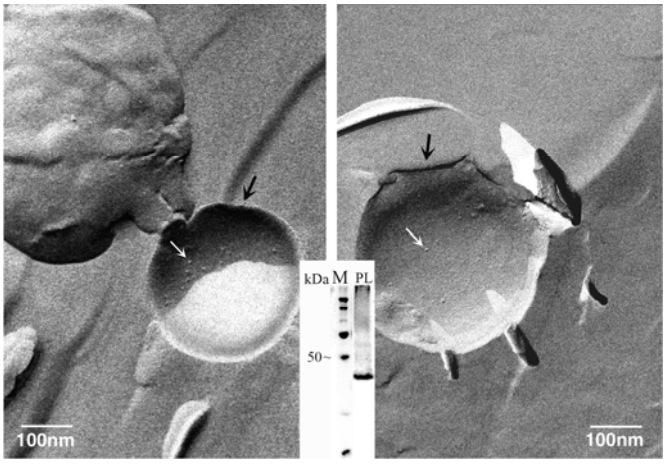


Fig. 28: Freeze-fracture analysis of ETA proteoliposomes. Freeze-fracture EM was performed with P-CF produced ETA reconstituted into *E. coli* polar liposomes enriched with cholesterol after sucrose gradient purification. Liposomes are indicated by black arrows. White arrows indicate reconstituted protein particles. Inset: Immunodetection of ETA in the analysed proteoliposome sample by western blotting using anti-His-tag antibodies after separation by SDS-PAGE. M, marker; PL, proteoliposome sample (10 µL).

Freeze-fracture analysis of reconstituted ETA revealed relatively low but detectable reconstitution efficiency with some 5–10 particles per liposome in approximately 10 % of total liposomes. The homogenously dispersed particles indicated non-aggregated insertion as should be expected for a functional reconstitution. The ETA receptor incorporation into the proteoliposomes was further verified by immunodetection of the C-terminal poly$(His)_{10}$-tag (Fig. 28). This observed low incorporation along with the likewise low activity (Table 12) illustrates the necessity of extensive optimization.

4.3.2. Ligand binding of ETA reconstituted into lipid bilayers: Homologous competition

According to the experimental set-up (**see 3.9.2.** and **3.9.3.**) and to the evaluation of the most promising reconstitution conditions, ETA was reconstituted into *E. coli* polar lipids supplemented with cholesterol at P/L-ratios of 1:1,000 and 1:350 (**see 4.3.1.**).

The functional reconstitution of ETA into proteoliposomes was analysed in radioassays by homologous competition experiments with unlabelled wild type ET-1. Three independent data sets were analysed to calculate the IC50 of cyclic wild type ET-1, needed to displace a given concentration of ^{125}I-ET-1 on CF produced ETA receptors.

Unlabelled ET-1 was able to inhibit ^{125}I-ET-1 binding to the ETA proteoliposomes in all three experiments. By using non-linear regression and a one-site binding model, half- maximal inhibition could be observed resulting in individual IC50 values of 1.675 µM, 2.218 µM and 2.614 µM with consistent R^2 values from 0.98 to 0.99, indicating that the used model fits well the data. By combination of the three data sets a final IC50 value of 2.17 µM ± 0.471 for the ETA receptor was determined (Fig. 29).

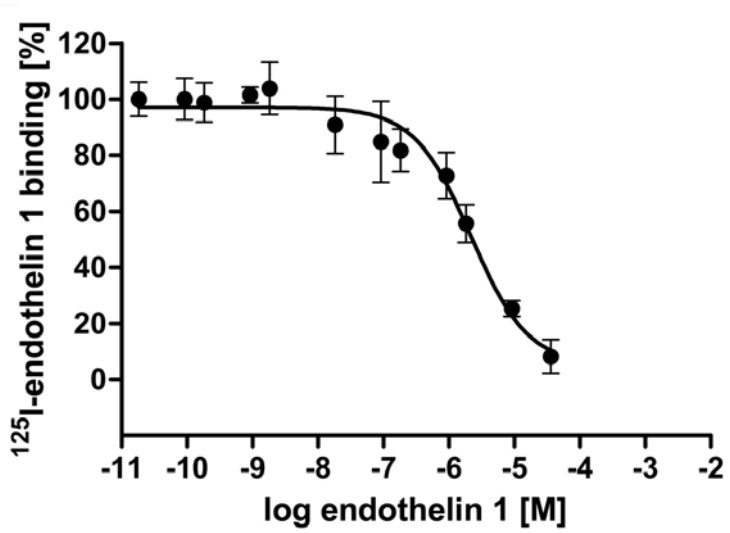


Fig. 29: Radioligand competition binding of CF produced ETA receptor. Homologous competition experiments were performed with P-CF produced ETA receptors reconstituted into *E. coli* polar lipids enriched with cholesterol. Proteoliposome preparations were incubated with 500 pM ^{125}I-ET-1 and with increasing concentrations of unlabelled ET-1 for 1 h at 22 °C. Data points out of three independent determinations.

This calculated value is approximately 10^3 times higher as compared with competition observed in experiments with *in vivo* produced, membrane-integrated human ETA, ranging in between 0.01-10 nM (Desmarets and Frelin 1999, Sakamoto et al. 1993).

As the specificity of the experimental set-up itself was already verified by an appropriate positive control (**see 4.3.**) a possible source of this low IC50 was associated with the quality of used competitor. In order to exclude experimental errors such as miscalculation of the competitor stock concentration caused by degradation, aggregation or misfolding due to false intra- and intermolecular cysteine bridges of the unlabelled ET-1, HPLC analysis of the peptide on an RP-18 column was conducted in cooperation with the group of Prof. Tampé (Institute of Biochemistry, Goethe University, Frankfurt/Main) (Fig. 30).

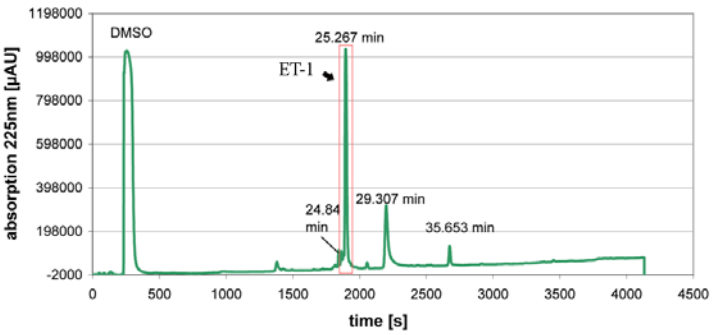


Fig. 30: HPLC analysis of unlabelled ET-1 competitor. The relevant peak for ET-1 is highlighted by a red frame. The additional peaks could be verified as column impurities by an empty control gradient. 25 µg of peptides were analysed in each run.

Non-aggregated, pure peptide ligand should elute at around 25 min (personal communication from Dr. Michael Beyermann, FMP, Berlin) which could be verified (Fig. 30). No aggregation and degradation were observed as the additional peaks at 29.303 min and 35.653 min were allocated to column impurities by an empty buffer gradient. Comparison of the peak areas under the curve of ET-1 with a standard peptide further supported the calculated stock concentration of the unlabelled ET-1.

4.3.3. Ligand binding of ETA proteoliposomes: Saturation binding of ^{125}I-ET-1

Saturation binding of ^{125}I-ET-1 to proteoliposomes was performed to investigate the equilibrium binding constant K_D of CF produced and *in vitro* reconstituted ETA. Significant unspecific background binding presumably caused by the hydrophobicity of the ligand ET-1 tremendously decreased the detected signal-to-noise ratio which caused problems during measurements and evaluation. However, three different *in vitro* reconstitution protocols (Fig. 27) resulted in signal/background ratios sufficient for further analysis (**see 4.3.1.**). Respective samples were generated of P-CF produced ETA, either resolubilized in LPPG or further detergent-exchanged to F_6-TAC upon IMAC purification. ETA was subsequently reconstituted at a P/L-ratio of either 1:350 (LPPG) and 1:1,500 (F_6-TAC) into EPL/C or at 1:1,000 (LPPG) into aso-PC liposomes from soybean. Increasing concentrations of radioactively labelled ET-1 from 0.05-5 nM were incubated with fixed amounts of ETA proteoliposomes.

Unspecific binding was determined in presence of 2 µM unlabelled ET-1 and subtracted from total ^{125}I-ET-1 binding observed in the absence of ET-1 (Fig. 31).

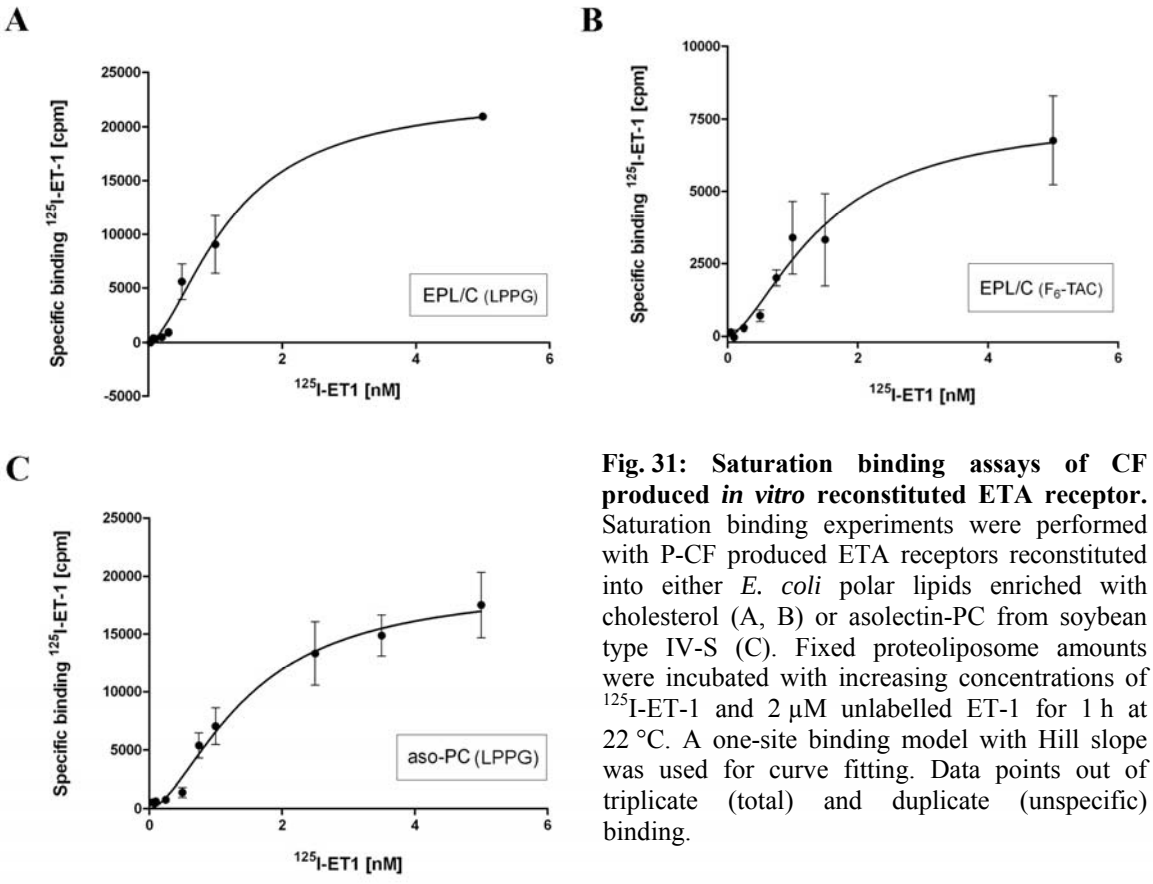


Fig. 31: Saturation binding assays of CF produced *in vitro* reconstituted ETA receptor. Saturation binding experiments were performed with P-CF produced ETA receptors reconstituted into either *E. coli* polar lipids enriched with cholesterol (A, B) or asolectin-PC from soybean type IV-S (C). Fixed proteoliposome amounts were incubated with increasing concentrations of ^{125}I-ET-1 and 2 µM unlabelled ET-1 for 1 h at 22 °C. A one-site binding model with Hill slope was used for curve fitting. Data points out of triplicate (total) and duplicate (unspecific) binding.

The three data sets were analysed by non-linear regression using a one-site model for specific binding in Graph Pad Prism 5. R^2 values of the determinations representing the reliability of the fit ranged from 0.89 (Fig. 31 B) to 0.99 (Fig. 31 A, C). Low values are likely due to the high calculated standard deviations. For all three preparations Hill slopes of ~1.6 were observed

indicative of positive cooperativity (Fig. 31 A-C). Models which do not account for a positive cooperative effect could not be used for reliable data processing. The individually calculated K_D of 1.23 nM (A), 1.47 nM (B) and 1.49 nM (C) were highly consistent among one another supposing that the ETA receptor displays similar binding characteristics in the three evaluated conditions. An average K_D of 1.39 nM± 0.14 could finally be determined which is comparable to data gained with *in vivo* crude membranes of human tissue, still being on average one to two orders of magnitude higher.

The detected positive cooperativity not present in human tissues, in which one ligand is supposed to bind to one receptor molecule, might hint to altered binding kinetics and characteristics of the CF produced ETA receptor in these artificial environments (**see 5.3.1.**).

4.4. Co-translational reconstitution of ETA by cell-free expression in presence of lipids: L-CF mode

The L-CF expression mode in presence of supplied lipids or whole preformed liposomes offers a unique option for the co-translational insertion of MPs. Like shown for lipid molecules which are the natural environment of MPs and which are known to often act as essential modulators of folding, stability and function (Opekarova and Tanner 2003), model biomembranes (liposomes) have recently been reported to induce a variety of potential functions under variable environmental conditions such as chaperone-like behaviour and membrane fusions. They were even shown to reactivate enzyme activity under oxidative stress (Tuan et al. 2008, Umakoshi et al. 2009).

MP integration into the provided bilayers may be more directed during L-CF mode expression if compared with the random orientation in classical post-translational *in vitro* reconstitution approaches. In addition, the synthesized target will be the MP inserted into the bilayers and measured activities could thus clearly be attributed. These characteristics can have considerable benefits for subsequent functional studies (Junge et al. 2010a). Due to these potential advantages preliminary investigations of the L-CF mode as an alternative for the *in vitro* reconstitution of ETA were hence started. Initial optimization trials of co-translational reconstitution were performed and co-precipitation of ETA and lipids after expression was analysed by sucrose gradient ultracentrifugation. An indication of functional insertion of L-CF samples was finally gained by radioactive ligand binding as described in **3.9.3.**.

4.4.1. L-CF expression set-up and analysis of co-translational reconstitution of ETA

Different basic considerations have been made concerning the application of the L-CF mode for the synthesis of ETA. To address the inherent problem of low functional insertion often encountered if adding pure artificial liposomes which might result from inefficient translocation

(Kalmbach et al. 2007), detergent-destabilized liposomes were supplemented only to the RM and incubated under standard conditions (Table 9). The addition of detergents to the liposomesprior to use is thought to relax the tightly packed liposome structure without complete disintegration thereby lowering the energy barrier for a spontaneous incorporation of MPs into an artificial lipid composed environment (Shimono et al. 2009). Lipids were supplied at final concentrations of 3.5 or 4.5 mg/mL. To destabilize the liposomes prior to their addition to the RM, final concentrations of 1 % CHAPS supplemented with 0.2 % CHS were mixed with the preformed liposomes and incubated for 30 min at RT before use. Due to its high CMC of 0.5 % and its small micellar size, CHAPS can be dialysed out during the reaction leaving pure proteoliposomes as shown for bacteriorhodopsin (Shimono et al. 2009).

The influence of the lipid head group's charge was also considered for the co-translational reconstitution of ETA as translation might be considerably inhibited by cationic lipids (Bui et al. 2008, Umakoshi et al. 2009). Neutral as well as anionic lipids were furthermore necessary for the functional tetramerization of the potassium channel KscA (Van Dalen et al. 2002). Therefore, different lipids from natural sources have been analysed such as *E. coli* polar lipids and aso-PC which already proved effective in the *in vitro* reconstitution of ETA (**see 4.3.2.** and **4.3.3.**). Pure artificial PC (DPPC) and a mixture of natural aso-PC with neutral PE (DOPE) and anionic PG (POPG) were tested as well.

ETA proteoliposomes of aso-PC were harvested at 23,000 x g for 30 min at 4 °C and subsequently subjected to sucrose gradient ultracentrifugation (Fig. 32). As positive control a GFP-fused small multidrug resistance protein SugE was L-CF expressed in parallel with ETA. Proteoliposomes of ETA and SugE were supplemented with sucrose to a final concentration of 60 % and overlaid with decreasing sucrose concentrations of 40-30-20 %. Liposome-associated SugE could easily be verified by the fluorescence of the GFP-tag in the liposome layer (data not shown). P-CF produced ETA which was expressed as NC to visualize the behaviour of ETA precipitates in absence of lipids totally accumulated at the bottom of the tube. Empty aso-PC liposomes moved to the top of the gradient. Proteoliposome ETA floated to the barrier between 40 and 60 % sucrose while non-associated ETA precipitated. The layer displayed a more dispersed character than the layer of SugE presumably indicating different intergration rates (Fig. 32 A). Immunoblotting of 5 µL of the respective sucrose gradient fractions further confirmed that it was possible to separate precipitated ETA from liposome-integrated or -associated protein. Taking the intensity of the western blot signal as a measure, 30 % of total ETA were estimated to be in liposome contact (Fig. 32 B).

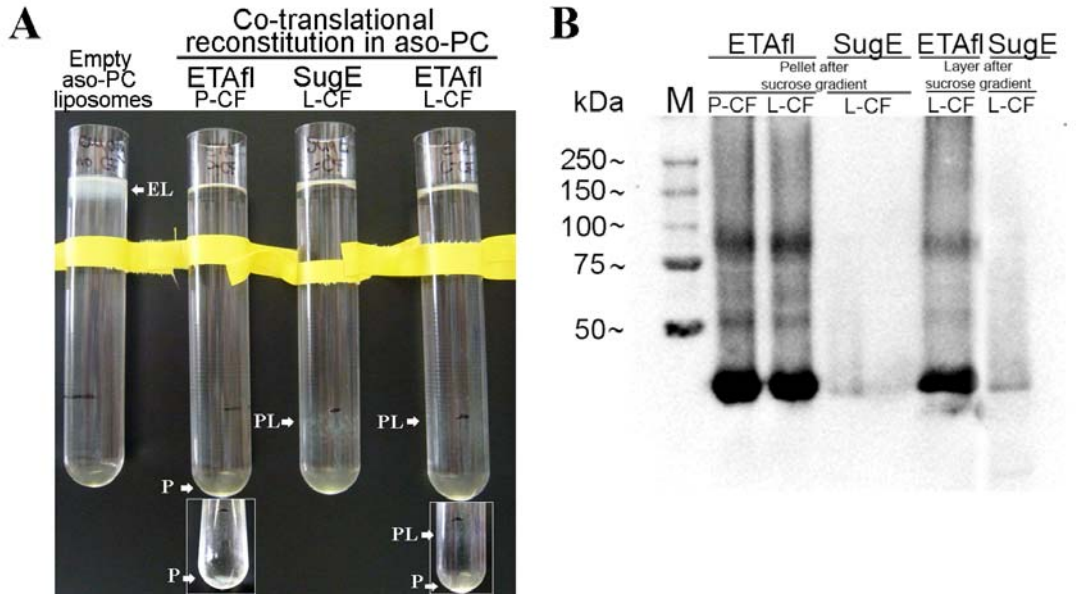


Fig. 32: Co-translational reconstitution of ETA in aso-PC liposomes. ETA was L-CF expressed in presence of 4.5 mg/mL aso-PC lipids, destabilized by 1 % CHAPS/0.2 % CHS. (A) Sucrose gradient ultracentrifugation of L-CF samples. SugE and ETA showed proteoliposome layers between 60 and 40 % sucrose. P-CF expressed ETA totally precipitated. Empty liposomes floated to the top of the tube. Insets: Maginification of respective sample tubes. (B) Verifcation of ETA in pellet and layer fractions by immunoblotting against the C-terminal poly(His)$_{10}$-tag. 5 µL were loaded on 12 % SDS-Tris-glycine gels and subsequently subjected to western blotting. By sucrose gradient ultracentrifugation it was possible to separate liposome-associated from free ETA. aso-PC, phosphatidylcholine from soybean, type IV-S; EL, empty liposomes; M, marker proteins; P, precipitate; PL, proteoliposomes.

4.4.2. Evaluation of co-translational reconstitution by ligand binding of ^{125}I-ET-1

A variety of L-CF expressed ETA samples were investigated by ligand binding in order to get information on the functional reconstitution efficiency. Since it has been experienced that the activity of ETA in proteoliposomes can significantly be decreased by sucrose gradient ultracentrifugation, samples for functional assays were only treated with centrifugation at 23,000 x g for 30 min at 4 °C. Precipitates were resuspended in either Tris buffer as used for purification (20 mM Tris, 150 mM NaCl, pH 7.0) or S30 C buffer (pH 8.2) used in the CF reaction (**see 3.3.2.**) to analyse the binding behaviour of ETA in dependency of pH and liposome composition (Fig. 33). Empty liposomes used in L-CF reactions served as NCs to determine the unspecific binding of the ligand to the liposomes themselves. ETA *in vitro* reconstituted into aso-PC-DDM (Fig. 27) characterized by a high signal/background-ratio was used as positive control. This sample displayed significantly lower specific binding after two weeks which might account for the fact that long-time storage at -80 °C might reduce the functionality of the ETA proteoliposomes (Fig. 33, first bar).

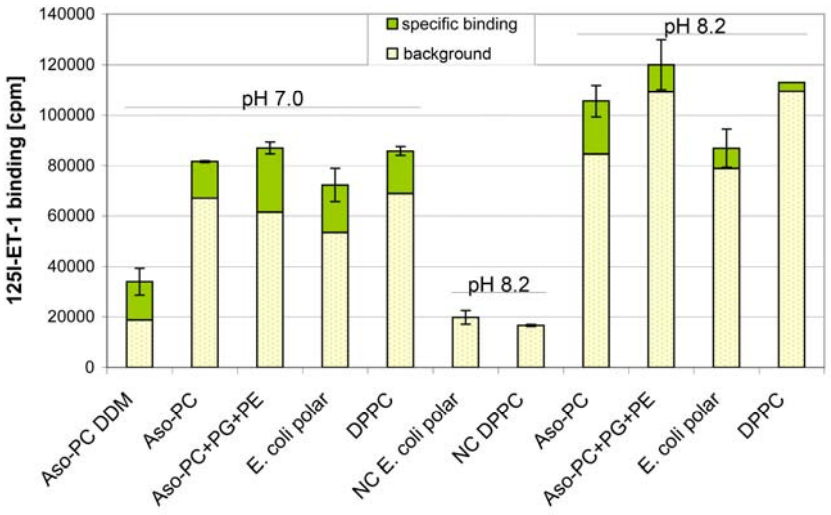


Fig. 33: Influence of lipid combinations and pH on the binding activity of L-CF expressed ETA. The abscissa describes the used lipids for the L-CF expression of ETA. A combination of neutral (PC and PE) with anionic (PG) at pH 7.0 generated the highest specific binding.

Highest specific binding was achieved for L-CF expressed ETA at pH 7.0 with a mixture of aso-PC, PG and PE which could be calculated to 30 % over background followed by *E. coli* polar lipids (26 %), DPPC (19 %) and aso-PC (17 %).

However, compared to the NCs of the empty liposomes the background binding showed significant values in all tested samples. As no sucrose gradient was performed in order to retain full binding activity, this high unspecific binding might be due to the remaining ETA precipitate. Sucrose gradient ultracentrifugation (**see 4.3.1.**) could serve as a means to separate precipitate from liposome-inserted receptors. ET-1 potentially displayed unspecific binding to non-integrated ETA. The resuspension of the proteoliposomes in S30 C buffer (pH 8.2) even aggravated the problem of high background delineated before. A high pH should therefore be avoided for the determination of ligand binding from L-CF produced ETA. Competition or saturation binding studies were excluded at this point since the observed signal/background ratio was too low to determine reliable activity data.

4.5. Functional analyses of the detergent-solubilized ETA

4.5.1. Ligand affinity chromatography of detergent-solubilized ETA receptor

Affinity chromatography of solubilized GPCRs with immobilized ligands is a key tool to quantify and to isolate the homogenous, ligand binding competent fraction of a sample. Amounts of 0.33-0.67 nmol of ETA samples were incubated with monomeric avidin agarose beads pre-coated with b-ET-1 ligand. The receptor was produced in the P-CF or in the D-CF mode, purified, subsequently incubated with the prepared b-ET-1 affinity material and competitively eluted with biotin after incubation for 2 h at 22 °C with gentle shaking at 180 rpm (Fig. 34).

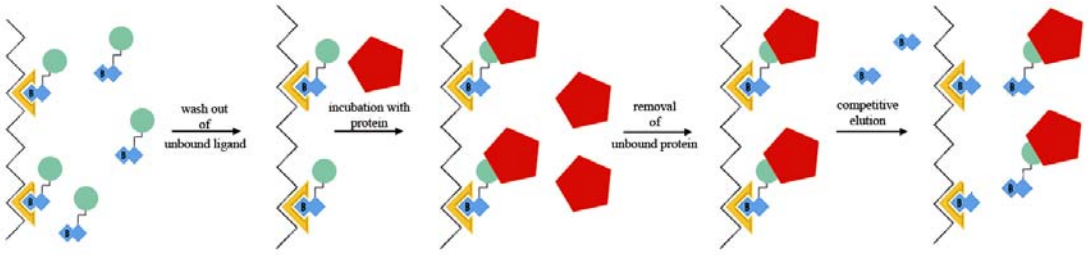


Fig. 34: Flowchart of the ligand affinity chromatography with biotinylated ligand and solubilized receptor. Firstly, monomeric avidin agarose beads were saturated with biotinylated ligand. Unbound ligand was subsequently washed out and the matrix was incubated with selected protein preparations. Unbound protein is removed by washing steps followed by the competitive elution of ligand-bound protein with an excess of biotin.

An array of NCs were performed to ensure the specificity of the binding assay prior to evaluation of different ETA receptor preparations. Those control experiments were focused on binding of the receptor to the empty matrix material, binding of unrelated MPs to b-ET-1 or interaction of ETA with unrelated biotinylated ligands (Fig. 35 A). ETA resolubilized in 1 % LPPG and IMAC purified in 0.1 % B35 did not bind to the empty, non-ligand saturated, monomeric avidin agarose beads since all protein could only be detected in the flow-through and the wash fractions. Therefore, unspecific cross-interaction of the matrix with the receptor could be excluded (Fig. 35 A, 1st line). The seven TMS containing light-driven proton pump proteorhodopsin (PR), kindly provided by Sina Reckel (AK Dötsch, Institute of Biophysical Chemistry, Goethe University, Frankfurt/Main), was D-CF produced in presence of its cofactor retinal, and exchanged to 0.04 % DDM. Functional folding of the obtained proteorhodopsin sample was indicated by its purple colour (data not shown). In two independent experiments the PR sample did not bind to the immobilized b-ET-1 peptide and the protein quantitatively eluted in the flow-through and wash fractions (Fig. 35 A, 2nd line). Accordingly, ETA P-CF expressed, LPPG resolubilized and IMAC purified to 0.1% B35 did not bind to the immobilized biotinylated peptide vasopressin (b-vasopressin) specific for the vasopressin receptor (Fig. 35 A, 3rd line). P-CF expressed ETB, generating low quality receptor (Klammt et al. 2007b, 2007c), was resolubilized in 1 % LPPG and exchanged to 0.1 % B78 upon IMAC purification. An estimate of only 5–10 % of the ETB sample bound to the column. The major unbound fraction was observed in the flow-through and in the washes (Fig. 35 A, 4th line).

Depending on the selected conditions for the ETA preparation, different degrees of apparent binding competence could be observed upon detergent evaluation (Fig. 35 B). High apparent ETA binding competences of approximately 50 % were found after D-CF expression in presence of 0.5 % B35 followed by exchange to 0.04 % DDM/0.0005 % CHS (Fig. 35 B, 1st line) and after P-CF expression and resolubilization in 1 % LPPG followed by an exchange to 0.1 % B35 (Fig. 35 B, 2nd line). In two and six independent experiments, respectively, about half of the receptor was removed in the flow-through fraction and during the washing steps, while the other half was competitively eluted with biotin. No elution at all was detected after D-CF

expression in 0.5 % B35 and reduced to 0.1 % B35 upon IMAC purification (Fig. 35 B, 3rd line). This experiment was repeated twice with different receptor preparations. The sample presumably remained on the matrix due to its apparent high aggregation state (Fig. 19 A, 21, 22). P-CF generated ETA, resolubilized in 1 % LPPG and exchanged to 0.005 % Fos-16 showed a binding competence of approximately 20-30 % (Fig. 35 B, 5th line).

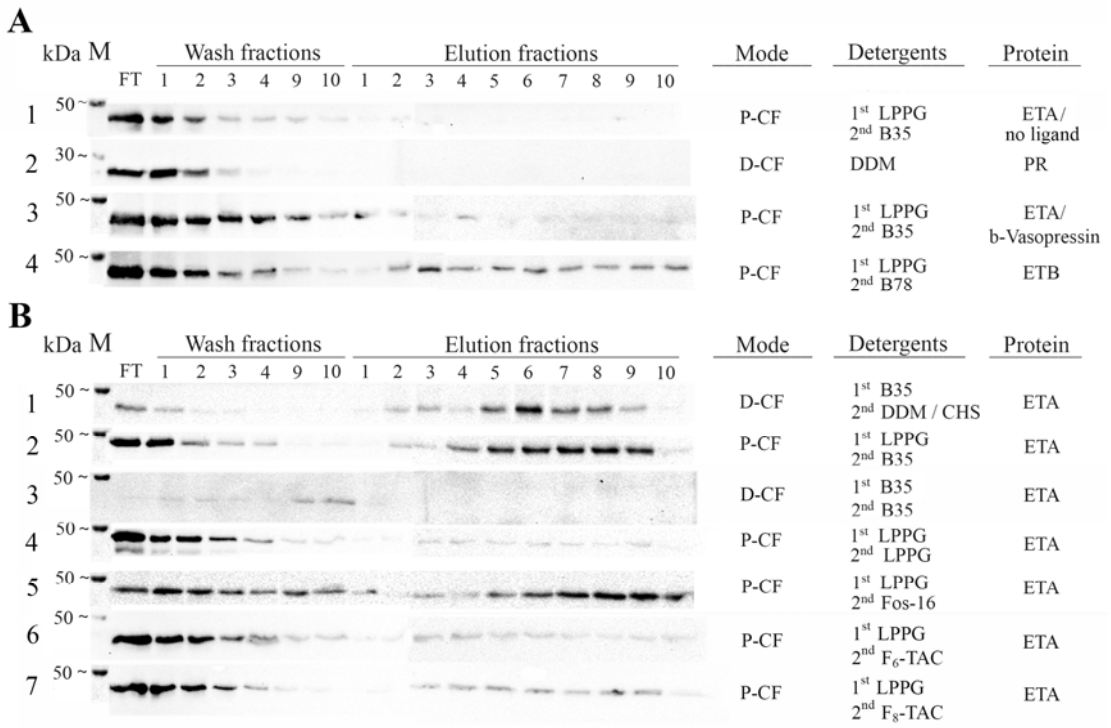
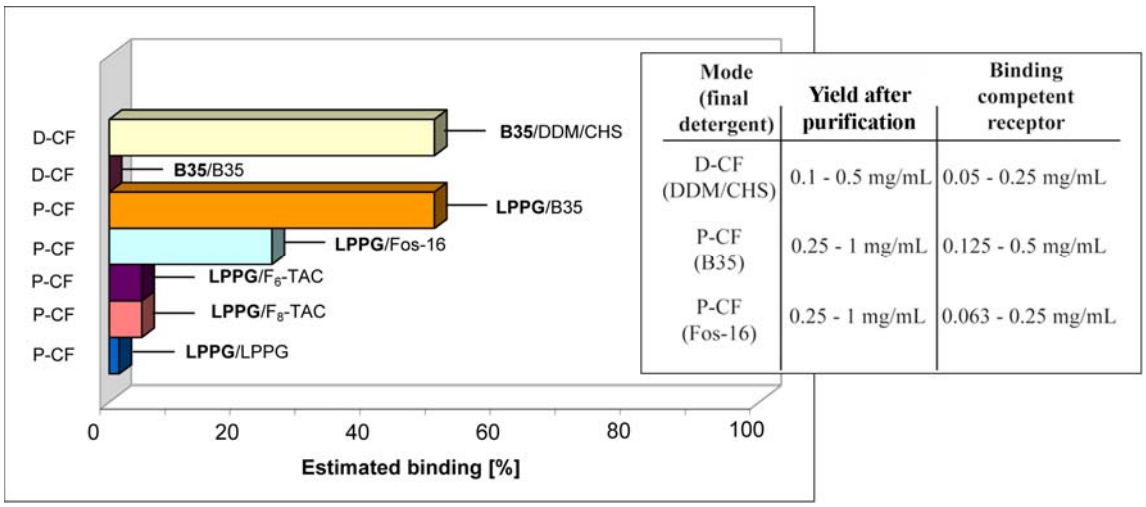


Fig. 35: Ligand binding immunodetection of CF produced ETA (51 kDa) and ETB (49 kDa). ETA was either expressed in the P-CF or in the D-CF mode and detergent exchanged upon IMAC purification. PR and ETB were both D-CF expressed and exchanged to the indicated 2nd detergent. Samples of 32.5 and 55 µL were separated by 12 % SDS–PAGE followed by western blotting and immunodetection with anti-His-tag antibodies. (A) lines 1–4: Controls for the specificity of the experimental conditions. Line 1: P-CF expressed ETA in 1 % LPPG/0.1 % B35 incubated with empty matrix material. Line 2: D-CF expressed proteorhodopsin (27 kDa) in 0.04 % DDM. Line 3: P-CF expressed ETA in 1 % LPPG/0.1 % B35 incubated with immobilized b-vasopressin. Line 4: P-CF expressed ETB in 1 % LPPG/0.1 % B78. (B) lines 1-7: ETA ligand binding efficiency after different CF expression and solubilization conditions (1st detergent/2nd detergent). Line 1: D-CF expressed ETA in 0.5 % B35/0.04 % DDM/0.0005 % CHS. Line 2: P-CF expressed ETA in 1 % LPPG/0.1 % B35. Line 3: D-CF expressed ETA in 0.5 % B35/0.1 % B35. Line 4: P-CF expressed ETA resolubilized in 1 % LPPG/0.05 % LPPG. Line 5: P-CF expressed ETA in 1 % LPPG/0.005 % Fos-16. Line 6-7: ETA P-CF expressed and resolubilized in 1 % LPPG/ 2 mM fluorinated surfactants, F_6-TAC and F_8-TAC, respectively. M, marker; FT, flow-through; CHS, cholesteryl hemisuccinate; PR, proteorhodopsin.

The binding competence decreased if the 1 % LPPG of the P-CF produced ETA receptor was reduced to 0.05 % LPPG upon purification. The vast majority of P-CF produced ETA in 0.05 % LPPG did not bind to the affinity material and was washed out before elution which was verified by two repetitions. Only an estimate of 1-2 % of the sample could be recovered in the elution fractions (Fig. 35 B, 4th line). The very mild fluorinated surfactants F_6-TAC and F_8-TAC did only allow a low binding competence of P-CF expressed ETA and resolubilized in 1 % LPPG. In two independent experiments a maximum of estimated 5 % of ETA was found to bind specifically to the ligand (Fig. 35 B, 6th and 7th line).



Mode (final detergent)	Yield after purification	Binding competent receptor
D-CF (DDM/CHS)	0.1 - 0.5 mg/mL	0.05 - 0.25 mg/mL
P-CF (B35)	0.25 - 1 mg/mL	0.125 - 0.5 mg/mL
P-CF (Fos-16)	0.25 - 1 mg/mL	0.063 - 0.25 mg/mL

Fig. 36: Ligand binding competence of the ETA receptor with different expression protocols. Upon calculation of expression and purification efficiencies in mg/mL the relative amount of active receptor in the three best conditions was determined.

Considering initial optimal yields of purified ETA of approximately 1 mg/mL after P-CF expression and 0.5 mg/mL after D-CF expression, corresponding amounts of some 500 and 250 µg ligand-binding competent ETA could finally be isolated out of 1 mL RM with the described optimal conditions (Fig. 36).

The difference in SDS-PAGE stability of the CF expressed ETA or ETB receptor/ET-1 complex which has been discussed in literature (Takasuka et al. 1992, 1994) was further analysed after ligand affinity chromatography. Therefore, selected elution fractions of both receptors were subjected to SDS-PAGE followed by immunoblotting against the biotin label of the ligand ET-1. In contrast to the CF expressed human ETB receptor, which formed a stable complex during SDS-PAGE with the b-ET-1 peptide as observed in present work, the ETA/b-ET-1 complex eluting from the monomeric avidin matrix could not be verified (Fig. 37). This observation is in accordance with published data suggesting a complex of higher stability during gel separation for ETB/ET-1 than for ETA/ET-1 and even the two bands appearing in the D-CF expressed ETB sample occurred by separation of native ETB receptor (Takasuka et al. 1992, 1994). These findings might point at a comparable behaviour of detergent-solubilized ET receptors obtained from either *in vivo* material or CF expression.

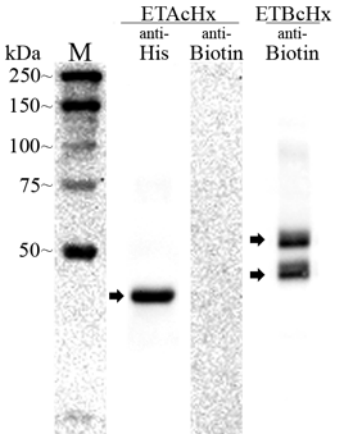


Fig. 37: Analysis of ligand-bound receptor complexes by immunodetection of the biotin-label. Elution fractions of momomeric avidin matrix affinity chromatography were subjected to 12 % SDS-Tris-glycine-PAGE and subsequently immunoblotted. M, marker proteins.

4.5.2. Iodination of the linear peptide 4-Ala-ET-1 with ^{125}I: Binding experiments with detergent-solubilized ETA by centrifugation-assisted gel filtration

A further aim was to determine the equilibrium binding constant of detergent-solubilized ETA/ET-1 complexes in order to compare CF synthesized protein to published functional data of ETA from *in vivo* crude membrane preparations. Centrifugation-assisted gel filtration with radioactive ligands appeared to be a promising approach for the ETA receptor as it was already successfully used GPCRs (Grisshammer et al. 1999).

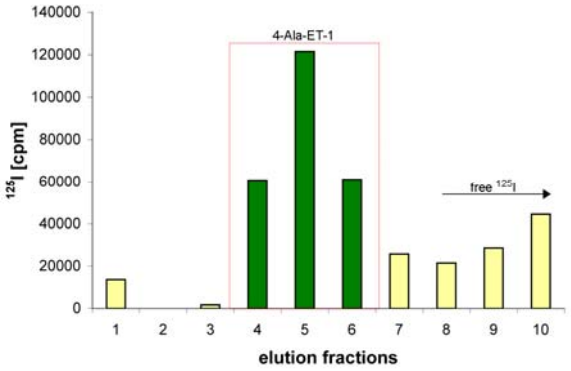


Fig. 38: Iodination of 4-Ala-ET-1. 2 µL of each collected elution fraction was measured in a gamma counter. Fractions with highest detected counts were pooled (green).

Introduced into iodination and supervised by PD Dr. Rupert Abele and Christian Schölz (group of Prof. Tampé, Institute of Biochemistry, Goethe University, Frankfurt/Main) the linear mutant of ET-1 in which all cysteines are replaced by alanines (4-Ala-ET-1) was iodinated with ^{125}I at the single tyrosine 13 of the ligand. In the iodination reaction, 4-Ala-ET-1 was chosen instead of wild type cyclic ET-1 as disulfide bridges were likely to be modified during the procedure which could introduce heterogeneity (**see 3.9.1.**).

^{125}I-4-Ala-ET-1 was separated from free ^{125}I by gel filtration on Sephadex G-10 spin columns and fractions with highest radioactive counts were pooled (Fig. 38). The initially applied ligand achieved a radioactive concentration of approximately 25-60 µCi/mL. The radioactively labelled 4-Ala-ET-1 was used for saturation binding experiments of detergent-solubilized ETA by centrifugation-assisted gel filtration.

50 nM of D-CF expressed ETA receptor purified in 0.04 % DDM supplemented with CHS were incubated with increasing concentrations of ^{125}I-4-Ala-ET-1 as delineated in detail in experimental procedures (**see 3.9.4.**).

The parameters for centrifugation such as speed and time were optimized with fluorescently labelled 4-Ala-ET-1 (data not shown). Proteomicelles in complex with bound ligand were separated from free ligand. While the free ligand should be retained on the matrix the receptor/ligand complex was expected in the flow-through of the centrifugation.

Using non-linear regression and a one-site binding model, total binding as well as unspecific binding could be determined in only one representative experiment resulting in a K_D of 614 nM which is in the range of literature values (Fig. 39). All the other attempts remained unsuccessful due to the tremendous background noise.

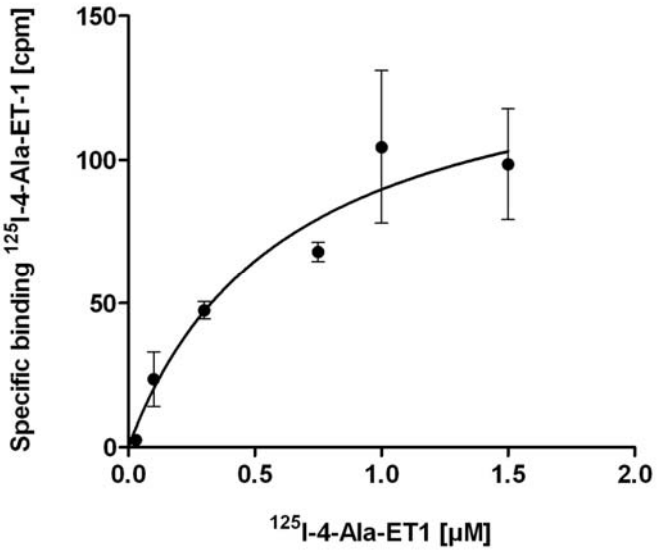


Fig. 39: Evaluation of ^{125}I-4-Ala-ET-1 binding to ETA/DDM/CHS proteomicelles. 50 nM of protein were incubated with increasing concentrations (0.03-1.5 µM) of labelled ligand. Unspecific binding was determined with 100-fold excess of unlabelled ligand. Each data point was determined in duplicates.

The observed specific binding over background was extremely low with high standard deviations resulting in R^2 values of 0.95 limiting the reliability of the acquisitioned data. This might on the one hand be attributed to a tremendously reduced amount of binding active receptor in DDM/CHS which is unlikely as around 50 % of ETA could be purified by ligand affinity chromatography (**see 4.5.1.**). More likely, experimental errors are causative for such a loss of signal intensity as this assay highly depends on the interplay of a variety of parameters like centrifugation efficiency and possible cross-interaction of proteomicelles or the hydrophobic ligand with the used matrix material which leads to ineffective harvesting of binding active receptor. Therefore, centrifugation-assisted gel filtration was not continued as it does not constitute a fast evaluation method of binding activity for the ETA receptor which is needed for quality control.

4.5.3. Ligand binding of detergent-solubilized ETA monitored by fluorescence anisotropy

Binding of fluorophore-containing ligands to their receptors should restrict the mobility of the ligand and consequently of the fluorophore label resulting in increasing anisotropy signals upon titration of receptor molecules.

The binding affinity of the linear fluorescein-labelled ligand f-4-Ala-ET-1 was determined by using non-linear regression and a one-site binding model for total binding without subtraction of the background (Fig. 40). A constant amount of 30 nM f-4-Ala-ET-1 was incubated at RT for 1.5 h with increasing concentrations of P-CF expressed ETA receptor purified in 0.1 % B35. Anisotropy control measurements were performed in presence of ETA receptor in 0.1 % B35 presaturated with b-ET-1 upon binding to b-ET-1 loaded monomeric avidin agarose. The ETA in the eluted ETA/b-ET-1 complex was controlled by Western blot

analysis against the C-terminal poly(His)$_{10}$-tag of ETA (Fig. 40). The observed anisotropy signal was normalized by subtraction of the anisotropy value of 0.038 obtained from free f-4-Ala-ET-1 in assay buffer.

The increase of anisotropy upon titration of ETA to the ligand enabled the calculation of an equilibrium dissociation constant K_D of 2.25 µM. Considering that approximately 50 % of ETA is in a ligand binding conformation, the corresponding binding constant could be corrected to a value of about 1.2 µM. Only a minor increase in anisotropy was detected with the b-ET-1 presaturated ETA sample.

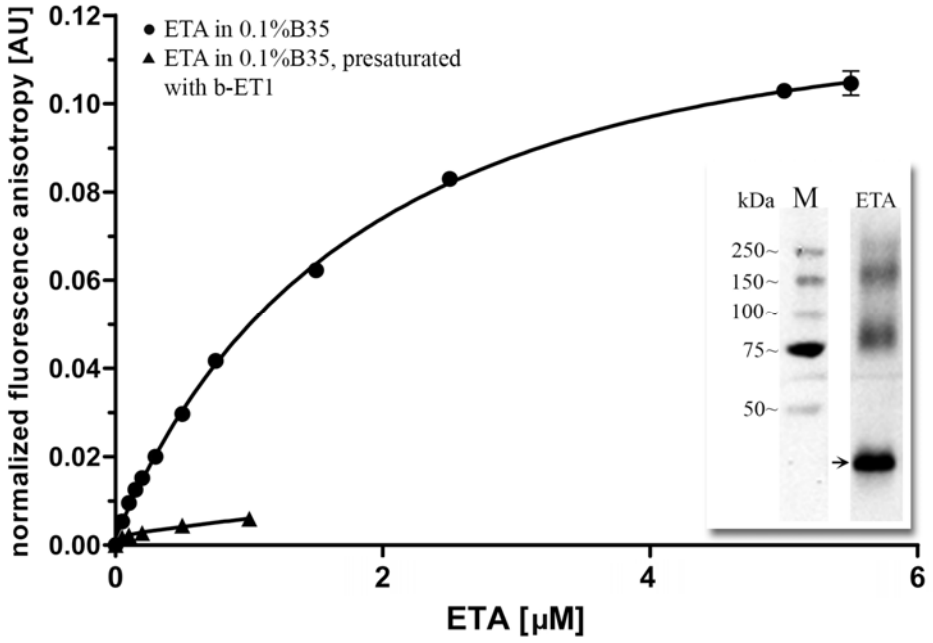


Fig. 40: Ligand binding competence of ETA/B35 proteomicelles analysed by fluorescence anisotropy. 30 nM of fluorescein-labelled 4-Ala-ET-1 was incubated with increasing concentrations of free (0-5.5 µM) (circles) or ligand-bound ETA receptor eluted from monomeric avidin agarose (0-1 µM) (triangles) for 1.5 h at RT in a final volume of 500 µL. Each data point represents a mean value of 7 repetitions. Fluorescence signals were normalized by subtracting the anisotropy value 0.038 from 30 nM of free fluorescein-labelled 4-Ala-ET-1 in assay buffer. The inset shows the detection of ETA in the elution fraction of the ligand binding column by western blotting.

4.6. Structural investigations of ETAcHx on an automated platform in 96-well formats

Crystallization trials of the CF expressed ETA receptor was performed in cooperation with Dr. Yvonne Thielmann (group of Prof. Michel, Department of Molecular Membrane Biology, Max-Planck Institute of Biophysics, Frankfurt/Main). The receptor was expressed and quality optimized by the author. The automated platform as well as crystallization expertise was provided by Dr. Yvonne Thielmann.

4.6.1. Concentration and homogeneity of CF expressed ETA

Basic crystallization parameters of ETA were analysed in 0.1 % B35 as highest yields were obtained after IMAC purification in this detergent. Therefore, starting from 6 mL P-CF reactions ETA was purified by Co^{2+}-NTA. An additional purification step by SEC or ligand affinity chromatography was first excluded in order to get sufficient amounts of protein even if homogeneity might be reduced if compared to SEC-purified protein. The protein preparation was subsequently concentrated to a volume of 100-150 µL with a protein content of 5-8 mg/mL by using ultrafiltration with a MWCO of 10 kDa. Successful concentration and homogeneity of the protein was analysed by SDS-PAGE and SEC (Fig. 41).

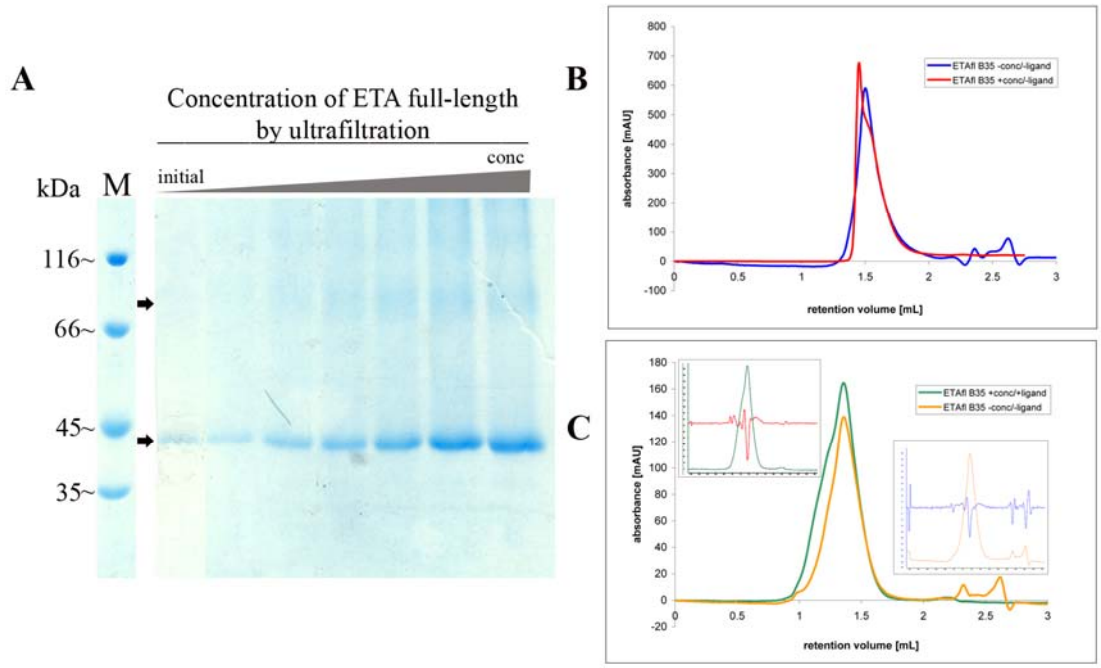


Fig. 41: Evaluation of ETA homogeneity after ultrafiltration. (A) Concentration by ultrafiltration was monitored by SDS-PAGE. Sample volumes of 1 µL of initial protein preparation and of samples collected during the concentration process were loaded on 12 % SDS-Tris-glycine gels and subsequently visualised by Coomassie staining. Arrows point at monomeric ETA and potential dimers. (B, C) The homogeneity of ETA before and after concentration was assessed by SEC on an analytical Superdex 200 3.2/30 column. Approximately 10-30 µg were analysed. (B) Concentration without addition of the native ligand ET-1. (C) Concentration in presence of ET-1. Insets: Second order differentiation of concentrated (left) and non-concentrated (right) protein. M, marker proteins.

Only a marginal loss of less than 5 % ETA could be observed upon average concentration. Furthermore, concentration of ETA to 3 mg/mL did not seem to result in excessive generation of higher- and lower-order aggregates as judged by SDS-PAGE (Fig. 41 A). SEC of ETA concentrated to 8 mg/mL in the presence of its ligand ET-1 further indicated only a minimal increase in lower-order aggregates by determination of second order differentiation if compared to the non-concentrated protein (Fig. 41 C). Those preliminary data suggest that addition of 1 % glycerol and equal moles of ligand prior to the concentration process seemed to stabilize the receptor, because concentration of ETA to over 3 mg/mL without ligand generated micro-

aggregation which could be observed by a SEC profile shift of the major fraction of protein to higher molecular weights (Fig. 41 B).

4.6.2. Set-up of basic crystallization trials for the evaluation of critical parameters for structural investigations of ETA

Initial crystallization screens were accomplished with 150 µL each of ETA samples either concentrated to 5.2 mg/mL without ligand or concentrated to 8.0 mg/mL in presence of ET-1. Using the robotic platform "CrystalMation" (Rigaku, Berlin, Germany) volumes of 100 nL protein solution were provided in 96-well plates and incubated with 100 nL drops of different buffer compositions (**see 3.10.**). 18 different commercial or custom screens in 96-well formats for soluble proteins and MPs as well as classical vapour diffusion and cubic phase trials were performed with both receptor preparations. None of them yielded crystals yet after 83 days (ETA without ligand) and 41 days (ETA with ligand). For further reference and listing of the applied screens **see 7.5.**.

At the given conditions of protein concentrations between 5 and 8 mg/mL, reduced to 50 % due to the addition of the respective buffer volumes, ETA seemed to stay soluble hardly ever reaching the point of supersaturation or precipitation. Only in about 20-30 % of the investigated conditions precipitates could be observed, suggesting that the protein concentration had to be increased in order to force ETA to precipitate by addition of various precipitants. Custom pH screens which were designed according to Koszelak-Rosenblum and collegues (2009) further did not reveal any pH preference of ETA in between pH 4.0 and pH 9.5 as precipitates occurred randomly, either at 4 °C or 18 °C. Similar observations were made for the cubic phase trials at 22 °C.

However, besides salt crystals which were obtained with the MemGold™ Screen (Molecular Dimensions Ltd., Suffolk, UK) (Fig. 42 B) changes in the precipitation state followed by the occurrence of structures of higher density could be detected in some of the drops over time, possibly pointing towards microcrystallization (Fig. 42 C, D, E). Those results were obtained with MemSys™, MemGold™ and a combination of MemStart™ and MemPlus™ (Molecular Dimensions Ltd., Suffolk, UK). Furthermore, some of the cubic phase wells showed changes in their surface which might also point to preferable crystal growth conditions over time (Fig. 42 A, time course).

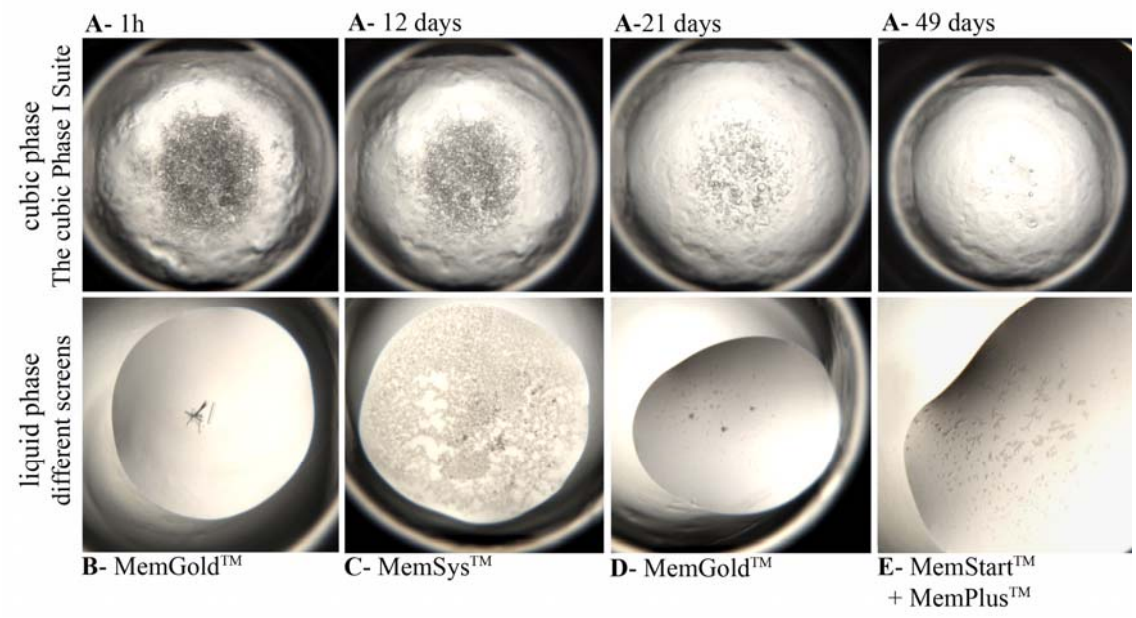


Fig. 42: ETA precipitation in different crystallization conditions. ETA was expressed in the P-CF mode, resolubilized in 1 % LPPG and subsequently exchanged to 0.1 % B35 in 20 mM Tris, 150 mM NaCl, pH 7.5 upon IMAC purification. (A) ETA was mixed with cubic phase material and incubated at 22 °C for 49 days. A clear change in the cubic phase surface could be detected. (B) Small needle shaped crystals arranged as a starlike complex indicating grown salt crystals. (C) Densified protein precipitate monitored after 83 days. (C, D,E) Possible formation of microcrystals after 83 days.

4.7. Analysis of the oligomerization potential of the CF expressed ET receptors

As delineated in the introduction, the traditional view of a GPCR monomer acting as single unit on downstream effectors has changed. Homo- as well as hetero-oligomerization is common amongst GPCRs and there is evidence of clear physiological functions for oligomerization (**see 1.3.4.** and **1.5.5.**). A variety of experimental data mostly determined by FRET and co-immunoprecipitation on ET receptors in membranes of cultured cells also propose homo- and heterodimerization of both ET subtypes (**see 1.5.5.**).

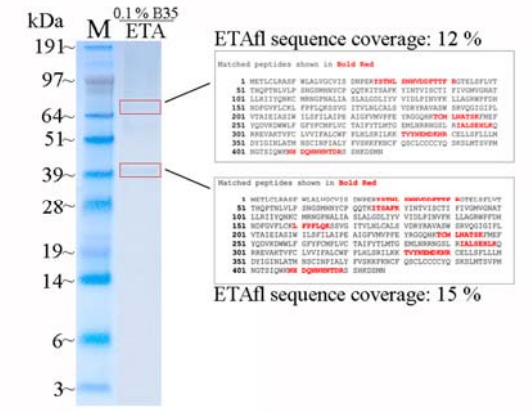


Fig. 43: Verification of ETA oligomers by ESI-mass spectrometry. 1.25 µg of purified ETA were loaded on precast 11 % Bis-Tris gels, bands excised, digested and subjected to mass spectrometry. M, SeeBlue Plus-2 marker proteins.

In this study, ETA seemed to form dimers and lower order oligomers in certain buffer conditions as observed in gel filtration profiles of LPPG and B35 or MALS analysis of ETA in B35 micelles (Fig. 20). Likewise, protein bands with molecular weights corresponding to lower order oligomers or dimers of full-length ETA which remain stable even during SDS-PAGE could be verified in cooperation with Dr. Julian Langer (group of Prof. Michel, Department of

Molecular Membrane Biology, Max-Planck Institute of Biophysics, Frankfurt/Main) by peptide mass fingerprinting (**see 3.7.4.**) in B35 with 12-15 % sequence coverage (Fig. 43).

To characterize oligomerization of CF expressed ETB, Dr. Christian Klammt prepared a variety of truncations of the receptor which he used to analyse *in vitro* homo-oligomerization by different pull-down assays (**see 1.6.**) (Klammt et al. 2007c). Following these experiments on the ETB receptor three different ETA truncations were generated in order to analyse both homo- and hetero-oligomerization of ETA and ETA with ETB by pull-down assays taking advantage of poly(His)$_{10}$-tag as well as strep-tag for cross-purification (Fig. 44 A).

4.7.1. Expression, purification and gel filtration analysis of CF expressed ETA truncations

Protein synthesis resulted in the ETA variants with C-terminal poly(His)$_{10}$- or strepII-tags. The truncations ETA197cHx (221 aa, 24.7 kDa) and ETA197strep (219 aa, 24.4 kDa) comprise the first three TMS of the full-length ETA receptor. ETA198strep (158 aa, 29.9 kDa) consists of the last four TMS.

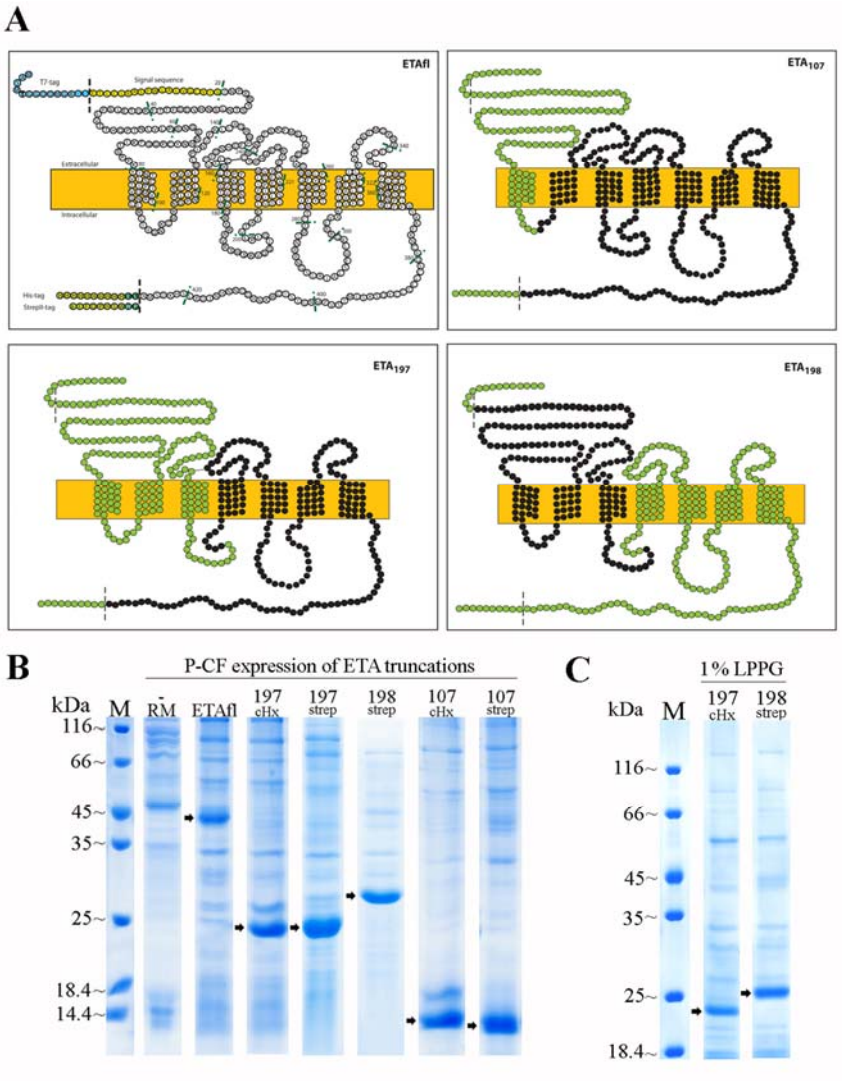


Fig. 44: Designed ETA truncations for the analysis of ETA/ETB oligomerization. (A) Predicted secondary structure of truncated ETA constructs compared with the full-length ETA receptor. The black part of the full-length protein, depicted in all truncations, was removed from the sequence leaving the green part. (B) P-CF expression screen of ETA truncations. 3 µL of RM were loaded, separated on 16 % SDS-Tris-glycine gels and subsequently Coomassie stained. (C) Resolubilization of P-CF expressed precipitates in 1 % LPPG. 3 µL were loaded on 12 % SDS-Tris-glycine gels and Coomassie stained. Arrows point at the expressed or resolubilized truncations. M, marker proteins; -RM, control RM before expression.

Expression of ETA107cHx (133 aa, 14.5 kDa) and ETA107strep (131 aa, 14.5 kDa) resulted in constructs comprising the N-terminal region and first TMS of the full-length receptor (Fig. 44 A).

All constructs were successfully expressed in the P-CF mode with final yields in the mg range per mL RM (Fig. 44 B). Exemplified with ETA197cHx as well as with ETA198strep, which were used in subsequent oligomerization studies, a resolubilization efficiency of 100 % was reached for all the truncated constructs thus displaying a solubilization behaviour similar to the full-length ETA receptor (Fig. 44 C). Sample preparation for further analysis was therefore performed according to the protocols established for ETAcHx (**see 4.2.**).

Resolubilization in 1 % LPPG and subsequent affinity purification for both ETA197cHx and ETA198strep resulted in pure protein with final yields of 530 µg/mL and 250µg/mL RM, respectively, which was sufficient for interaction studies and gel filtration analysis on an analytical Superdex 200 3.2/30 column (Fig. 45 A, B, SDS-gel inset). The sample of ETA197cHx in 0.1 % B35 (~10 x CMC) displayed a very broad peak pattern after four days storage at 4 °C covering almost one third of the whole retention volume (Fig. 45 A). Likewise, it started to elute right following the void volume. The elution profile centred at approximately 1.44 mL corresponding to ~200 kDa. A prominent shoulder at 1.25 mL (~520 kDa) indicated high molecular weight which might account for the formation of higher order oligomers of a considerable part of the protein. This assumption was supported by second order differentiation of the elution curve calculated by the Unicorn 5.11 software which detected one major minimum below the peak at 1.44 mL and a couple of smaller minima below the shoulder at 1.25 mL (Fig. 45 A, inset on the right hand side).

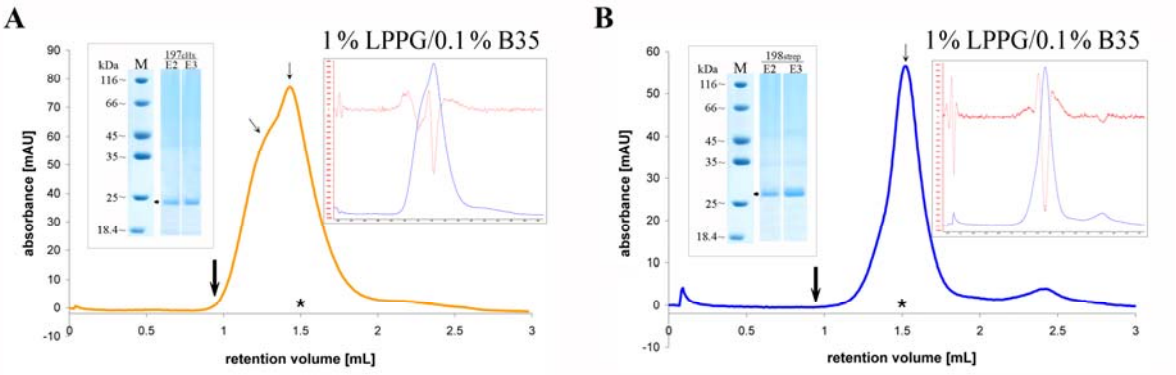


Fig. 45: Homogeneity of P-CF expressed ETA truncations exchanged to B35 upon purification. SEC elution profiles of purified ETA197cHx (A) and ETA198strep (B) in 0.1 % B35. The analysed detergents are indicated (1st detergent/2nd detergent). Samples containing 5-20 µg of protein were analysed on a Superdex 200 3.2/30 column. Protein absorbance was recorded at 280 nm. The elution peaks of ETA are indicated by thin arrows. The bold arrow indicates the void volume of the column in between 0.9 and 1.0 mL. The retention volume indicated by an asterisk corresponds to approximately 150 kDa. Grey framed insets: 10 µL of elution fractions were loaded on 16 % SDS-Tris-glycine gels and subsequently Coomassie stained. The right hand side insets comprising blue and red curves represent initially smoothed curves (blue) subjected to second order differentiation (red) in the Unicorn 5.11 software. M, marker proteins; E, elution fraction.

Due to the apparently reduced stability of the ETA197 fragment over time it was only used in fresh preparations for subsequent experiments.

ETA198strep in 0.1 % B35 showed a homogenous elution profile even after four days storage at 4 °C which centred at approximately 1.53 mL (~120 kDa) (Fig. 45 B). Supposing a micellar contribution of B35 of 50-60 kDa ETA198strep could be present as lower order oligomer and potentially display dimeric character. Second order differentiation resulted in only one major protein fraction indicating a high degree of sample homogeneity (Fig. 45 B, inset on the right hand side).

4.7.2. Pull-down experiments with full-length ET receptors and truncations

Two affinity pull-down approaches were used to investigate the oligomerization potential of the ETA and ETB receptors. On the one hand, the full-length strep-tagged ETA receptor was co-expressed in the D-CF mode with 0.5 % B35 either together with full-length his-tagged ETA and ETB or with available his-tagged ETA and ETB truncations. Those preparations were incubated with the equilibrated matrix for 2 h at RT followed by o. n. incubation at 4 °C prior to purification by strep-affinity chromatography (**see 3.5.4.**).

On the other hand, full-length receptors with poly(His)$_{10}$- and strepII-tag were expressed separately either in P-CF mode and subsequently resolubilized or produced in D-CF mode using 0.5 % B35. The single purified receptors were mixed and further treated as described above for the co-expression.

In the co-expression assay, the synthesis of the receptors and the corresponding truncations as well as the subsequent pull-down analysis was always visualised by immunoblotting against the C-terminal poly(His)$_{10}$-tag (Fig 46 A).

After loading of the co-expressed RMs on the strep-matrix, the full-length receptors ETAcHx and ETB-signcHx which lacks its signal sequence (Fig. 46 A) as well as all analysed poly(His)$_{10}$-tagged ETB truncations like ETB93a (15.4 kDa, TMS1-TMS3), ETB131 (14.4 kDa, first TMS with N-terminal part), ETB308 (18.8 kDa, TMS6-TMS7 with C-terminus) and the ETA fragment ETA197 (24.7 kDa, TMS1-TMS3 with complete N-terminal domain) (Fig. 46 B) were co-eluted with ETAstrep.

No specificity could be detected in the co-expression approaches with respect to the TM domains potentially involved in heterodimerization. All TMSs seem important for the interaction of ETA and ETB since all fragments either with or without N- or C-terminal domains bound to the ETAstrep receptor. Therefore, no special interaction interface could be confined for ETA/ETB heterodimerization at this point like it was possible for the homodimerization of ETB (Klammt et al. 2007c).

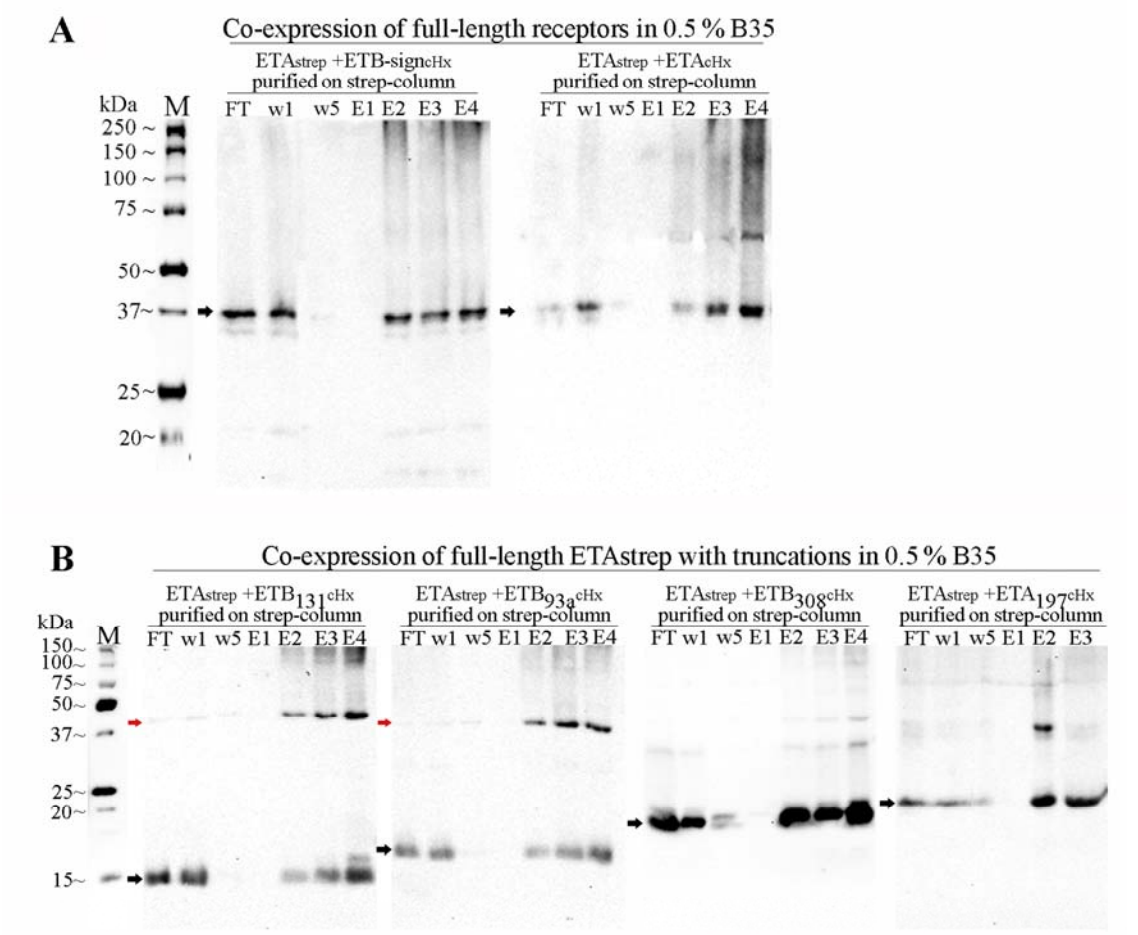


Fig. 46: Homo- and heterodimerization analysis of full-length receptors and of ETA and ETB fragments with ETAstrep. Co-expressed receptors and fragments containing a poly(His)$_{10}$-tag have been analysed by pull-down experiments on strep-tactin matrix. Co-expression of ETAstrep, ETAcHx and ETB-signcHx (A) as well as of ETAstrep and various truncations (B) was performed in presence of 0.5 % B35 and purification of the soluble part was achieved by strep-affinity chromatography. 5 µL of acetone precipitated material was subsequently loaded on 12 % (A) and 16 % (B) SDS-Tris-glycine gels and immunodetected by antibodies against the C-terminal His-tag. Black arrows indicate the relevant co-eluted proteins. Co-detection of strep-tagged ETA by a specific anti-strep antibody is represented by red arrows. M, marker proteins; FT, flow-through; w, wash fractions; E, elution fractions.

A variety of NCs were accomplished in order to investigate the specificity of the co-expression experiments. The cross-reactivity of the his-tagged full-length receptors (ETAcHx and ETB-signcHx) and a selection of truncations (ETA197cHx, ETB93acHx, ETB308cHx) with the applied strep-matrix was analysed by loading separate protein preparations of the respective receptors on equilibrated matrix material (Fig. 47 A, B). None of the analysed fragments showed any affinity to the strep-matrix alone excluding the possibility of false positives. Every poly(His)$_{10}$-tagged protein sample was already removed in the flow-through and the wash fractions.

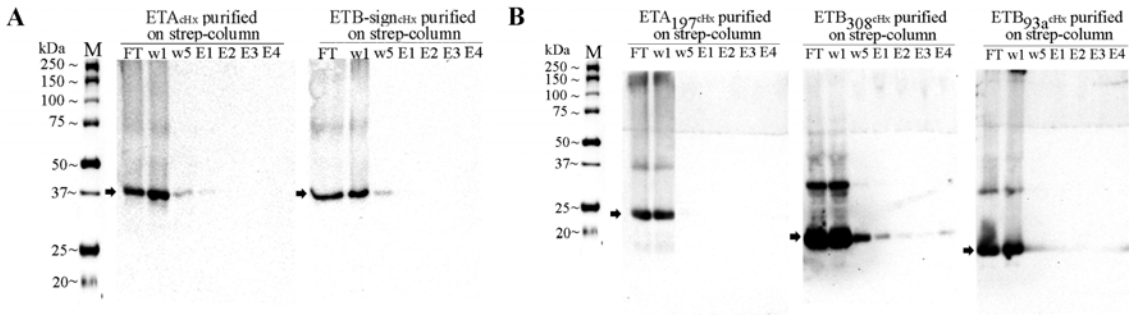


Fig. 47: Analysis of cross-reactivity of poly(His)$_{10}$-tagged ET receptor fragments with the strep-affinity matrix. Neither full-length receptors (A) nor ETB and ETA truncations (B) bound to the matrix if lacking the necessary strepII-tag. His-tagged ETA, ETB and their various truncations have been expressed in the D-CF mode in presence of 0.5 % B35. The soluble fractions of the RM have been purified by strep-tag affinity chromatography as performed with the co-expressed samples. All fractions were precipitated with acetone. 5 µL of resuspended protein samples were loaded on 12 % (A) and 16 % (B) SDS-Tris-glycine gels, respectively and subsequently immunodetected by western blotting against the C-terminal poly(His)$_{10}$-tag. Receptors and fragment monomers are indicated by arrows. M, marker proteins; FT, flow-through; w, wash fractions; E, elution fractions.

In a second NC, different unrelated poly(His)$_{10}$-tagged MPs were co-expressed with ETAstrep to analyse if the detected protein-protein interactions were restricted to the ET receptors hinting at a specific interaction mechanism or if those co-elutions relied on a more general mechanism which would also be observable upon co-expression with other MPs. The poly(His)$_{10}$-tagged N-terminal fragment of presenilin 1 (NTF, 33.25 kDa) as well as the N-terminal membrane domain of TAP-like (TMD0, 19.8 kDa), belonging to the ATP-cassette (ABC) transporter family, were chosen for the control experiments with ETAstrep.

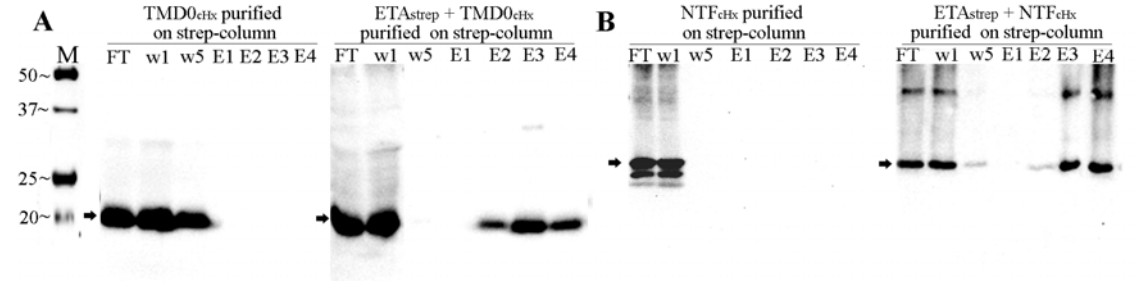


Fig. 48: Co-expression of unrelated his-tagged MPs with ETAstrep. D-CF co-expressed MPs in 0.5 % B35 containing a poly(His)$_{10}$-tag have been analysed by pull-down experiments on strep-tactin matrix. Both TMD0 (A) and NTF (B) did not display any interaction with the matrix itself but with ETAstrep as they could be co-eluted like observed for the ET receptors. All fractions were precipitated with acetone. 5 µL of resuspended protein samples were loaded on 12 % (A) and 16 % (B) SDS-Tris-glycine gels, respectively and subsequently immunodetected by western blotting against the C-terminal His-tag. MPs are indicated by arrows. M, marker proteins; FT, flow-through; w, wash fractions; E, elution fractions.

Cross-reactivity with the strep-matrix itself could again be excluded for all of the proteins (Fig. 48 A and B, left illustration). Nevertheless, co-expression resulted in a similar elution pattern for the unrelated MPs like it was already observed for the ET receptors. The selected

proteins all interacted with ETAstrep suggesting a more general mechanism underlying the detected interactions between ETA and ETB (Fig. 48 A and B, right illustration).

When expressed separately either in P-CF mode and resolubilized in 1 % LPPG or in D-CF mode in presence of 0.5 % B35, then subsequently mixed in equal volumes of RM and incubated with the strep matrix as mentioned above for the co-expression assay, only minor protein-protein interaction of the full-length ET receptors ETAstrep/ETB-signcHx could be observed (Fig. 49 A). An estimated fraction of 1-5 % of ETB-signcHx could be co-eluted with ETAstrep. Addition of the wild type ligand, often supposed to stabilize the oligomerization interface in GPCRs, did not increase the interaction potential of single ET receptors in this assay and addition of CHS during incubation did further reduce the binding capacity to the strep column for both assays (data not shown).

The separately P-CF produced truncations ETA197cHx and ETB198strep, resolubilized in 1 % LPPG and purified by Co^{2+}-chelate or strep-affinity chromatography were also analysed with respect to their mutual interaction. Following their separate purification and subsequent dialysis to remove the competing imidazole and biotin, both truncations were mixed at equal concentrations and the pull-down of ETA198strep was assessed with ETA197cHx bound to Co^{2+}-NTA beads (Fig. 49 B).

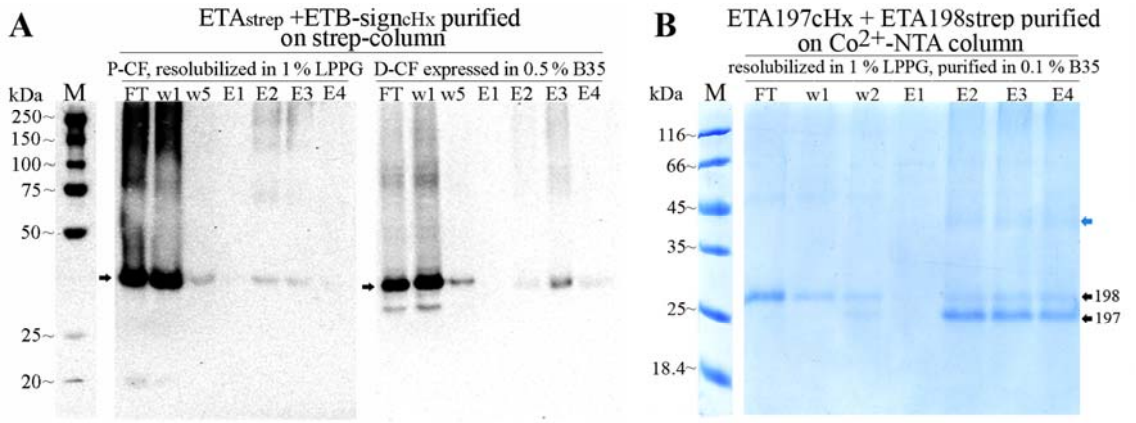


Fig. 49: Oligomerization studies with separately expressed ET receptors and ETA truncations. (A) Analysis of interaction of full-length receptors. The soluble fractions of the RM or the resolubilized samples were purified by strep-tag affinity chromatography as performed with the co-expressed samples. 5 µL of acetone precipitated fractions were loaded on 12 % SDS-Tris-glycine gels. The gels were subsequently immunoblotted and the C-terminal His-tag of ETB-signcHx was detected. Co-eluted bands of ETB-signcHx are indicated by black arrows. (B) Pull-down of ETA truncations. The purified receptor fragments were mixed at a molar ratio of 1:1 and subsequently purified by Co^{2+} affinity chromatography. 13 µL of acetone precipitated fractions were loaded on 16 % SDS-Tris-glycine gels and stained with Coomassie Blue. Black arrows point at co-eluted proteins. The blue arrow might indicate a 197/198 heterodimer. M, marker proteins; FT, flow-through; w, wash fractions; E, elution fractions.

ETA197cHx showed interaction with ETA198strep since both fragments were detected at their respective molecular weights of 24.7 kDa (ETA197cHx) and 29.9 kDa (ETA198strep) in the elution samples. In addition a third faint band at approximately 45 kDa undetectable in flow-through and wash fractions was observed in the elution fractions possibly indicating an SDS-

resistant heterodimer of ETA197cHx and ETA198strep. However, a remarkable fraction of ETA198strep was already removed in the flow-through and in the more stringent washing steps suggesting that not every ETA197cHx was able to bind to ETA198strep at the given reaction conditions (Fig. 49 B).

4.8. Cell-free expression of the human ETB receptor: Functional analysis by SPR

As SPR measurements were successfully applied for the functional investigations of CF produced detergent-solubilized ETB receptor and various truncations (Klammt et al. 2007c) this technique was used to compare ETB either generated in *E. coli* or in wheat germ extracts. SPR measurements were performed in cooperation with Dr. Michael Beyermann (FMP, Berlin, Germany) who provided all his expertise.

4.8.1. Wheat germ extract versus E. coli: Comparison of full-length ETB solubilization behaviour in the D-CF expression mode

The presented work was performed in cooperation with the lab of Vladimir Shirokov (Institute of Protein Research, Russian Academy of Sciences, Pushchino, Moscow Region, Russia). Introduced into the technique of WGE preparation and expression of ETB in the WG system by Vladimir Shirokov, the initial set-up and all detergent screenings were further carried out by Gelina Kopeina and Zhanna Afonina (PhD students, Institute of Protein Research). Wheat germ CF expression of ETB for functional studies by SPR measurements was accomplished by the author of current thesis.

The full-length ETB receptor was cloned into the pOBE-TMV vector as described in detail in experimental procedures (**see 3.1.1.**). CF synthesis was carried out in an uncoupled transcription/translation CECF system achieving highest expression rates at 3 mM Mg^{2+} and 105 mM K^{+} for the translation reaction (**see 3.3.5.**). A selection of detergents at various concentrations which already proved suitable for the D-CF expression of various MPs as well as of ETB in the *E. coli* CF system (Klammt et al. 2005, 2007b, 2007c) was analysed (Table 13).

In contrast to the *E. coli* system the long chain phosphoglycerol LMPG could be used for direct soluble expression of ETB at concentrations up to 0.04 % (Fig. 50) but apparently resulted in either degradation or two different conformational states of ETB upon synthesis as the two detected bands upon western blotting might indicate (Fig. 50, black and red arrow). An increase in LMPG concentrations further reduced the overall expression yield tremendously. Synthesis of ETB in presence of DDM almost totally abolished its expression in the WGE, supposing a hampering effect of DDM on the translation process of ETB in wheat germs. In the *E. coli* system, DDM did likewise only result in insoluble expression of human ETB. However, in contrast to the WGE system the *E. coli* system displayed a high degree of tolerance for DDM

or similar alkyl glycosides in terms of protein synthesis for a variety of MPs (Klammt et al. 2005) as well as for ETA (Junge et al. 2010b).

Table 13: Detergent tolerance of WGE for the expression of full-length human ETB. Concentrations of the respective detergents in % and x CMC as well as the detected expression level are indicated.

Detergent	Concentration		Expression level[a]	
	(%)	(x CMC)	pellet	supernatant
none	-	-	+++	-
Polyethylene glycol derivates				
Triton X-100	0.2	13.4	+	++
	0.4	26.8	++	++
	0.8	53.6	++	+
	1.3	87.1	++	+/-
Steroid derivates				
Digitonin	0.1	1.12	+++	++
	0.5	5.62	+	+++
	1.0	11.25	+/-	+++
Alkyl glycosides				
DDM	0.1	15.0	+	+/-
	0.2	30.0	-	-
	0.4	60.0	-	-
Long chain phosphoglycerols				
LMPG	0.01	4.2	+++	++
	0.02	8.4	++	++
	0.04	16.8	+/-	+
Polyoxyethylene-alkyl-ethers				
Brij 35	0.01	1.04	+++	++
	0.05	5.2	+	++(+)
	0.1	10.4	+	++(+)
	0.5	52	+	+++
	1.0	104	+	++
Brij 78	0.01	1.89	++	++
	0.05	9.45	+	++
	0.1	18.9	+/-	++
	0.5	94.5	-	+
	1.0	189.0	+/-	++
Brij 98	0.01	3.48	+++	+(+)
	0.05	17.4	++	++
	0.1	34.8	+	+++
	0.5	174.0	+	+++
	1.0	348.0	+/-	+++

[a] expression level: +++, high; ++, ++(+), medium; +, low; +/-, spurious detection; -, no yield

Best expression with highest yield of up to 1.5 mg/mL of soluble ETB receptor was performed in presence of the steroid derivative digitonin and a variety of polyoxyethylene-alkyl-ethers (Brij). 1 % of digitonin, B78 and B98, respectively as well as 0.1 % and 0.5 % B35 resulted in the highest soluble amount of ETB (Fig. 50). These data are well in accordance with the D-CF expression of ETB in the *E. coli* system in which highest amounts of detergent-solublilized ETB

were produced with 0.4-0.6 % digitonin, 2 % B58, 1 % B78 and 0.1 % B35 (Klammt et al. 2007b).

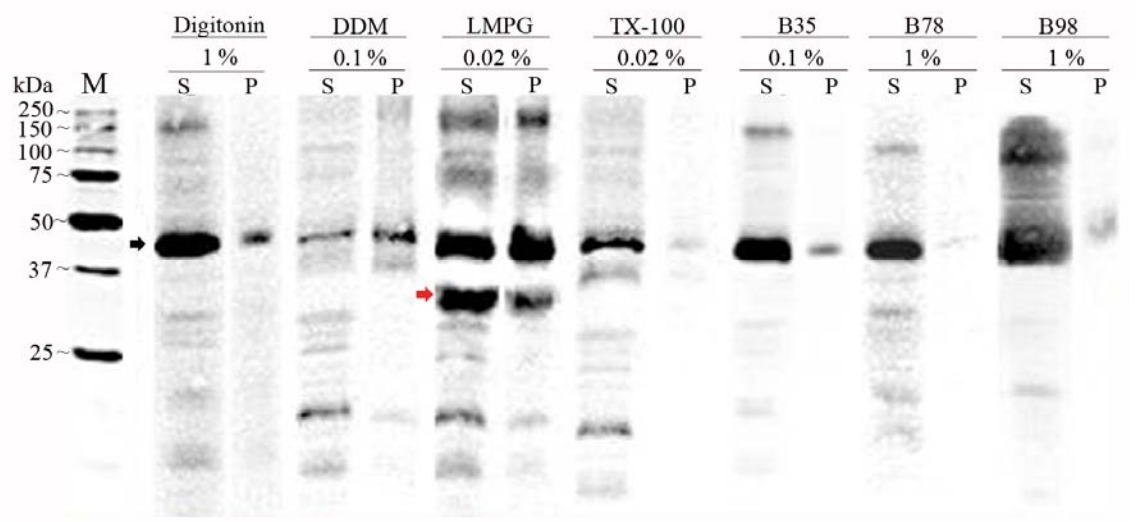


Fig. 50: CF production of soluble human ETB in an individual WGE. Western blot analysis of ETB synthesized in presence of the optimized concentrations of the respective tested detergents. Samples of supernatant and pellet fractions (3 µL) were loaded on 12 % SDS-Tris-glycine polyacrylamide gels and subsequently immunodetected by antibodies against the C-terminal poly(His)$_{10}$-tag. Black arrows indicate monomeric ETB. The red arrow points at possible degradation products or different conformations of ETB in LMPG. M, marker proteins; S, supernatant; P, pellet.

In order to compare generated functional data of WGE expressed ETB with the published and analysed data of the ETB receptor expressed in the *E. coli* system (Klammt et al. 2007b, 2007c) 1 % B78 was chosen as detergent for the expression of ETB in the WGE.

4.8.2. Functional analysis of human ETB receptors produced in different CF systems by SPR measurements

To quantitate its ligand binding affinity in real time by calculation of the equilibrium dissociation constant (K_D) via determination of both association (k_a) and dissociation (k_d) constants ($K_D = 1/K_A = k_d/k_a$) SPR measurements of detergent-solubilized ETB receptor were performed by Dr. Michael Beyermann (FMP, Berlin, Germany) as described in the experimental procedures (**see 3.9.5.**). Therefore, the human ETB receptor was D-CF expressed in presence of 1 % B78 in both implemented CF systems, *E. coli* as well as WGE. Both different receptor preparations were subsequently purified to 0.1 % B78 upon Ni^{2+}-chelate chromatography and analysed on biosensor CM-5 chips on which ET-1 was immobilized by amide coupling.

Both receptors, D-CF expressed either in *E. coli* or in WGE, seem to behave similarly with respect to their affinities to the wild type ET-1 ligand. K_D values in between 10-100 nM were determined suggesting that the ET-1 ligand binds to the receptors with high affinity (Fig. 51 A, B).

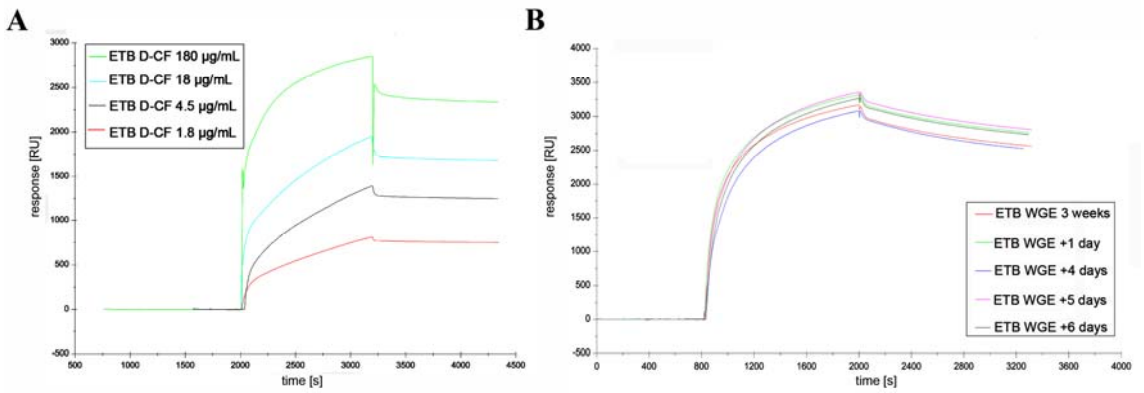


Fig. 51: SPR measurements of human ETB CF expressed in *E. coli* or WGE extracts. SPR response curves for the ET-1/*E. coli* ETB and ET-1/WGE ETB interaction using ET-1 coated CM-5 chips. 700 RU (A) and 1200 RU (B) of ET-1 have been immobilized on the chips via amide coupling and respective proteins were analysed in 20 mM Tris, 100 mM NaCl, 0.005 % P-20, pH 8.0, 0.1 % B78 at 25 °C using a flowrate of 2 µL/min. (A) ETB was D-CF expressed in *E. coli* in presence of 1 % B78, purified and titrated between 1.8 µg/mL and 180 µg/mL to investigate the concentration dependency of ligand binding. (B) ETB was expressed in presence of 1 % B78 in WGE. 120 µg/mL of purified receptor were subsequently loaded on ligand-coated chips. The stability of binding over time was analysed in a time range of days to weeks.

Moreover, the WGE receptor was very stable over time since a repetition of measurements of the same preparation after approximately three weeks resulted in comparable binding constants (Fig. 51 B). While association seems to be fast, dissociation of the ligand occurred very slowly assuming a tight binding to ETB. These values for the K_D only display approximately 1-2 orders of magnitude less affinity than those obtained from crude membrane preparations which are in between 40 pM-10 nM (Elshourbagy et al. 1993, Schiller et al. 2000, Taylor et al. 2003). Published SPR data of CF expressed ETB by Christian Klammt are comparable with values of about 7 nM (Klammt et al. 2007c). In order to analyse the consistency of the determined values and to prove the applicability of SPR to the ET receptor system, different NCs were conducted in parallel to the respective measurements. Cross-binding of *E. coli* CF expressed ETB receptor to unrelated GPCR-specific peptide ligands such as sauvagine (40 aa, 4.6 kDa) and urocortin (40 aa, 4.7 kDa), known ligands of the CRF receptor, as well as unspecific binding of the chip-bound ET-1 to two other CF expressed detergent-solubilized GPCRs, CRF and V2R, were investigated (Fig. 52 A, B, C). None of the employed, ET-1 substituting peptide ligands, showed any remarkable binding to the ETB receptor suggesting specific binding of ETB to ET-1 (Fig. 52 A).

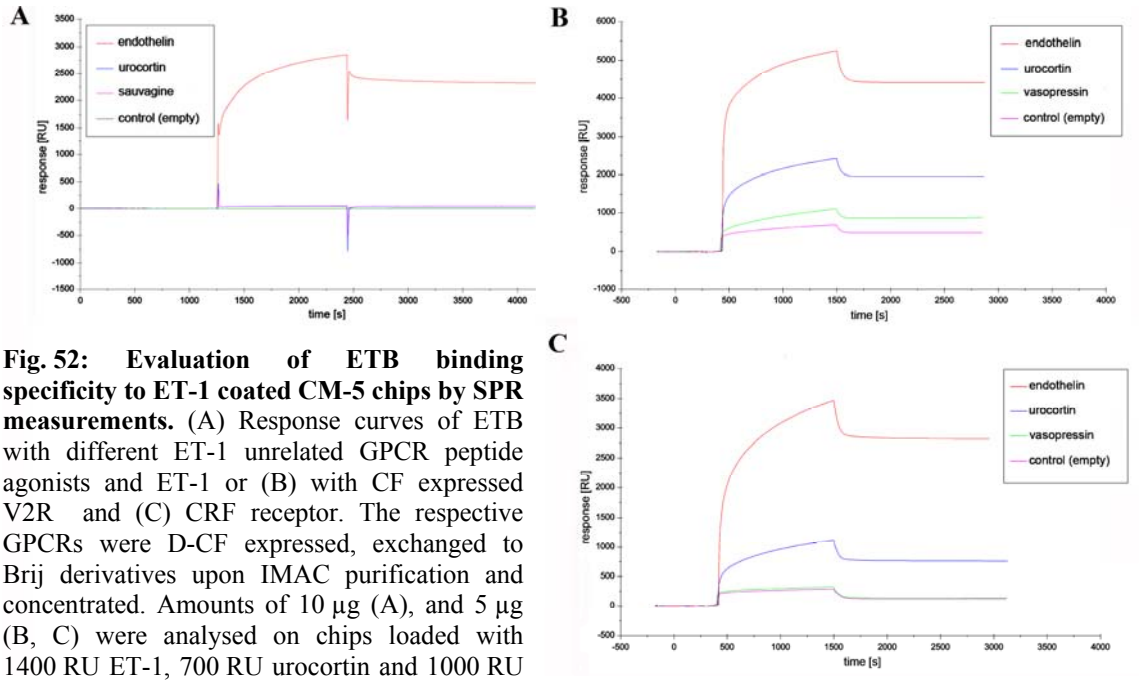


Fig. 52: Evaluation of ETB binding specificity to ET-1 coated CM-5 chips by SPR measurements. (A) Response curves of ETB with different ET-1 unrelated GPCR peptide agonists and ET-1 or (B) with CF expressed V2R and (C) CRF receptor. The respective GPCRs were D-CF expressed, exchanged to Brij derivatives upon IMAC purification and concentrated. Amounts of 10 µg (A), and 5 µg (B, C) were analysed on chips loaded with 1400 RU ET-1, 700 RU urocortin and 1000 RU vasopressin. Experiments were performed at 25 °C and a flowrate of 2 µL/min.

However, upon loading of the ET-1 coated chips with different GPCRs high cross-interaction between ET-1 and unrelated GPCRs was observed displaying comparable association and dissociation behaviour as seen for both ETB receptor preparations (Fig. 52 B, C). Those detected side-effects considerably hampered the evaluation and further questioned the reliability of the above gained binding data. Therefore, SPR data were not further used to determine binding constants, and the experimental set-up was so far excluded for future functional analysis of the ET-receptom.

4.9. CF expression of ETB intein fusion constructs for segmental isotope labelling

With regard to the reasonable quality of the first recorded liquid-state NMR [$^{15}N^{1}H$]-TROSY-HSQC spectra of the LMPG-solubilized ETB truncation ETB93a (15.4 kDa, TMS1-TMS3) performed by Dr. Christian Klammt (PhD thesis) which showed the least spectral overlap of all tested truncations, this fragment represented a potential candidate for structural investigations of a ligand-binding competent GPCR fragment (Klammt et al. 2007c) by liquid-state NMR spectroscopy.

In order to further minimize the observed peak overlap, segmental labelling of single TMSs in combination with the CF expression technique was considered for ETB93a. An interesting approach for the introduction of selectively labelled TMSs into a MP is the use of so called split inteins (Fig. 53). Those inteins are protein introns which can self-excise in an autocatalytic manner *in cis* as well as *in trans*, rejoining two flanking peptides or proteins via native peptide bond formation (Brenzel et al. 2006, Hong et al. 2008, Mathys et al. 1999, Sun et

al. 2004, Wu et al. 1998). This type of labelling would allow the structural characterization of a single protein domain in the context of a full-length protein without extensive signal overlaps generated by the other domains.

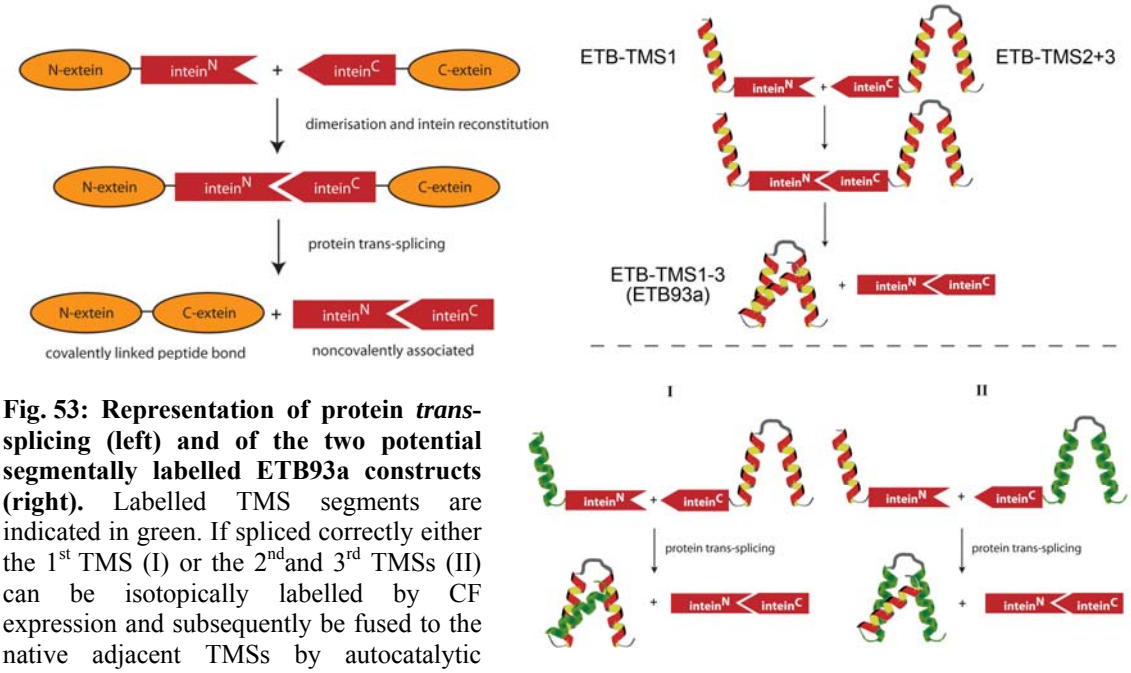


Fig. 53: Representation of protein *trans*-splicing (left) and of the two potential segmentally labelled ETB93a constructs (right). Labelled TMS segments are indicated in green. If spliced correctly either the 1st TMS (I) or the 2nd and 3rd TMSs (II) can be isotopically labelled by CF expression and subsequently be fused to the native adjacent TMSs by autocatalytic *trans*-splicing.

4.9.1. CF expression of ETB intein fusions and evaluation of optimal solubilization

The CF expression of the ETB93a intein fusion constructs pVSO 4.1 and pVSO 4.2 plasmids was accomplished in cooperation with the group of Prof. Mootz who provided cloning and splicing expertise (Technical University Dortmund, Germany) (**see 3.11.**). Expressed proteins from split intein fusions for the *trans*-splicing reaction were TMS 1 plus $DnaE^N$ peptide from *Nostoc punctiforme* (ETB 4.1, ETB-TMS1 intein) with a molecular weight of 19.1 kDa as well as the corresponding split intein $DnaE^C$ peptide plus TMS2+3 (ETB 4.2, ETB-TMS2+3 intein) with a calculated mass of 15.1 kDa. Both intein fusion constructs harboured an N-terminal T7-tag and a C-terminal poly$(His)_{10}$-tag and could be produced in the P-CF mode at final yields of more than 1 mg/mL of RM (Fig. 54 A).

D-CF expression screens of ETB 4.1 revealed a low overall solubilization potential of all detergents at the analysed concentrations of 0.1 % DDM, 0.1 % B35, 1 % B58, 1 % B78 and 1 % B98, although Brij detergents were shown to result in quantitative solubilization of the full-length ETB receptor and various truncations (Klammt et al. 2007b, 2007c). All tested detergents failed. Only 1 % B98 generated marginally soluble ETB 4.1 at estimated values of less than 1 % (Fig. 54 B). ETB 4.2 displayed a higher detergent compatibility with regard to its solubilization behaviour. While 0.1 % DDM completely abolished CF synthesis, the best

quantitative soluble expression was achieved in 1 % 58 and 1 % B78 with 30-50 % followed by 0.1 % B35. B98 could not be used for the soluble production of ETB 4.2 (Fig. 54 C).

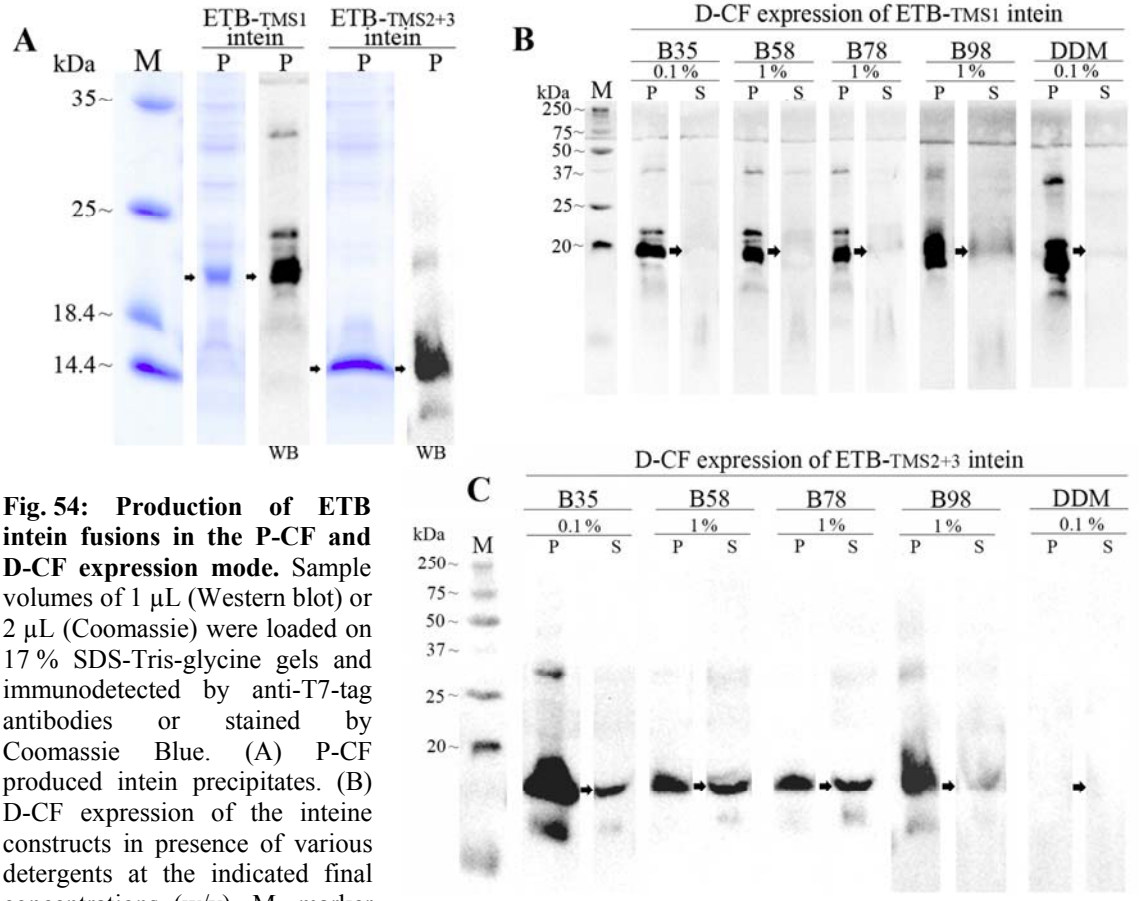


Fig. 54: Production of ETB intein fusions in the P-CF and D-CF expression mode. Sample volumes of 1 µL (Western blot) or 2 µL (Coomassie) were loaded on 17 % SDS-Tris-glycine gels and immunodetected by anti-T7-tag antibodies or stained by Coomassie Blue. (A) P-CF produced intein precipitates. (B) D-CF expression of the inteine constructs in presence of various detergents at the indicated final concentrations (w/v). M, marker proteins; P, pellet; S, supernatant.

Since D-CF expression resulted in very low final yields of both soluble ETB intein constructs this mode was not further considered. Instead, post-translational resolubilization was analysed to evaluate the overall amount of detergent-solubilized intein fusions (Fig. 55).

Detergents were chosen with respect to their applicability in solution-state NMR spectroscopy. The lyso-phospholipid and long-chain phosphoglycerol LPPG, the phospholipid derivative Fos-12 as well as the short-chain phospholipid diC_6PC are well accepted in the area of structural investigations by NMR (Arora et al. 2001, Krueger-Koplin et al. 2004). TX-100 was used as a reference as it proved already successful in the resolubilization of MPs such as EmrE and Tsx (Klammt et al. 2005).

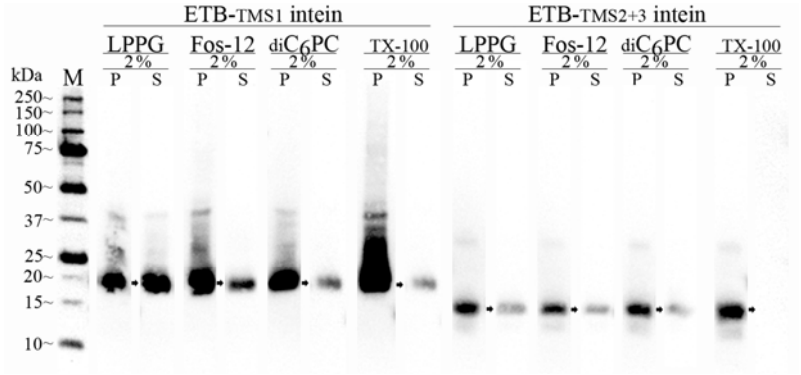


Fig. 55: Resolubilization of ETB intein fusions. Sample volumes of 1.5 µL in splicing buffer and detergent at the indicated concentrations (v/w) were loaded on 10 % Tris-Tricine-SDS gels and subsequently immunoblotted against the C-terminal poly(His)$_{10}$-tag. Arrows indicate soluble fractions. M, marker proteins; P, pellet; S, supernatant.

Resolubilization after P-CF mode resulted in higher recovery of solubilized intein fusions as compared to the co-translational solubilization in the D-CF mode (Fig. 54 and 55). ETB 4.1 (ETB-TMS1 intein) could be produced as precipitate in high amounts. However, quantitative solublilization proved to be more difficult than expected. TX-100 and diC_6PC resulted only in marginal yield of soluble ETB 4.1, followed by Fos-12 and LPPG which exhibited a resolubilization efficiency of 20 and 50 %, respectively (Fig. 55, left). A similar preference of detergents for resolubilization could be detected with ETB 4.2 (ETB TMS2+3 intein) although the overall expression rate was diminished compared to ETB 4.2 (Fig. 55, right). Summarizing the resolubilization experiments for both inteins from the P-CF mode, a list of suitable detergents (LPPG > Fos-12 > diC_6PC >> TX-100) could be defined as basis for subsequent *trans*-splicing approaches.

4.9.2. Trans-splicing of detergent-solubilized ETB intein fusions

Correct complete splicing of the two precursor ETB intein fusions should result in the potential products ETB93a consisting of 15.4 kDa with N-and C-terminal tags as well as the residual split intein DnaE^N peptide (13.1 kDa, without T7-tag, plus poly(His)$_{10}$-tag) and the split intein DnaE^C peptide (5.6 kDa, characterized by the possession of a remaining T7-tag but lacking the poly(His)$_{10}$-tag).

In agreement with Prof. Mootz and under consideration of the published data of Hong et al. (2008), the parameters for the investigations of *trans*-splicing were chosen as follows. The intein cleavage buffer consisted of 50mM Tris, 100mM NaCl, 2mM DTT, 1mM EDTA and 2 % LPPG or 2 % Fos-12 showing the highest solubilization efficiency. The pH, optimal for the splicing between 6.5 and 7.0, was kept neutral at 7.0 as this reflects best the overall physiological conditions perceived by human GPCRs. The respective ETB intein fusions precursors ETB 4.1 and ETB 4.2 were expressed separately in P-CF mode, the precipitate was harvested by centrifugation, washed and resolubilized in the chosen detergents (**see 3.11.**). The single resolubilized targets were subsequently mixed at equal volume ratios (1:1, v/v) and subjected to *trans*-splicing reactions.

Three temperatures of 16 °C, 22 °C and 40 °C in combination with a variety of incubation times from 45 min to 65 h were tested (Fig. 56). Splicing was followed by immunodetection against both N-terminal T7-tag and C-terminal poly(His)$_{10}$-tag as well as by autoradiography after CF expression in presence of ^{35}S-methionine (Fig. 57).

One of the most difficult tasks was the differentiation between the uncleaved precursor ETB 4.2 with a mass of 15.1 kDa and the potential splice product ETB93a with 15.4 kDa since both proteins only differed in approximately 200 Da which can hardly be resolved by SDS-PAGE. Furthermore, remaining inteins after complete cleavage could fuse again resulting in a protein with a molecular weight of 18.7 kDa again hardly distinguishable from the ETB 4.1 precursor with 19.1 kDa. Due to those similarities in protein mass it was almost impossible to evaluate the optimal time span and temperature for the splicing reactions for both chosen detergents by immunodetection (Fig. 56).

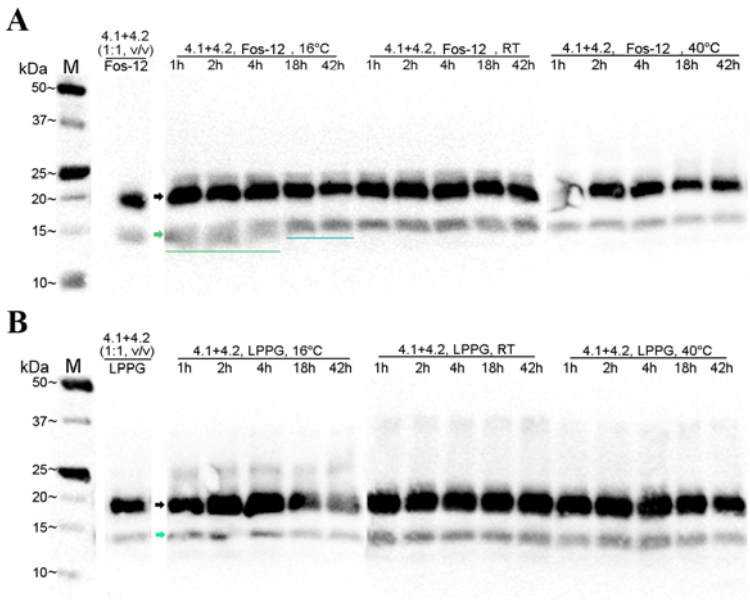


Fig. 56: Evaluation of *trans*-splicing parameters for P-CF produced ETB intein fusions. ETB intein precursors were either resolubilized in 2 % Fos-12 (A) or 2 % LPPG (B). Sample volumes of 2.5 µL of a total reaction volume of 30 µL were taken at different time points, loaded on 12 % Tris-Tricine-SDS gels and subsequently subjected to immunoblotting against the C-terminal His-tag. Black arrows point to ETB 4.1 bands while green arrows illustrate ETB 4.2. Possible mass shifts are underlined in green and cyan. M, marker proteins; v/v, volume/volume.

Only a slight difference in the observed mass of the lower band at 15.1 kDa could be detected with 2 % Fos-12 at 16 °C. This observation was interpreted as a possible indication for ongoing splicing which resulted in the product after 18 h (Fig. 56 A, underlined in green and cyan). Since no such change in molecular mass was detected at 22 °C it was suggested that splicing might occur earlier at higher temperatures. Similar results pointing at successful *trans*-splicing were not achieved with 2 % LPPG as all protein bands remained at the same positions (Fig. 56 B).

As 2 % Fos-12 seemed suitable for further studies it was chosen for the investigation of splicing by a transfer of radioactive methionine from either ETB 4.1 or ETB 4.2 to the product during cleavage. P-CF expression of both precursors was successfully performed in presence of ^{35}S-methionine. Single protein bands in the autoradiogram further demonstrated the specificity of the methionine incorporation during CF reaction as no background was observed (Fig. 57).

Combination of equal volumes of radioactively labelled ETB 4.1 (^{35}S 4.1) with unlabelled ETB 4.2 resulted in an additional band at around 15 kDa arising upon incubation under splicing conditions after 1 h which increased in intensity over time reaching highest signal intensity after 65 h. Simultaneously, the precursor band at 19.1 kDa became fainter suggesting a quantitative turnover in line with the occurrence of the lower band. Since the detected additional band displayed a mass of ~15 kDa, comparable to the mass of the precursor ETB 4.2 with 15.1 kDa, this band could rather be explained by the formation of ETB93a (15.4 kDa) than with the peptide split intein DnaE^{N} splicing intermediate (13.1 kDa) (Fig. 57). Radioactive labelling of intein constructs resolubilized in 2 % LPPG did not show any turnover (data not shown) which is in consistency to the above delineated results (Fig. 56).

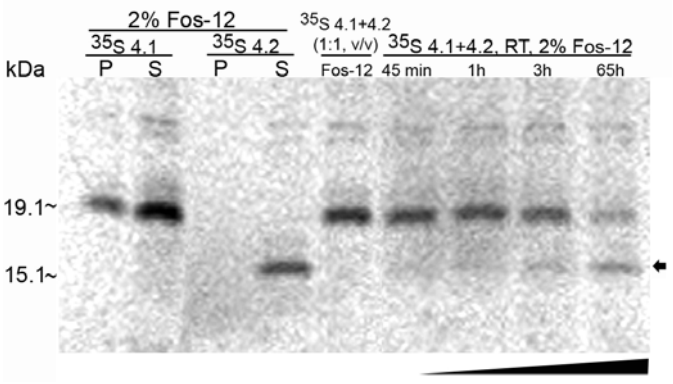


Fig. 57: Autoradiogram of ETB intein *trans*-splicing monitored by incorporation of ^{35}S-methionine by CF expression. Sample volumes of 5 µL from single CF expression or various time points were loaded on 10 % Tris-Tricine-SDS gels and analysed after incubation on phosphoimager plates. Gel exposition was performed at RT for two days. The black arrow and triangle indicate the potential arising product ETB93a.

In order to further monitor the occurrence of splicing intermediates in the ongoing process, the existence of different immunotags depending on which intermediate was formed was assessed by immunodetection against both tags (Fig. 58). The splicing process seemed to proceed under the evaluated conditions as the turnover of precursors as well as the formation of intermediates could be detected. The band at 19.1 kDa, representing ETB 4.1, decreased in intensity after 24 h which could be observed by immunodetection of both tags (Fig. 58, red arrows). Furthermore, a slight upward shift in the band at 15 kDa might indicate the formation of the product ETB93a (Fig. 58, green arrows). The band at a low molecular mass of estimated 6 kDa, which was only detectable by an antibody against the T7-tag, suggests the occurrence of the peptide split intein DnaE^{C} intermediate (5.6 kDa) (Fig. 58, blue arrow). The second peptide intermediate of the split intein DnaE^{N} was monitored at 13 kDa only by the antibody against the C-terminal poly(His)$_{10}$-tag

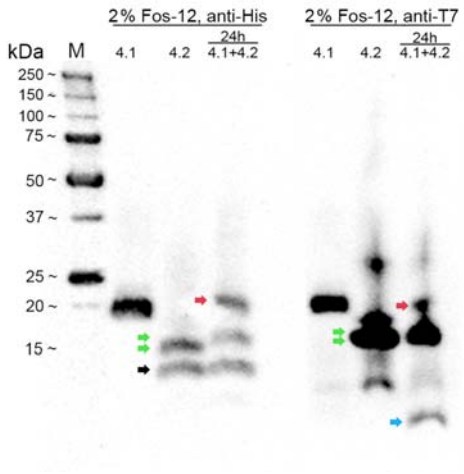


Fig. 58: Detection of splicing intermediates. Protein samples of 3 µL were applied to 12 % Tris-Tricine-SDS gels and immunoblotted against N- and C-terminal tags. Various arrows point at splicing intermediates and potential products. M, marker proteins.

which is well in accordance with the calculated molecular weight of the intermediates. However, this band was also detectable in the single precursor sample ETB 4.2 which might be explained by an unstable state, in which the intein already starts to excise itself without the presence of its counterpart (Fig. 58, black arrow).

Although no complete assembly of the TMSs of ETB93a occurred by *trans*-splicing because precursors, intermediates and splice product seemed to persist in parallel in the RM, it was demonstrated with high probability that *trans*-splicing could successfully be applied to CF expressed MPs in a detergent environment. However, its applicability to NMR spectroscopy at this point remains questionable since the turnover was rather qualitative than quantitative and high amounts of product are needed for structural investigations which could not be achieved with the constructs and conditions at hand. Furthermore, the ETB93a product as well as the precursors displayed highly similar molecular masses together with the modification by the same immunotags which considerably hampered straightforward detection and purification methods.

5. Discussion

5.1. Protocol development for the preparative scale expression of functionally folded GPCRs in individual cell-free systems

5.1.1. Selecting CF expression systems based on E. coli for GPCR production: Basic considerations

CF expression as alternative option for the preparative scale production of MPs is emerging for approximately the last five years with first reports starting in 2004 (Berrier et al. 2004, Elbaz et al. 2004, Klammt et al. 2004). Evident advantages of CF expression reside in the reduced system complexity eliminating many critical steps of conventional cell-based systems which allows fast protein production in small scale volumes with easy and reliable labelling techniques (Schwarz et al. 2007b, Koglin et al. 2006, Liguori et al. 2007, Reckel et al. 2008). PTMs of expressed targets such as glycosylation, lipidation or phosphorylation can a priori not be expected in CF systems based on *E. coli* extracts. This might affect stability and/or function of individual proteins, but on the other hand could result in more uniform sample qualities in terms of structural aspects. Specific chaperone systems or cellular quality control mechanisms that might scrutinize and remove misfolded MPs are also most likely absent in CF systems. Due to the lack of those cell-internal machineries, CF synthesis of MPs requires extensive evaluation of protein quality.

Two major configurations exist for the CF production of proteins known as batch or CECF delineated in detail (**see 1.2.2.**). The highest productivity at the mg/mL of RM level is still obtained with the CECF system (Kigawa and Yokojama 1991, Shirokov et al. 2007, Spirin et al. 1988). Since the batch mode resulted in very moderate expression levels of GPCRs of approximately 160 µg/mL as analysed for the β2-adrenergic (AR), the muscarinic acetylcholine 2 (AChM2) and the neurotensin receptors (NTR) (Ishihara et al. 2005) the CECF configuration is likely to be favoured for GPCRs with regard to relative production efficiencies. It was not only successfully used to achieve expression levels of up to 3 mg/mL of RM for human ETB but also for other GPCRs such as the human V2R, the rat corticotropin releasing factor receptor 1 (CRF), the human melatonin (MTN) and neuropeptide Y receptors (NPY) (Klammt et al. 2007b, 2007c), for the human histamine H1 receptor (HRH1) (Kamonchanok et al. 2008) and for olfactory receptors (Kaiser et al. 2008). Therefore, this configuration was chosen for the preparative scale expression of both members of the ET receptom in the current work.

ETA and ETB constructs were designed by using expression vectors of the pET or the pIVEX series since they possess T7 promotors which rely on the highly processive and specific T7RNAP. Since low expression rates can mostly be connected to problems with peptide

elongation or especially translation initiation in which the approximately first ten nucleotides following the start codon play an important role (Gonzalez de Valdivia and Isaksson 2004), different strategies have been used to effectively circumvent initiation problems with GPCRs in CF systems. Most problems of initiation have successfully been avoided by fusion of small expression tags like the T7-tag (Klammt et al. 2005) or larger fusion partners such as Trx to the N-terminus of the target protein (Ishihara et al. 2005, Kamonchanok et al. 2008). Especially valuable for expression of GPCRs in our hands proved the T7-tag whose addition solely allowed their production in sufficient amounts (Klammt et al. 2007b, Schneider et al. 2010). This tag is advantageous over larger fusion partners like Trx due to its small size of only 1.5 kDa and it might not even be necessarily removed for structural evaluations by X-ray crystallography. Choosing C- or N-terminal poly(His)$_x$-tags or strep-tags additionally facilitate rapid purification by IMAC or other affinity matrices and has been applied to all CF expressed GPCRs. Beneficial for an increase in recovery of detergent-solubilized MPs seems an extension of the poly(His)$_6$-tag provided by most of the standard CF expression vectors to a poly(His)$_{10}$-tag which proved valuable for the purification of CF ETB achieving amounts of 1.8 mg/mL final purified receptor (Klammt et al. 2007c). Furthermore the addition of immunodetectable tags on both ends simplifies the verification of full-length synthesis of expressed MPs as they tend to run faster than their predicted molecular weight on SDS-PAGE. The functional folding of MPs in general might furthermore not be affected by terminal fusion tags (Massotte et al. 1997, 1999).

CF expression of ETA and ETB was conducted with an N-terminal T7-tag and a C-terminal poly(His)$_{10}$-tag in the present thesis. Extensions with peptide tags like Flag-tag (1.0 kDa) or c-myc-tag (1.2 kDa) at the N-terminus were previously reported to have neglectable effects in the ETA receptor's ligand binding affinity upon overexpression in *Pichia pastoris* (Cid et al. 2000). Likewise, the addition of poly(His)$_6$-tags at both ends of the ETB receptor did not show a pronounced influence on functionality either (Doi et al. 1997) indicating that an addition of terminal tags can be regarded as acceptable strategy for functional receptor studies within the ET receptom. Both tags were successfully used in immunodetection to verify the full-length synthesis of ETAcHx and ETAstrep (Fig. 10).

Regarding the choice of the optimal detergent there is no general rule as to which type is most suited for the functional conservation of individual GPCRs. Detergents should therefore be subject of intense investigations and optimization processes as discussed in detail (**see 5.2.**). However, some detergents have been shown to be preferred for the direct solubilization of GPCRs by D-CF expression as well as for the resolubilization out of P-CF precipitates. For the D-CF mode, the steroid derivative digitonin as well as representatives of the long chain polyoxyethylene-alkyl-ethers B35, B58, B78 and B98 resulted in the highest soluble yield of the majority of analysed GPCRs such as both human ET receptors ETA and ETB, human and rat V2R, the rat CRF 1, NPY4R and MTNR1B (Junge et al. 2010b, Klammt et al. 2007b), the

human β2AR-GαS fusion, human AChM2 and the rat NTR (Ishihara et al. 2005) as well as the olfactory receptors human OR17-4 and mouse OR23 (Kaiser et al. 2008). The presence of those detergents yields soluble GPCRs of more than 3 mg/mL RM (Klammt et al. 2007b). Whereas Brij derivatives are applicable in a broad range of concentrations for the expression of GPCRs (Klammt et al. 2005), digitonin displays very defined optima, and increasing concentrations might impair production levels (Kaiser et al. 2008, Klammt et al. 2005). Other detergent classes such as polyoxyethylene sorbitane esters (Tween derivatives) and alkyl glucosides such as DDM, HDM and TDM resulted in considerably lower yield or even no co-translationally solubilized receptors (Ishihara et al. 2005, Kaiser et al. 2008, Klammt et al. 2005, Junge et al. 2010b). Most effective resolubilization of P-CF produced GPCR precipitates was obtained in detergents such as LMPG and LPPG (Junge et al. 2010b, Klammt et al. 2005) and DDM in case of the human HRH1 (Kamonchanok et al. 2008).

5.1.2. Cell-free expression of the ET receptom using E. coli extracts: Comparison of protein production rates to cell-based systems

Aiming at structural determination of proteins requires sufficient protein yields combined with functionality and intrinsic stability of the target receptor preferably in a detergent-solubilized state. In *in vivo* expression systems most of the MPs are either expressed purely in membranes or in form of IBs (Tate 2001). A high protein quality is an essential prerequisite for structural studies of the ET receptors. Different production strategies have been explored but none of them has apparently been successful in generating suitable samples in reasonable amounts as no structural data have been published so far.

Heterologous expression hosts of mammalian background like COS, CHO or HEK293 cells (Arai et al. 1990, Adachi et al. 1992, Gregan et al. 2004a, 2004b, Evans and Walker 2008) are mostly used for clinical studies and pharmacological characterization of novel receptor agonists and antagonists. Although those systems represent the natural environment of the receptors they do usually not allow for extensive overexpression in the range of mg amounts of soluble receptor and therefore are hardly suited for structural studies (Arai et al. 1990, Mondon et al. 1998, De Leon and Garcia 1995). Further heterologous *in vivo* expression systems like *E. coli* (Haendler et al. 1993), the methylotrophic yeast *Pichia pastoris* (Schiller et al. 2000, 2001, Saravanan et al. 2004, 2007), and *Spodoptera frugiperda* Sf9 cells (Doi et al. 1997) have already been investigated with respect to overproduction efficiencies of ET receptors with varying degrees of success in terms of high yield, receptor purity, stability and receptor function (Table 14).

With 41 receptors per cell ETB was overexpressed at low levels in *E. coli*. Membrane fractions were analysed for their binding behaviour. The receptor displayed equal binding properties of all three ligands with a dissociation constant to ET-1 of 100 pM which is in the

range of native receptors (Haendler et al. 1993). However, if comparing its overexpression rate to other GPCRs in *E. coli*, fusion of the NTR to MBP and Trx for instance resulted in the detection of up to 700 binding sites per cell (Tucker and Grisshammer 1996) which is considerably higher, implying that direct expression of ETB into *E. coli* membranes might not be a straightforward approach if high protein yield is required.

The expression of N- and C-terminal tagged mutated ETB receptors in *P. pastoris* resulted in high expression levels of stacked membrane inserted receptor which could be up-scaled by fermentation with final yields of 9 mg/L. In its membrane environment it exhibited comparable pharmacological properties to the native ETB (Schiller et al. 2000, 2001). The expression in yeast therefore seems to be very promising for structural approaches. However, upon its extraction from ET-1 pre-saturated membranes at RT in DDM approximately 40 % of the receptor was lost although retaining monomeric character and high stability over a wide pH range. The determination of ligand binding capacity of 30 % final receptor yields (~3 mg/mL) in a detergent-solubilized state was only roughly determined. Radioactive ET-1 found in the supernatant after cell lysis was correlated to totally bound ligand (Schiller et al. 2001).
However, as membrane solubilization is far from being a very mild procedure it might be critical to assume, that all membrane bound receptors keep the agonist and its functional conformation.

Table 14: CF and heterologous non-mammalian expression systems for the production of ET receptors. Representative selection of published data on the production level as well as on functional ET receptor yield of the human ET system.

Receptor subtype	Expression system	Production levels[A]	Final functional receptor yield in soluble form	Assay[B]	Reference
ETB	*E. coli*	41 r/c	n. a.	m	Haendler et al. 1993
ETB	*P. pastoris*	4-9 mg/L	n. a.	m	Schiller et al. 2000
ETB	*P. pastoris*	9 mg/L	3 mg/mL	m	Schiller et al. 2001
ETB	*P. pastoris*	-	2.3–2.8 mg/L	s	Saravanan et al. 2007
ETB	Sf9 cells	~0.7-1 mg/L	~0.3 mg/L	s	Doi et al. 1997, 1999
ETB	*E. coli* CF	3 mg/mL	0.9 mg/mL	s	Klammt et al. 2007b
ETA	*P. pastoris*	~0.6 mg/L	0.6 mg/L	s	Saravanan et al. 2004
ETA	Sf9 cells	~0.25 mg/L	< 0.05 mg/L[C]	s	Doi et al. 1999
ETA	*E. coli* CF	1.5 mg/mL	0.5 mg/mL	s	Junge et al. 2010b

[A] Production levels either expressed as total amount of receptor per litre culture (mg/L), receptors per cell (r/c) or as mg/mL of CF RM.
[B] Assays either performed on solubilized receptor preparation (s) or on membrane preparation (m)
n. a., not analysed
-, not stated
[C], estimated value determined based on the statement of 5-10 times lower efficiency for ETA as for ETB

Likewise, the data by Saravanan et al. in *Pichia* seem very promising but they were produced using a completely new assay based on ligand affinity chromatography via hydroxyl apatite gels coupled to colour development (Saravanan et al. 2004). They could indeed show that binding of different derivatives of ET-1 was comparable to *in vivo* data, even higher than the binding of the

ligand to native membranes. However, this assay was not applied to other GPCRs so far and therefore lacks a well-founded comparison to related receptors. In this work the immobilized ligand ET-1 has been shown to bind unspecifically to other detergent-solubilized GPCRs with very high affinity in SPR measurements, therefore tending to generate false positives in micellar environments (**see 4.8.2.**).

ETA and ETB receptors as well as mutated variants were further expressed in considerable amounts in Sf9 cells, subsequently detergent-solubilized and ligand affinity purified to homogeneity with a loss of protein of approximately 90 % (Doi et al. 1997, Satoh et al. 1997). Furthermore, ETB was expressed more efficiently in insect cells than ETA. Besides their inherent ability to promote glycosylation, Sf9 cells also allowed Ca^{2+} signalling indicating that, upon ligand binding, the ET receptors were capable of inducing downstream processes comparable to the wild-type environment (Doi et al. 1999, Satoh et al. 1997). Upon affinity purification, mostly performed by using a biotinylated (Lys9) ET-1 wild type variant (Doi et al. 1997, 1999, Saravanan et al. 2004, 2007, Satoh et al. 1997), ligand binding activity of the receptors could be preserved to the same levels as in insect cell membranes (Satoh et al. 1997).

CF expressed ETB receptor was purified to levels of up to 1.8 mg/mL of RM in 0.1 % B78 and its ligand binding capacity upon co-elution studies by gel filtration with Cy3-labelled ET-1 was calculated to be approximately 50 % which would result in ~0.9 mg/mL of functional receptor (Klammt et al. 2007c). This is comparable to *in vivo* data from Sf9 cell produced receptor (Table 14).

In the present study, final CF expressed ETA receptor concentrations of up to 1 mg/mL after IMAC purification in 0.1 % B35 could be achieved under optimal conditions. Calculating with a loss of 50 % upon ligand affinity chromatography (**see 4.5.1.**) still 0.5 mg/mL of functional receptor can be obtained (Fig. 36). These data of final receptor yield of ETA produced in a self-made *E. coli* extract are highly competitive to most of the published values for ETA from *in vivo* expression (Doi et al. 1999, Saravanan et al. 2004) and therefore represents a promising approach for structural investigations.

5.1.3. Cell-free expression of ETB using wheat germ extracts

Preparative scale expression achieving mg amounts of protein per mL of RM is currently possible by using *E. coli* or WGE (Endo and Sawasaki 2005, Zubay 1973). *E. coli* as well as wheat embryo extracts have similar efficiencies in the production of prokaryotic and eukaryotic MPs and even proteins far exceeding 100 kDa in size can be synthesized in both systems (Liguori et al. 2007, Schwarz et al. 2010). It is in discussion if eukaryotic MPs should be expressed in eukaryotic CF systems. Prokaryotic sources do not provide eukaryotic chaperone systems eventually necessary for a higher quality of eukaryotic target proteins (**see 1.2.2.**). Disulfide bond formation is generally feasible in all CF systems (Goerke et al. 2008) but other

PTMs like directed proteolytic processing, phosphorylation, prenylation and glycosylation can principally only take place if modifying enzymes or corresponding microsome fractions have been supplemented (Swartz 2006).

ETB was chosen as target MP as it has been most extensively studied by *E. coli* CF expression before, and functional assays were readily available (Klammt et al. 2007b, 2007c). Basic investigations of production efficiencies as well as of detergent tolerances were performed along with a final comparison of the functionality of the ETB receptor isolated from both systems. In cooperation with Vladimir Shirokovs lab, the WGE system was established for the expression of the ETB receptor. In terms of productivity as well as detergent compatibility for high yield D-CF expression of ETB no major differences were revealed by intensive screenings (**see 4.8.1.**). The average yield of D-CF expressed ETB receptor using Brij derivatives in both systems achieved mg amounts per mL of RM being slightly reduced in WGE with 1.5 mg/mL as compared with 3 mg/mL in CF *E. coli* (Klammt et al. 2007b). Moreover, similar binding kinetics were determined by SPR measurements (**see 4.8.2.**). However, the detection of ETB glycosylation as an example for PTMs remained negative in our lab indicating that necessary modifications might not be performed in WGE either (data not shown). This is also in accordance with observations of Endo an co-workers who stated that glycosylation generally does not occur during CF expression using WGE (Takai et al. 2010). In WGE N-terminal lipoylation may only be possible if the necessary substrate is supplied (Lingappa et al. 1997). Insect cell as well as rabbit reticulocyte and *Leishmania* extracts might perform required PTMs if used for analytical scale protein production in the µg/mL range (Arduengo et al.2007, Ezure et al. 2006, Mureev et al. 2009, Ren et al. 1993, Tie et al. 2005).

So far, the expression of ETB did not profit from any obvious advantages in a self-made WGE CF system. Since WGE preparation has the disadvantages of being relatively laborious and time consuming (Endo and Sawasaki 2005, Madin et al. 2000) and as its production efficiency is highly dependent on the choice of the wheat source, the more reliable *E. coli* extract was preferred for further functional and structural investigations of the ET receptors.

5.2. Development of target specific quality control protocols for CF expressed GPCRs: Case study ETA receptor

The continuously growing diversity of options to design and modulate expression environments, protocol compositions and folding kinetics increasingly require rational strategies for the optimization of protein sample quality in CF expression systems. Quality control of the synthesized proteins is fundamental in order to obtain a necessary feedback on the design of appropriate expression protocols (Wagner et al. 2006, Junge et al. 2010a). MPs are likewise more difficult to handle than soluble proteins as detergents and lipids are major determinants in defining their overall quality (Seddon et al. 2004). Appropriate final protocols are hard to

predict as even closely related MPs seem to behave very differently like observed for CF expressed ETA and ETB (Junge et al. 2010b, Klammt et al. 2007b, 2007c).

5.2.1. Primary solubilization of CF expressed MPs: Considerations for the conservation of structural and functional features

The preparative scale expression of MPs in the D-CF and P-CF modes are unique approaches for the rapid production of proteomicelles and it is tempting to speculate which of the two expression modes is more likely to result in correctly folded protein.

The D-CF mode appears to have certain advantages as it avoids any initial precipitation of the synthesized MPs, provides an efficient solubilization approach independent from specific translocation systems and delivers MP samples that can directly be used for a variety of functional and structural assays. Although this mode appears to be more likely in resulting in non-aggregated MPs, some potential disadvantages have to be considered. Expression yields are usually lower if compared with the P-CF mode. Reduced translation rates caused by the detergents and incomplete solubilization of the synthesized MPs are possible reasons. With ETA, 50 % less purified protein was obtained after D-CF expression in presence of B35 if compared with P-CF expression and resolubilization in LPPG (**see 4.2.3.**). Furthermore, harvesting a P-CF produced MP precipitate by centrifugation already eliminates most contaminating cell extract proteins which stay in the supernatant. This represents already a first purification step and resolubilization of the precipitate can sometimes even result in apparently pure protein (Klammt et al. 2004). For instance, D-CF produced ETA was considerably less pure after IMAC than the P-CF expressed and purified receptor (Fig. 17 A, C).

ETA was efficiently produced in the P-CF (**see 4.1.1.**) and in the D-CF mode (**see 4.1.2.**). However, while post-translational solubilization of ETA in 1 % LPPG already generated a homogenous elution profile if compared with other analysed detergents (Fig. 15 A), the D-CF expression of ETA in 0.5 % B35 seemed to be less suited. In this case, the reduction in detergent concentration upon IMAC purification to 0.1 % B35 resulted in a Gaussian shaped peak which still covered a very broad range of the elution volume comprising at least four different oligomeric states (Fig. 19 A). Reduced B35 concentrations might cause the exposure of TMS parts which could then more easily form intermolecular aggregates. This behaviour suggests that the initial structure of ETA upon expression in B35 was not correctly packed or is unstable, so that a loss of detergent molecules increases aggregation. All other detergents used for the D-CF expression of ETA, including digitonin, resulted in a predominant loss of soluble receptor with the exception of B35 still yielding ~50 % of the P-CF production efficiencies. Therefore, they were considered less suited for the production of ETA due to the reduction in final receptor yield (**see 4.1.2.**).

One of the most frequent associations if working with P-CF produced proteins is that P-CF precipitates are similar to bacterial IBs. IB formation is a common mechanism observed upon expression of proteins in cell-based systems, especially in *E. coli*, and many GPCRs can be targeted into IBs upon overexpression (Michalke et al. 2009). The proteins in IBs are mostly inactive (Vallejo and Rinas 2004) and their functional folding has to be restored by often harsh refolding procedures which usually require solubilization in either a strong detergent, in a chaotrope or in organic solvents (Kiefer 2003). Those aggregates are therefore often undesired as successful refolding procedures in particular of GPCRs still remain the exception (Baneres et al. 2003, 2005, Bazarsuren et al. 2002, Dahmane et al. 2009).

Although structural analyses of P-CF produced MP precipitates are still rare, accumulating evidence indicates that they behave differently if compared with MP inclusion bodies. Maslennikov et al. could show by magic angle spinning solid-state NMR that P-CF precipitates of the *E. coli* histidine kinase receptors ArcB and KdpD are characterized by α-helical folding. The recorded spectra were highly comparable to those recorded after lyophilization of the detergent-solublilized receptors. Proton-deuterium exchange measurements by solution NMR led the authors to the unambiguous interpretation that secondary structure elements must be generated during P-CF expression (Maslennikov et al. 2010). In cooperation with Karsten Mörs, similar preliminary evidence was obtained with P-CF expressed ETA receptor in current work (**see 4.2.8.**). Judged by the signal intensity of the CαCβ cross-peaks of alanine and isoleucine in two independent experiments, an estimated amount of 30-40 % of the secondary structure might already contain α-helical elements (Fig. 24). Those values are marginally lower than the predicted (51 %) and the determined values of samples in 0.1 % B35 (46 %) which exhibited ligand binding capacity (**see 4.2.7.** and **4.5.1.**). These findings support the potential of P-CF expression to result in correctly folded MPs after adequate solubilization.

P-CF precipitates solubilize in a variety of relatively mild detergents without prior denaturation (Klammt et al. 2004, Schwarz et al. 2007b). The lyso-phosphatidylglycerols LMPG as well as the related LPPG were found to be almost universal in the efficient P-CF solubilization of diverse structural classes of MPs ranging from transporters to GPCRs (Keller et al. 2008, Klammt et al. 2005). P-CF expressed ETA was also found to be most effectively resolubilized in LPPG and moreover exhibited the highest degree of homogeneity in this detergent (**see 4.2.2.**). The reasons to choose lyso-phosphaditylglycerols for resolubilization of ETA are suggestive in that (I) they might be well suited for the solublilization of MPs due to their close structural resemblance to native membrane lipids although displaying micellar character (Williams et al. 1996). (II) Lyso-phosphatidylglycerols were used to isolate large MPs in a native and functional form before and are well suited to analyse small MPs by NMR spectroscopy (Krueger-Koplin et al. 2004, Williams et al. 1996). A survey of 25 different detergents singled out lyso-phosphaditylglycerols as superior in stabilizing native MP conformations and in preventing their aggregation at high concentrations (Krueger-Koplin et al.

2004). (III) The anionic character of the phosphatidylglycerol might be necessary for the native folding of ET receptors. The zwitterionic lysophosphocholine LMPC generated a much broarder elution profil upon gel filtration analysis (Fig. 15 A) suggesting a variety of oligomeric states. As many cell types predominantly expressing ET receptors contain PG to an average value of 5-10 % (Christie 1985) ETA might not only need zwitterionic phosphatidylcholine but also a negatively charged headgroup for homogenous folding. Predominant fatty acids in endothelial cells were additionally found to be oleate, stearate and palmitate (Cansell et al. 1997). Therefore, LPPG (- palmitoyl) is likely preferred over LMPG (-myristoyl). (IV) Retrospectively, BN-PAGE (Schägger et al. 1994, Wittig et al. 2006) performed on ETA directly after resolubilization in 1 % LPPG additionally supported the choice of this detergent. Most of the receptor seemed to display monomeric character with a faint band pointing at dimerization (Fig. 15 B). This behaviour is reported for native ET receptors as well as for other GPCRs (**see 1.3.4.** and **1.5.5.**). Likewise, a number of further GPCRs and complex transporters have already been functionally reconstituted from solubilized P-CF precipitates. The Trx-β2 AR in fusion to its Gsα protein (Ishihara et al. 2005) as well as the HRH1 (Kamonchanok et al. 2008) showed activities comparable to *in vivo* data. Keller et al. revealed the functional reconstitution of P-CF produced eukaryotic drug transporters solubilized in 2 % LMPG (Keller et al. 2008). In contrast human P-CF expressed ETB was highly aggregated in LMPG and did not show any co-elution with fluorescently labelled ET-1 upon gel filtration, while D-CF expressed ETB in B78 exhibited ligand binding (Klammt et al. 2007c).

Further reports of non-related MPs support that not only the D-CF but also the P-CF mode result in functionally folded proteins as shown for EmrE (Elbaz et al. 2004, Klammt et al. 2004), the bacterial mechanosensitive channel MscL (Berrier et al. 2004) and a bacterial light-harvesting protein (Shimada et al. 2004). Furthermore, synthetic amphipathic polymers appear to have a considerable potential in the functional solubilization of GPCRs even out of IB material (Dahmane et al. 2009) The P-CF mode might therefore become increasingly important for the rapid production of MP samples and optimized resolubilization conditions implementing an increasing variety of compounds could significantly improve success rates in future.

5.2.2. Detergent exchange of ETA: Effects of secondary detergents on the quality of the receptor

The type and the properties of detergents play an important role in the recovery of an active state MP as they effect homogeneity and secondary structure elements (Le Maire et al. 2000). Two options for detergent screening can be considered. The first screen focuses on the detergent selected for initial MP solubilization in the D-CF or P-CF mode (**see 5.2.1.**). Any selected primary detergent in the P-CF or D-CF mode can be exchanged with almost any other detergent preferred for downstream analyses upon MP immobilization during the initial IMAC purification, dialysis or gel filtration (Arnold and Linke 2008, Linke 2009). Hence, in the

second screen upon MP purification, the detergents most suitable for the desired downstream assays are selected.

An important parameter for sample quality is its homogeneity monitored by different oligomeric states. SEC profiling is an established and relatively universal tool for the initial estimation of protein aggregation and heterogeneity, ideally suited to investigate the effect of detergents on MPs. The homogeneity of different ETA samples could be correlated to the different production conditions. P-CF produced samples generally displayed a more symmetric, narrower peak shape after detergent exchange. The broad elution profile of D-CF produced ETA in B35 is in strong contrast to the homogeneity of P-CF produced samples (Fig. 18 and 19). Negative staining electron micrographs of different ETA sample preparations generated from D-CF or P-CF mode supported those assumptions (**see 4.2.6.**). Purified P-CF produced ETA was characterized by a high degree of monodispersity with equally sized particles in LPPG/B35. D-CF ETA instead displayed a high degree of heterogeneity (Fig. 22). For the CF expressed ETB receptor the opposite picture was revealed by negative staining. Whereas LPMG resolubilized P-CF produced ETB was highly aggregated, no detectable signs of aggregation were found for D-CF synthesized ETB in B78, which indicated a high sample quality (Klammt et al. 2007b).

For P-CF produced ETA precipitates, resolubilization with LPPG followed by exchange to B35 or Fos-16 was best suitable. Highest yield of purified protein in line with homogenous gel filtration profiles indicated both detergents for further analysis (**see 4.2.3.** and **4.2.4.**). The obtained results in the present study show that P-CF expression of GPCRs in combination with appropriate solubilization detergents can result in apparently homogenous sample qualities. The expression mode (**see 5.2.1.**) as well as the secondary detergent upon detergent exchange could therefore be identified as major determinants for the quality of the ETA receptor. These findings correlate with previous results of secondary structure analysis of CF expressed Tsx protein and the porcine GPCR V2R by CD spectroscopy. They revealed considerable variations of the α-helical and β-sheet contents which were dependent on the mode of expression or on the added detergent during IMAC purification (Klammt et al. 2005).

Another effect of detergents is their influence on the oligomerization potential of MPs. DDM and CHAPS for instance influenced the oligomer-dimer-monomer equilibrium of rhodopsin (Jastrzebska et al. 2004) and CF expression in presence of different detergents affected the apparent dimerization of porcine V2R and human ETB observed upon SDS-PAGE analysis (Klammt et al. 2007b).

The oligomerization state of P-CF produced ETA samples were analysed with MALS (Slotboom et al. 2008). ETA appeared to be predominantly monomeric in Fos-16 micelles whereas monomeric as well as dimeric particles were present in B35 micelles (**see 4.2.5.**). D-CF produced ETA in B35 displayed such a high molecular mass with over 1 MDa that no reliable data collection was possible. The equilibrium between monomeric and dimeric ETA forms can

therefore be triggered by the surrounding micelles but is also dependent on the expression mode. This option might be of primary importance to study GPCRs more closely *in vitro*. The formation of molecular assemblies composed of dimeric or higher oligomeric units among GPCRs is discussed as a common mechanism potentially involved in modulating receptor function (Milligan 2004, Rios et al. 2001). The possibility of individual *in vitro* manipulation of the oligomeric state of a GPCR by a micelle of distinct composition might therefore represent an interesting tool to study their structure in order to develop therapies for diseases caused by oligomerization (**see 1.3.4.**).

5.2.3. Evaluation of secondary structure stability of CF expressed ETA

Thermostability is an essential prerequisite of MPs which are subjected to structural investigations by X-ray crystallography and liquid-state NMR spectroscopy. Therefore, the stability of ETA proteomicelles was analysed by gel filtration as well as by CD spectroscopy. The stability of P-CF synthesized ETA appeared to be considerably high as almost identical SEC elution profiles could be obtained in several conditions after one week storage at 4 °C. Even for the very broad peak of D-CF produced ETA the elution profile did not change over time (Fig. 18 and 19). Accordingly, the thermal stability of the secondary structural elements of D-CF produced ETA in B35 and of P-CF produced ETA exchanged to B35 was notably high and observable denaturation started above 50 °C with T_m values of approximately 72 °C and 85 °C, respectively (**see 4.2.7.**). These values correspond to the thermal stability of dark-adapted rhodopsin in disc membranes of around 72 °C (Khan et al. 1991).

Enhancing thermostability from initial values of 37 to 47 °C or from 23 to 40 °C upon mutation was an important parameter towards crystallization of recombinant bovine rhodopsin and human adenosine A2a receptor, respectively (Magnani et al. 2008, Standfuss et al. 2007). Alteration of receptors by point mutation also enhanced the thermostability of the β1-AR to ~53 °C or of the NTR 1 to about 50 °C compared with its highest stability in ligand bound form (Serrano-Vega et al. 2008, Shibata et al. 2009). However, those denaturation temperatures are not directly comparable to the data obtained with CF expressed ETA as temperature induced loss of radioligand binding versus thermal unfolding were determined. Those measurements indicate the thermal stability of the active tertiary structure of a protein. Potentially different sensitivities of the applied techniques must therefore be considered. For ETA, the high thermal stability only characterizes its secondary structure but nothing is so far known about its tertiary structure stability.

To investigate the thermostability of a GPCR by ligand binding requires a straightforward technique and an easy to handle ligand. Due to its complex structure with two disulfide bridges and a highly hydrophobic character, ET-1 is only limitedly suited to establish a technique which can be used as throughput method. An interaction of the ET-1 with artificial

hydrophobic surfaces might occur, generating false positives, and the disulfide bridges might break in certain buffer conditions rendering this ligand unreliable for throughput evaluation approaches (**see 5.3.3.**).

Alternatives to assess the tertiary structure thermostability of CF expressed ETA without using radioligand binding experiments would be differential scanning calorimetry (DSC) which measures the enthalpy of unfolding due to heat denaturation and was already successfully used for rhodopsin (Kahn et al. 1991). However, the major drawback of this technique is, that a standard DSC instrument needs large amounts of protein in a concentration range required for structural determination. With regard to quality optimization of recombinant MPs, this technique is not suited for throughput screening purposes. Differential scanning fluorimetry (DSF), a more recent approach to determine the thermal stability of a protein's tertiary structure, neither requiring the monitoring of enzyme activity nor high protein concentrations, might therefore be more suited for the investigation of the CF expressed ETA receptor. DSF has been adapted to high throughput formats and bases on environmentally sensitive dyes whose emission properties change upon interaction with thermally denaturated proteins (Pantoliano et al. 2001, Poklar et al. 1997). It has already been successfully applied to more than 200 soluble human proteins (Vedadi et al. 2006) and although specific detergents might interact with the fluoroprobe (Ericsson et al. 2006, Senisterra et al. 2010), evaluation of the right detergent/protein ratio might enable the implementation on MPs.

5.2.4. Membrane insertion of CF expressed ETA by co- or post-translational reconstitution

The functionality of MPs is commonly explored in native membranes or lipid bilayers as they represent their natural environment and are associated with correct folding of the respective target proteins. Comprehensive studies of either native or recombinant ET receptors in crude membrane fractions obtained from eukaryotic homologous or heterologous host cells have already been performed (Adachi et al. 1994, Arai et al. 1990, Doi et al. 1997, 1999, Haendler et al. 1993, Schiller et al. 2000, 2001). However, invesitgations of *in vitro* reconstituted ETA or ETB are still scarce with reports of incorporation efficiency by freeze-fracture (Klammt et al. 2007b) or extrinsic fluorescence intensity (Saravanan et al. 2004, 2007). However, ligand binding of *in vitro* reconstituted CF expressed ET receptors has not been evaluated so far.

Reconstitution of MPs, with special focus on CF expressed MPs and GPCRs, can be accomplished in two different ways. In the classical post-translational reconstitution approach, P-CF or D-CF mode generated MP proteomicelles are *in vitro* inserted into lipid bilayers by dilution of the solubilizing detergents below their CMC upon addition of liposome stocks (Rigaud et al. 1995). Preformed liposomes may alternatively be pre-destabilized by moderate detergent incorporation. Controlled detergent removal can be achieved by dialysis or adsorption

to hydrophobic resins like Biobeads (Rigaud et al. 1998). The reconstitution of CF expressed detergent-solubilized MPs and GPCRs by those conventional techniques is feasible and its success has been monitored by freeze-fracture analysis or ligand binding assays (Berrier et al. 2004, Elbaz et al. 2004, Kamonchanok et al. 2008, Keller et al. 2008, Klammt et al. 2004, 2007b).

Alternatively, the L-CF expression mode in the presence of supplied lipids, which is unique for CF expression, offers a new option for the co-translational insertion of MPs. In this mode, lipids can be supplied into the CF RM in a variety of formulations (**see 1.2.3.**). One clear advantage of the L-CF mode might be a prevalent inside out orientation of the target MP as the translation happens outside of the vesicles (Schwarz et al. 2008) in contrast to the random orientation of conventional *in vitro* reconstitution approaches in which detergent to lipid ratios and types have to be carefully evaluated to trigger the direction of insertion (Knol et al.1998). Such a unidirectional insertion would simplify functionality assays and enhances their sensitivity. However, a couple of parameters have to be considered if applying the L-CF mode because the charge of the lipids can greatly affect transcription and translation efficiency in the CF systems (**see 1.2.3.**). Lipid and lipid mixtures are tolerated at often high concentrations in CF expression (Klammt at al. 2004) but their concentration may furthermore influence the obtained expression level as shown for GFP (Bui et al. 2008). In contrast to the commonly used P-CF and D-CF mode, the L-CF mode is just emerging, but recent reports of successfully integrated MPs in different lipid environments including nanodiscs clearly point at the high potential of this technique (**see 1.2.3.**).

For both approaches, protocols have to be adapted to the respective target proteins as efficient translocation is target dependent and can be considered as major challenge that has to be overcome. The rate of successful MP integration can for instance be monitored by electron microscopic freeze-fracture analysis, immunodetection or ligand binding assays if available. Protease protection assays can further support the insertion of MPs by topological information but their implementation was not successful for ETA in the current work (data not shown).

Freeze-fracture analysis revealed a homogenously dispersed character of the inserted ETA particles from post-translational insertion (Fig. 28) as is expected for functional reconstitution (Lindstrom et al. 1980). The observed reconstitution efficiency was however very low with only 5-10 particles per vesicle. Furthermore, the majority of the protein precipitated during reconstitution causing high background signals in subsequent radioligand experiments. Change of reconstitution conditions by evaluation of expression modes, protein detergents as well as liposome destabilizing detergents, lipid-to-protein ratios and lipid compositions enabled the suppression of high background in some cases and highlighted that reconstitution of ETA and ETB samples was only successful with P-CF produced samples out of LPPG or LMPG micelles (Table 12 and Fig. 27). This is in line with published results of other CF expressed

GPCRs which were most effectively reconstituted from P-CF mode solubilized protein samples (Ishihara et al. 2005, Kamonchanok et al. 2008, Klammt et al. 2007b).

A functional assay was further developed which allowed a correlation between insertion rate of liposomes either gained by co- or post-translational reconstitution and specifically bound ligand (**see 4.3.** and **4.3.1.**). Highest activity of ETA was always measured at 1:1,000 molar protein-lipid ratio with artificial lipids composed of either eukaryotic asolectin PC, PC/PE mixtures or *E. coli* polar lipids (with major components PE and PG).

L-CF expressed ETA, whose synthesis was always performed in presence of CHAPS pre-destabilized liposomes, was also evaluated by radioligand binding (**see 4.4.2.**). The best signal/background ratio was determined for a mixture of asolectin PC, PE and PG, which was higher than the apparent with pure aso-PC (Fig. 33). These results are in accordance with the evidence that lipid charge influences CF translation and that anionic lipids might be required for functional reconstitution of specific targets (Bui et al. 2008, Umakoshi et al. 2009, Van Dalen et al. 2002). Anionic LPPG resulted in the most homogenous profile of ETA supporting the assumption that ETA might need surrounding negative charges (**see 5.2.1.**). However, overall binding efficiencies were lower as compared with post-translational reconstitution. Whereas for the latter approach binding values of up to 60 % over background were calculated, only 30 % were reached with the best conditions in the L-CF mode. Protein precipitation or partial integration caused by detergent removal along with inefficient translocation through the lipid bilayer is most probably the reason for the observed high background. Approaches to wash the L-CF pellet with urea in order to remove attached proteins from the surface of the liposome failed, as the same high background was observed with or without urea treatment (data not shown).

Reasons for problems with translocation are evident. Efficient insertion of MPs into the membrane is generally hampered by large terminal domains, extended hydrophilic loop regions or tags since those enlarge the hydrophilic MPs parts and with it the energy barrier which has to be surpassed to become inserted (Hessa et al. 2005). Furthermore, hydrophobic protein parts should be completely covered by detergent molecules which is essential for functional reconstitution (Jung et al. 1998, Padan et al. 2008) especially at the stage of detergent removal. Loosely covered hydrophobic patches will tend to aggregate faster resulting in precipitation instead of reconstitution. GPCRs are MPs with large hydrophilic domains whose integration in the membrane is processed by eukaryotic translation machineries (**see 1.1.1.**). The CF expressed ETA additionally comprises hydrophilic N- and C-terminal tags which enlarge those domains. Furthermore, detergent removal by Biobeads might not be suited for ETA with regard to rapidity or detergent compatibility. Careful evaluation of the right detergent and of the detergent removal technique in combination with changes in the speediness of removal might increase the efficiency of classical reconstitution.

In order to improve the functional reconstitution of ETA in the L-CF mode, one of the first steps is the reduction of precipitated protein by lowering the amount of DNA template or by decreasing the expression temperature thus slowing down production kinetics. Addition of agents like non-delipidating detergents such as fluorinated surfactants (Park et al. 2007, Polidori et al. 2006), Brij derivatives or the addition of lipids like cholesterol, all likely stabilizing the MP prior to liposome insertion or acting as specifically bound co-factors for functional folding (Hunte and Richers 2008, Opekarova and Tanner 2003), might be further options to control and to enhance functional reconstitution in the L-CF mode. However, it is questionable if the L-CF mode is suitable for ligand binding assays of GPCRs. If one prevalent orientation of the MP upon insertion is generated (Schwarz et al. 2008), the N-terminus as well as all native ECLs of GPCRs could face the interior of the liposome. With most peptide ligands binding to extracellular domains and the upper parts of the TMSs (Ji et al. 1998, Kobilka 2007) effective ligand binding might then not be expected for the ET receptors if the ligand is not transported through the membrane. But even if functionality could not be approached, the reconstitution by L-CF mode would still be a promising option for techniques that require quantitative reconstitution as a prerequisite such atomic force microscopy (AFM) to study protein folding and stability. If efficient co-reconstitution of ETA and/or ETB can be obtained by L-CF expression this would further be a very fast, straightforward and competitive sample preparation method to investigate the oligomerization potential of CF expressed receptors by FRET measurements (**see 5.5.**).

5.3. Functional characterization of the ET receptors

5.3.1. Lipid based functional studies

Functional studies of GPCRs have mostly been performed in native membranes or in artificial liposomes of defined composition. Specific antagonist binding for CF expressed GPCRs was measured with reconstituted synthetic Gsα-fusions of the human β2-AR expressed in the P-CF mode in *E. coli* extracts (Ishihara et al. 2005). The human HRH1 was synthesized in *E. coli* extracts in the P-CF mode, solubilized in DDM and reconstituted into proteoliposomes. Competition binding assays revealed pharmacological properties similar to the protein isolated from Sf9 cells or measured in mammalian COS-7 cells, although the binding affinity of one inverse agonist was found to be 100-fold reduced (Kamonchanok et al. 2008). Likewise, alprenolol showed up to 1000-fold reduced inhibition and an altered binding mechanism to the human β2-AR with thioredoxin and Gsα in two-site competition analyses if compared to the Sf9 positive control which showed one-site binding (Ishihara et al. 2005). The determined IC50 values of reconstituted ETA and ETB in the present study with 2.17 µM are in between 10^3- and 10^4-fold higher if compared with previously reported data (Table 15 and **see 4.3.2.**), indicating

only moderate ET-1 binding affinities (Desmarets et al. 1996, Desmarets and Frelin 1999, Saeki et al. 1991). Nevertheless, the IC50 values are in the same order of magnitude for both ET receptors (Junge et al. 2010b) which is in good agreement with previous studies, suggesting equal inhibition competence of unlabelled ET-1 (Saeki et al. 1991). K_D values with CF expressed ETA receptors in artificial liposomes of varying composition were calculated by saturation binding of radioactively labelled ET-1. Equilibrium dissociation constants with positive cooperativity were determined at around 1.4 nM (**see 4.3.3.**). These binding data are on average only one or two orders of magnitude higher than in other heterologous membrane preparations (Table 15) and therefore well comparable to *in vivo* data except for the detection of cooperativity.

Table 15: Heterologous expression systems for the production of ET receptors. K_D determined by radioactive binding of ^{125}I-ET-1. K_i or IC50 values were determined with native peptides. The table was modified after Schiller et al. 2000.

Receptor subtype	Expression system	Production levels[A]	Affinity (binding)	Assay[B]	Reference
ETB	*E.coli*	41 r/c	100 pM [K_D]	m	Haendler et al. 1993
ETB	*P. pastoris*	20-60 pmol/mg	42 pM [K_D][a] 200-700 pM [K_i][b]	m	Schiller et al. 2000
ETB	*P. pastoris*	9 mg/L	40 pM [K_D]	m	Schiller et al. 2001
ETA	Sf9 cells	~0.25 mg/L	50 – 80 pM [K_D][a]	m	Doi et al. 1999
ETB	CHO cells	0.82 pmol/mg	310 pM [K_D]	m	Takagi et al. 1995
ETB	CHO cells	0.02 pmol/mg	25.5 pM [K_D]	m	Buchan et al. 1994
ETA	CC139 fibroblasts	-	30-400 pM [IC50] 20 pM [K_D]	m	Desmarets et al. 1996, 1999

[A] Production levels either expressed as total number of binding sites (pmol/mg), as B_{max} value calculated from the binding data (pmol/mg protein), total amount of receptor per litre culture (mg/L) or as receptors per cell (r/c)
[B] Assays performed on membrane preparation (m)
[a] saturation binding with ^{125}I-ET-1 (Schiller et al. 2000, 2001; Doi et al. 1999)
[b] competition with ET-1, ET-2 or ET-3
-, not indicated

Prominent differences in IC50 and K_D values of the same ligands to CF expressed receptors were observed for the human β2-AR with thioredoxin and Gsα (Ishihara et al. 2005) as well as for different ET receptor membrane preparations (Desmarets et al. 1996, Desmarets and Frelin 1999). Copy number effects resulting from difficult to determine receptor concentrations might account for those diverging results in obtained ligand binding data (Desmarets et al. 1996). Competition experiments with ET-1 and ETA commonly result in a broad range of apparent K_D values in between 20 pM and 1 nM. This oberservation is attributed to inaccuracy in the determination of receptor concentration rather than to the binding itself, as complex second-order rate kinetics and ligand depletion control ligand association employing receptor concentrations higher than the apparent K_D (Desmarets et al. 1996, Desmarets and Frelin 1999, Jacobs et al. 1975). The concentration of CF reconstituted ETA receptor was commonly only estimated according to the reconstitution efficiency detected in freeze-fracture electron

micrographs (Fig. 28) as all other assays failed due to the residual precipitate presumably attached to the liposomes (**see 5.2.4.**). Therefore, imprecise determination of ETA concentration might be an explanation for the high difference of IC50 values from *in vivo* material. Similarly, positive cooperativity with Hill slopes of ~2 was found in saturation binding experiments of purified NTR 1 at high receptor concentrations whereas at low receptor concentrations Hill slopes of ~1 prevailed. The authors concluded that the receptor must exist as dimers or higher-order oligomers at high concentrations to explain the positive cooperative effect (White et al. 2007). Such oligomerization effects might cause the positive cooperativity observed in CF expressed ETA.

Further explanations for those variations are related to the absence of a eukaryotic cell environment. PTMs such as glycosylations and palmitoylations are known as potential modulator of GPCR binding properties and could affect the interactions of ETA with ligands (Shraga-Levine and Sokolovsky 1998, Zhang et al. 2001). The C-terminal domain and the third cytoplasmic loop of ETA and ETB contain several putative phosphorylation sites and glycosylations and palmitoylations are further proposed modifications of both receptors. Quite pronounced is the role of G-protein coupling and association with GPCR interacting proteins in enhancing ligand binding (Fitzsimons et al. 2004, Hay et al., 2006). Dynamic interactions of ligand/receptor/G-proteins can be necessary for high affinity agonist binding. The production of unmodified receptors in the *E. coli* based CF system in combination with the absence of any accessory proteins or specific lipids after reconstitution into defined *E. coli* polar lipids could therefore contribute to modified binding properties or to incorrectly folded protein.

5.3.2. Working with detergent-solubilized ET receptors

The most common objection against working with MPs in a micellar environment is the assumption, that their functionality is considerably changed or even completely abolished due to the lacking membrane environment. The absence of lateral pressure and the exposure of hydrophobic surfaces to aqueous solutions during MP solubilization are thought to considerably perturb their structures (Rosenbusch 2001). This is supported by the fact that ligand binding profiles of ETB in intact cells already differ from corresponding membrane fractions (Hara et al. 1998).

However, a variety of GPCRs has already been synthesized in CF systems in a detergent-solubilized state and their functional activity has subsequently been evaluated by *in vitro* analysis. Ligand binding of a human olfactory receptor was reported after D-CF production in WGE (Kaiser et al. 2008). Full-length and truncated derivatives of the human ETB receptor have been D-CF synthesized in presence of B78 and ligand binding of the agonist ET-1 was shown by several complementary techniques. Their binding kinetics, determined by

SPR measurements, were shown to be only marginally different from *in vivo* data (Klammt et al. 2007b, 2007c).

A first indication that CF expressed ET receptors in detergent micelles might behave similarly to receptors extracted out of native membranes has been gained in present work. Both CF expressed receptors displayed different receptor-ligand complex stabilities with b-ET-1 upon separation via SDS-PAGE (Fig. 37). In contrast to ETA whose binding to b-ET-1 could not be detected by western blotting, human CF expressed ETB seems to form a tight complex with its ligand ET-1 which remains stable upon treatment with SDS. The same observations were made with receptors isolated from crude canine membranes and human placenta samples (Takasuka et al. 1992, 1994).

Since detergents do not display static structures and the polydispersity of micelles commonly varies (Garavito and Ferguson-Miller 2001, Hauser 2000), functional folding of MPs most likely is considerably effected by the micellar environment generated by different types of detergents. Besides heterogeneity in oligomeric states, the presence of different folding conformations can become a major bottleneck for structural approaches. Purification of solubilized GPCRs by their ligand binding properties is therefore an essential step in quality control and represents a valuable tool to receive homogenous sample qualities. The ligand binding competence of solubilized ETA to immobilized b-ET-1 ligand ranged from approximately 1 % up to 50 % in B35 or DDM micelles (**see 4.5.1.**). Those relatively high binding capacities could be obtained from ETA expressed in the D-CF mode as well as in the P-CF mode, the P-CF quality in particular being in accordance with the comparable sample qualities obtained by SEC and MALS (**see 4.2.4.** and **4.2.5.**). However, high batch variations of DDM along with decreasing yield of purified ETA in the course of the presented work made it impossible to proceed working with this detergent (**see 4.2.3.**).

Corresponding GPCR ligand binding capacities after cellular expression in *E. coli* cells revealed 40 % for the human peripheral cannabinoid 2 receptor and only less than 1 % for the human α2b-AR (Magnin et al. 2009). Large-scale fermentation of a NTR 1 fusion protein yielded in 90 mg functional fusion protein / 200 L of culture (White et al. 2004). Considering initial yields of purified ETA of approximately 1 mg/mL after P-CF expression and 0.5 mg/mL after D-CF expression, corresponding amounts of some 500 and 250 µg ligand-binding competent ETA could be isolated out of 1 mL RM with the described optimal conditions (**see 4.5.1.**). With P-CF expressed ETB, the ET-1 binding competence was found to be significantly lower within the range of less than 10 %. This corresponds to previous findings indicating that P-CF produced solubilized ETB still contains a high degree of aggregated inactive protein (Klammt et al. 2007c). It is therefore important to note that the ETA receptor behaves differently from ETB after P-CF expression in showing non-aggregated sample quality combined with high ligand binding competence. The P-CF as well as D-CF mode can thus be

implemented for the preparative scale production of functional ETA. However, while the D-CF mode should be preferred for ETB, the P-CF mode is highly suited for ETA.

The dissociation constant of the linear f-4-Ala-ET-1 ligand in ETA proteomicelles was, corrected by a factor of 50 % binding capacity, determined at around 1.2 µM by fluorescence anisotropy, compared with 0.57 µM in crude membranes of human tissue (Saeki et al. 1991) or 4.52 µM reported by Molenaar in cardic membranes (Molenaar et al. 1992). This marginal variation might be explained by different assay conditions, fluorescence anisotropy versus radioligand binding to membranes, and the use of differently labelled ligand derivatives. Specificity of the binding was controlled with the ETA receptor/b-ET-1 complex eluted from the monomeric avidin matrix, which did not show any increase in anisotropy (**see 4.5.3.**). Recent reports show that fluorescence anisotropy was also successfully applied to membrane preparations of melanocortin 4 (Veiksina et al. 2010) and muscarinic M1 receptors (Huwiler et al. 2010) and might therefore represent a valuable alternative to SPR measurements to study ligand binding of detergent-solubilized GPCRs.

5.3.3. Troubleshooting for functional studies: Structural complexity of ET-1

One of the most prominent problems working with ET peptide ligands is their intrinsic hydrophobicity along with a very complex structure formed by two disulfide bridges (**see 1.5.2.**) and a high aggregation potential in aqueous solution (Bennes et al. 1990, 1991), so that the peptide has to be solubilized in an organic solvent like DMSO. The physiological necessity of the ET-1 peptide of being partly hydrophobic might reside in the mechanism of receptor-ligand binding. It might at least partially need to integrate into the membrane to reach its binding region as peptide ligands smaller than 40 aa are thought to bind to parts of ECLs and the upper parts of the TMSs of GPCRs (Ji et al. 1998, Kobilka 2007). For ET-1 and ETB it has been proposed that TMS 1 (Klammt et al. 2007c) and parts of the fifth TMS (Boivin et al. 2005) are essential. ET-1 might interact with ETA at parts of ECL 1, IL 3 and TMS 4 and 6 (Aubin et al. 2008) with a histidine residue in the sixth helix binding to the essential tryptophan 21 of the ligand. Modelling studies further proposes a hydrophobic binding cleft for the C-terminus of the ligand confined by aromatic residues in helices 3, 5 and 6 (Huggins et al. 1993).

Difficulties which presumably arise from the ligand's complex structure have been encountered in this thesis. Scintillation proximity assays (SPA) with ET-1 and CF expressed ETB failed due to unspecific interactions of the ligand with empty scintillation beads of different types (cooperation with Dr. Michael Beyermann, data not shown). Likewise, the competitive displacement of monomeric avidin matrix bound ETA/b-ET-1 with ET-1 tried as alternative to elution with biotin was not possible, most probably due to a high interaction of ET-1 with the matrix material alone, monitored with ^{125}I-ET-1 (Fig. 59). This interaction might

have considerably reduced its quantitative potency to displace the b-ET-1 derivative from its binding site on ETA.

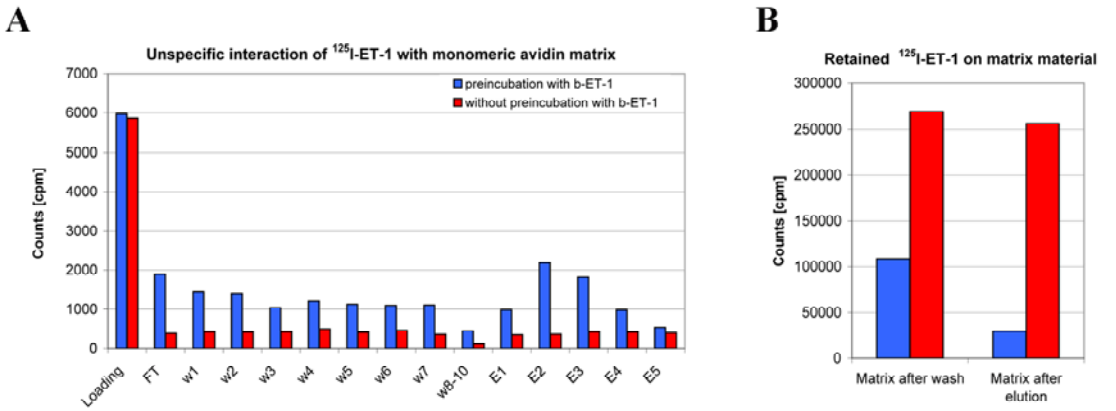


Fig. 59: Unspecific interaction of ^{125}I-ET-1 with the monomeric avidin agarose beads. (A) Elution profile of ^{125}I-ET-1 from MAM. (B) Radioactive ligand retained on MAM beads. The fraction of ligand, removed by washing and elution with biotin is very small (300-1000 cpm) although no receptor interaction partner is bound to the matrix. The retained total counts on the matrix after washing and elution are suggestive for high unspecific matrix/ligand interaction (25000-260000 cpm).

Unspecific interaction of ET-1 with the surrounding micelles or with similar hydrophobic binding pockets of the prepared CF expressed GPCRs, due to its hydrophobic C-terminus, might have been one of the reasons why cross-interactions with V2R (aromatic residues at similar positions, see above), CRF and ETB at equal binding kinetics were observed upon SPR measurements (**see 4.8.2.**). Those encountered difficulties rendered it highly necessary to perform excessive NCs for all presented functional assays with detergent-solubilized ETA.

High background binding which was always observed when using liposome reconstituted CF expressed ETA (Fig. 27 and 33) might also result from hydrophobic interactions of ET-1 with partially integrated, misfolded receptor or with liposome-attached receptor precipitate. Due to detergent removal, the hydrophobic patches of precipitated ETA are completely accessible and may offer many unspecific binding sites for ET-1.

5.4. Structural investigations of CF expressed human ETA receptor

5.4.1. Using CF expression for structural studies of GPCRs by X-ray crystallography

CF expression could offer a high potential for the structural analysis of MPs by NMR spectroscopy as well as by X-ray crystallography. The set-up is ideally suited for rapid screens of optimal expression and resolubilization conditions, and protein samples can be obtained in less than 24 hours. Besides the efficient incorporation of isotopically labelled amino acids (Ozawa et al. 2005, Reckel et al. 2008, Trbovic et al. 2005), non-natural and chemically modified amino acids can become incorporated quantitatively into CF-synthesised proteins (Kanda et al. 2000, Kiga et al. 2000, Sengupta et al. 2003). This can also be utilised in order to

include selenomethionine in protein samples for X-ray crystallography (Kigawa et al. 2002). In addition, beneficial supplements which can enhance protein stability for crystallization attempts like ligands, cofactors or substrates can be added directly, without considering transport or metabolic conversion problems. Furthermore, problems of heterogenous sample quality upon inefficient or differing glycosylation patterns often present in *in vivo* systems are avoided (Okamoto et al. 1998, Shraga-Levine et al. 1998). Crystals of CF expressed MPs were recently obtained with EmrE and VDAC1 (Chen et al. 2007, Deniaud et al. 2010, Nguyen et al. 2010) clearly demonstrating that the quality of CF-produced MP samples can be sufficient for crystallization.

ETA was judged suitable for initial screenings of critical parameters for structural analysis by X-ray crystallography as it fulfilled a variety of essential prerequisites. (I) In the present work, ETA could be expressed in the P-CF mode in high amounts of 1.5 mg/mL average yield. (II) After resolubilization in 1 % LPPG and subsequent IMAC purification to 0.1 % B35 a purity of approximately 98 % was achieved (Fig. 17 A and Table 11). (III) SEC and MALS analyses pointed at homogenous sample qualities (**see 4.2.4.** and **4.2.5.**) which was supported by negative staining EM (**see 4.2.6.**). Samples are furthermore stable after purification for at least one week at 4 °C. (IV) Its secondary structure exhibited high thermostability at around 85 °C investigated by CD spectroscopy (**see 4.2.7.**). This enabled crystal screening not only at 4 °C but also at 18 °C as well as at 22 °C required for lipid-based cubic phase trials. Higher temperatures than 4 °C were prevalent in solving most of the recent crystal structures of GPCRs (Cherezov et al. 2007, Hanson et al. 2008, Jaakola et al. 2008, Rasmussen et al. 2007, Warne et al. 2008). (V) The ligand binding competence of ETA in 0.1 % B35 was successfully proven by fluorescence anisotropy and ligand affinity chromatography (**see 4.5.1**. and **4.5.3.**). Most of the GPCR structures (Cherezov et al. 2007, Hanson et al. 2008, Jaakola et al. 2008, Warne et al. 2008) needed co-crystallization of agonists or antagonists to stabilize the active and homogenous receptor conformation. Binding of ETA to its ligand ET-1 at up to 50 % which could be performed in 0.1 % B35 might therefore be a requirement for successful crystallization. (VI) ETA could be concentrated in absence and in particular in presence of the ligand to >5 mg/mL without loosing its overall homogeneity as monitored by gel filtration (**see 4.6.1.**).

Detergents in type and concentration are one of the most critical parameters in MP crystallization as they have to keep the protein in a soluble form, provide the necessary stability, allow an active conformation and should not impair the formation of rigid crystal contacts largely based on hydrophilic domains of the protein protruding from the proteomicelle (Hunte and Michel 2002). Commonly used detergents such as DDM or Foscholine-derivatives (Fos-16 in this case) which were investigated in this study upon detergent exchange of CF expressed ETA did not provide the necessary combination of all parameters like long-term stability, homogeneity and ligand binding capacity of the receptor. The choice of the most promising

detergent used for crystallization of ETA was therefore primarily not guided by those detergents which most often yield ordered crystals but rather based on the fact that the Brij derivative B35 showed the best overall compatibility with ETA. Initial crystallization screens were accomplished with 150 µL each of ETA either concentrated to 5.2 mg/mL without ligand or concentrated to 8.0 mg/mL in presence of ET-1 with the sitting drop vapour diffusion method. The screens were performed on the CrsytalMation™ automatic platform in 96-well formats (in cooperation with Dr. Yvonne Thielmann) with crystal drops of 200 nL volume. Therefore, it was possible to apply over 18 commercial as well as custom screens not only comprising classical vapour diffusion but also cubic phase trials with only two ETA sample preparations of 150 µL each (**see 7.5.**).

So far, no crystals were observed in any of the analysed conditions. Neither a certain pH nor certain precipitants or additives could be qualified as essential until now but some tendency towards microcrystallization of ETA could be observed with MemSys™, MemGold™ and MemStart+MemPlus™ (**see 4.6.2.**).

5.4.2. Getting CF expressed human ETA to crystallize

Engineering MPs by the insertion of mutations to enhance the receptor's thermostability, integration of T4 lysozyme or binding of antibody fragments to the receptor in order to enlarge the available crystal contact surface was essential for crystallization of all recent recombinant GPCR structures (**see 1.3.5.**) (Hunte and Michel 2002, Rosenbaum et al. 2009, Serrano-Vega et al. 2008). Those approaches should be among the first choice if the non-engineered ETA receptor does not crystallize as they base on extensive fundamental research and high expertise.

There are however still a variety of options to improve the quality of non-engineered ETA. Crystallization is a result of supersaturation. 5-8 mg/mL is considered as the lower limit of an initial desirable concentration for crystal growth. This could hence be one of the first steps to be approached, as ETA did not show any remarkable tendency to high-order aggregates upon concentration which could further be modulated and controlled by the addition of ET-1 during concentration (**see 4.6.1.** and **4.6.2.**). The choice of detergent also plays an important role. B35 is not commonly used in crystallization since it generates very large micelles tending to impair crystal contact formation. There is a long list of common non-ionic and zwitterionic detergents used for crystallization which have to be tested in future in terms of quantity, quality and functionality of the receptor like the preferred alkyl maltosides with the CYMAL series, the alkyl glucosides as well as alkyl dimethylamine-oxides like LDAO (Hunte and Michel 2003). However, due to the observation that the shortest of the long-chain Brij derivatives B35 seemed optimally suited for the generation of crystallization-grade ETA by CF synthesis, a variety of short-chain alkyl polyoxyethylenes like C8E5, C8E4, C12E9 or C12E8, also commonly utilized in crystallization trials, could further be suited for the receptor. Around 7-12 carbons in length

are at least needed to mimic the thickness of the hydrophobic part of native membranes (Wiener 2004). It might therefore be an interesting option to mix short- and long-chain Brij derivatives for the crystallization of ETA. Overall, it is crucial to retain its ligand-binding capacity which might be reduced the shorter the alkyl chains get. Successful exchange to those detergents should therefore be analysed first by SEC and ligand affinity chromatography which is a valuable tool that will greatly facilitate the evaluation of the alternative detergents.

Other options to enhance the receptor's stability and homogeneity -which were not investigated so far due to a pronounced loss in sample quantity of up to 50 %- are secondary purification steps of ETA either by ligand affinity chromatography or by gel filtration which are also commonly applied techniques in receptor preparation for crystallization (White et al. 2004). The upcoming method of lipid cubic phase crystallization (Caffrey and Cherezov 2009), doped with cholesterol, was successfully applied in the structural determination of the human β2-AR (Cherezov et al. 2007, Hanson et al. 2008) and the human adenosine A2a receptor (Jaakola et al. 2008). This approach might further be a very interesting alternative to the classical vapour diffusion crystallization of ETA as it stabilizes the receptor in a more native-like environment and the addition of cholesterol could influence ETA receptor stability (Burger et al. 2000, Couet et al. 1997). Brij derivatives as well as fluorinated surfactants which showed reasonable (fluorinated surfactants) to high ETA receptor quality (B35) (**see 4.5.1.**) might in this case be ideally suited for the partitioning of the protein into the provided lipid bilayers since they are very mild and do not disintegrate membranes (Park et al. 2007, Polidori et al. 2006).

5.5. Interaction of the individual CF expressed ET receptors

5.5.1. Oligomerization behaviour of CF expressed ET receptors in the context of current models

Homo- and hetero-dimerization/oligomerization of rhodopsin-like GPCRs is an increasingly recognized mechanism and investigations thereof are a growing field in science. It has been shown in different receptor systems that both types of interaction have enormous physiological and pathophysiological implications (Chabre and Le Maire 2005, Jastrzebska et al. 2006, Lee S.P. 2004) and might therefore represent an important platform for the modulation of GPCR activities such as ligand binding, signalling or trafficking (Rios et al. 2001, Schöneberg et al. 1999). Understanding the mode in which different or the same protomers of the complex interact as well as revealing their interaction interface is essential for the development of totally new therapies for a variety of diseases related to receptor complex formation (George et al. 2002, Milligan 2007).

The underlying structural interaction mechanism in homo- and heterodimers of the ET receptors has not been elucidated yet and is still a matter of debate. Bivalent ligands have been discussed, possibly forming a bridge between ETA and ETB dimers (Harada et al. 2002) but

this hypothesis has been contrasted by the findings that indeed neither agonists nor antagonists did show any influence on dimer formation or dissociation (Evans and Walker 2008, Gregan et al. 2004a, 2004b) and single-particle analysis strongly suggests ETB dimer formation in the absence of the ligand (Klammt et al. 2007b). In the rhodopsin-like family, to which the ET receptom belongs, the model of robust hydrophobic interaction between the TMSs is favoured as SDS-resistant oligomers are often observed (Lemmon et al. 1992) and involvements of almost all TM helices (1, 2, 4, 5, 6) including parts of extracellular domains have been analysed for a variety of different GPCRs (Milligan 2007).

Recently, it has been proposed by pull-down experiments of D-CF expressed full-length human ETB receptor and truncations thereof that the TMS 1 is involved in the essential interface for intermolecular interactions between single ETB monomers as the two fragments ETB131 and ETB93a, which overlapped in that region, did still form dimers with full-length ETB (Klammt et al. 2007c).

In accordance with the studies performed by Dr. Christian Klammt, investigations of CF expressed human full-length ETA and ETB as well as various designed truncations should allow further characterization of the molecular interaction and possible interfaces between ETA homodimers as well as ETA/ETB heterodimers in the current study. Synthesizing truncated derivatives of MPs for the analysis of receptor interactions is a very straightforward approach and largely facilitated by using CF expression. Truncations might be toxic in cell-based systems resulting in low expression yields or even cell-death. Alternatively, they might be recognized by cellular regulatory machineries as aberrant or damaged, subsequently degraded by the proteasome or single proteases. Small-sized MPs, like truncations in this case, are readily synthesized in CF systems in high amounts often surpassing the yields of large proteins (Schwarz et al. 2010). This has already been shown for the human ETB receptor (Klammt et al. 2007c). Similar results were obtained with the synthesis of ETA truncations in this work (**see 4.7.1.**). All derivatives were expressed at high levels and were characterized by the same resolubilization behaviour as the full-length ETA construct (Fig. 44).

The potential of CF produced full-length ETA to homodimerize was already observed by different techniques during quality analysis. Apparently stable SDS-resistant dimers of ETA in 0.1 % B35 were detected by ESI-mass spectrometry of in-gel trypsin digested receptor bands and by MALS combined with SEC (**see 4.7.** and **4.2.5.**). Furthermore, protein bands at the approximate size of the calculated dimers were also observed by BN-PAGE analysis of directly resolubilized ETA in 1 % LPPG (**see 4.2.2.**).

Pull-down experiments with differently tagged receptors served as tool for the analysis of ETA and ETB interactions in the present thesis (**see 4.7.2.**). Pull-down assays require stable interactions with low dissociation constants to ensure that the interacting partners do not disassemble during the washing steps. This stable interaction might be hampered by large surrounding micelles. Homo-and hetero-oligomerization of both ET receptors were therefore

analysed by two different CF expression approaches followed by pull-down via strep-affinity chromatography. Either the full-length receptors were co-expressed during CF synthesis or they were expressed separately and subsequently mixed as individual components. The co-expression assay was favoured, as it has been hypothesized that at least homo-oligomerization already occurs at the level of protein synthesis in the ER or during maturation of the GPCR prior to cell surface delivery (Bulenger et al. 2005). B35 was chosen as most promising detergent since ETA already showed possible dimerization in it (Fig. 20, 40 and 41) and since it further allowed the expression of all interaction partners.

Co-expression resulted in a quantitative pull-down of the his-tagged ETB and ETA receptor with full-length ETAstrep indicating homo- and heterodimerization of both ET receptors (Fig. 46 A). Likewise, receptor interaction could also be observed with the combination ETAstrep/ETA197cHx which would suppose that at least the first three TMSs with adjacent loops and the N-terminal region might be sufficient for homodimerization (Fig. 46 B). Since all tested ETBcHx truncations, some harbouring N-terminal segments (ETB131, ETB93a), some only consisting of C-terminal receptor parts (ETB308) could further be co-eluted with the full-length ETAstrep receptor under the chosen conditions, it is likely that more than only one defined region may be necessary for the heterodimerization of ETA with ETB. The interaction interface might be allocated to different parts without any detectable preference for distinct TMSs. This is in accordance with the existing model of 'contact' dimers for a variety of other GPCRs (**see 1.3.4.**) indicating involvements of almost all TMS (1, 2, 4, 5, 6) for their interaction (Milligan 2007). In a co-expression approach of single helix bundles of rhodopsin it was further possible to reassemble a functional receptor (Yu et al. 1995) indicating a specific interaction within the helix bundle itself, most likely due to hydrophobic interactions (Yeagle and Albert 2007).

The obtained results are however highly questionable at this point. Supposed NCs of ETAstrep with unrelated his-tagged MPs (NTF as well as TMD0), which were performed in parallel, also resulted in co-elution of latter proteins with the ETA receptor showing the same elution behaviour like the interacting ET derived partners (Fig. 48). Analysis of all single his-tagged components with regard to their affinity to the strep-matrix could in fact exclude cross-reactivity as possible explanation of the results (Fig. 47). Therefore, it was assumed that hydrophobic interactions between the TMSs themselves might be the reason for the obtained results since those associations can readily occur either in a specific (for the ET receptor combinations) or a totally unspecific (for ETA and unrelated MPs) manner. Other possible molecular mechanisms like the formation of covalent disulfide bridges or ionic interactions in the loop regions would be too specific, most likely requiring a special amino acid motif, than to occur as artefacts with unrelated MPs.

5.5.2. Troubleshooting and suggestions for future experiments

The problem which clearly arose from such false positives was the question how those nonspecific artefacts were generated. One possible explanation might reside in the choice of co-expression for the analysis of oligomerization. Co-expression of MPs in presence of high concentrations of detergents such as 0.5 % B35 used in the present study (~46 x CMC) might be ideally suited for the promotion of hydrophobic interactions. Unrelated proteins might be co-translationally expressed into the same micelle due to the excess of detergent. The possibility to form hydrophobic interactions might be caused by their spatial proximity that is kept tight by the micellar environment. Native interaction partners will show the same behaviour, rendering it impossible to differentiate between specific interaction and nonspecific association.

The idea of artificially generated spatial proximity by the surrounding micelle being responsible for those artefacts is supported by the observation that the interactions gained from individually CF expressed ET receptors mixed directly from separate RM (Fig. 49) or truncations which were purified beforehand (Fig. 49 B), is reduced to only estimated 1-10 %.

The combination of separately expressed interaction partners might however also have draw-backs with regard to the decreased interaction intensity with ETA. While the co-expression assay might generate artificial proximity, the individual expression of supposed interaction partners might create such large micelles around the single receptors, tumbling in three dimensions, that they can hardly find each other. In addition, the necessary interaction interfaces, especially if those contact areas are confined to the TMSs, are completely surrounded by detergent molecules. Not only the micellar dimensions which can be very large with B35 (additional 50-100 kDa), but also the net charge of the detergent would play an important role in this case. Negatively charged micelles surrounding the protein such as LPPG might electrostatically repel each other with a high probability (Krueger-Koplin et al. 2004) so that the interacting proteins could hardly come into such a close contact necessary for homo- or hetero-oligomerization (Fig. 49 left illustration).

In order to identify specific interactions between the CF-expressed detergent-solubilized ET receptors with the given pull-down assay, different approaches are possible. (I) If the detergent itself creates spatial proximity in the co-expression approach and keeps the MPs tightly associated by its presence, detergent exchange breaking the influence of the previous detergent might remove unspecific association while stable specific interactions are preserved. (II) Specific interactions are supposed to be more stable than nonspecific association. Displacing an unspecifically bound MP by an interaction partner with a predicted higher affinity might therefore be a further option.

Given the problems caused by the micellar environment, a more reasonable approach for this type of investigation is to go into membranes. FRET measurements already show a potential for the analysis of GPCR oligomerization (Overton and Blumer 2002). Physiological insertion

levels are usually sufficient for FRET sensitivity (Patel et al. 2002), so that moderate reconstitution could already meet the requirements. The interaction between the single protomers would then occur in the 2-dimensional space of the natural lipid bilayer, in which the mobility of the receptors is much more directed than in the 3-dimensional space of the micelles. Specific interactions of ET receptors were already determined in crude membranes by FRET (Evans and Walker, 2008, Gregan et al. 2004a, 2004b). Single molecule FRET with co-reconstituted CF expressed ET receptors either obtained by classical post-translational or by co-translational reconstitution using the L-CF mode might therefore more reliably allow the determination of their specific homo- or hetero-oligomerization potential.

6. References

Adachi, M., Yang, Y. Y., Trzeciak, A., Furuichi, Y. and Miyamoto, C. (1992) **Identification of a domain of ETA receptor required for ligand binding.** *FEBS Lett.* 311, 179-183.

Akermoun, M., Koglin, M., Zvalova-Iooss, D., Folschweiller, N., Dowell, S. J. and Gearing, K. L. (2005) **Characterization of 16 human G protein-coupled receptors expressed in baculovirus-infected insect cells.** *Protein Expr. Purif.* 44, 65-74.

Andre, N., Cherouati, N., Prual, C., Steffan, T., Zeder-Lutz, G., Magnin, T., Pattus, F., Michel, H., Wagner, R. and Reinhart, C. (2006) **Enhancing functional production of G protein-coupled receptors in *Pichia pastoris* to levels required for structural studies via a single expression screen.** *Protein Sci.* 15, 1115-1126.

Angers, S., Salahpour, A. and Bouvier, M. (2002) **Dimerization: an emerging concept for G protein-coupled receptor ontogeny and function.** *Annu. Rev. Pharmacol. Toxicol.* 42, 409-435.

Arai, H., Hori, S., Aramori, I., Ohkubo, H. and Nakanishi, S. (1990) **Cloning and expression of a cDNA encoding an endothelin receptor.** *Nature* 348, 730-732.

Arduengo, M., Schenborn, E. And Hurst, R. (2007) **The role of cell-free rabbit reticulocyte expression systems in functional proteomics.** In: *Cell-free protein expression*, Kudlicki, W., Katzen, F. and Bennett, R. (ed.), Landes Bioscience, Austin, TX

Arnold, T. and Linke, D. (2008) **The use of detergents to purify membrane proteins.** *Curr. Protoc. Protein Sci.* Chapter 4, Unit 4. 8. 1.-4. 8. 30.

Arora, A., Abildgaard, F., Bushweller, J. H. and Tamm, L. K. (2001) **Structure of outer membrane protein A transmembrane domain by NMR spectroscopy.** *Nat. Struct. Biol.* 8, 334-338.

Aubin, J., Letourneau, M., Francoeur, E., Burgeon, E. and Fournier, A. (2008) **Identification of ETA and ETB binding domains using ET-derived photoprobes.** *Biochimie* 90, 918-929.

Azzi, M., Charest, P. G., Angers, S., Rousseau, G., Kohout, T., Bouvier, M. and Pineyro, G. (2003) **Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors.** *Proc. Natl. Acad. Sci. U S A* 100, 11406-11411.

Bakker, R. A., Dees, G., Carrillo, J. J., Booth, R. G., Lopez-Gimenez, J. F., Milligan, G., Strange, P. G. and Leurs, R. (2004) **Domain swapping in the human histamine H1 receptor.** *J. Pharmacol. Exp. Ther.* 311, 131-138.

Ballesteros, J. and Palczewski, K. (2001) **G protein-coupled receptor drug discovery: implications from the crystal structure of rhodopsin.** *Curr. Opin. Drug Discov. Devel.* 4, 561-574.

Baneres, J. L., Martin, A., Hullot, P., Girard, J. P., Rossi, J. C. and Parello, J. (2003) **Structure-based analysis of GPCR function: conformational adaptation of both agonist and receptor upon leukotriene B4 binding to recombinant BLT1.** *J. Mol. Biol.* 329, 801-814.

Baneres, J. L., Mesnier, D., Martin, A., Joubert, L., Dumuis, A. and Bockaert, J. (2005) **Molecular characterization of a purified 5-HT4 receptor: a structural basis for drug efficacy.** *J. Biol. Chem.* 280, 20253-20260.

Battistini, B., D'orleans-Juste, P. and Sirois, P. (1993) **Endothelins: circulating plasma levels and presence in other biologic fluids.** *Lab Invest* 68, 600-628.

Bazarsuren, A., Grauschopf, U., Wozny, M., Reusch, D., Hoffmann, E., Schaefer, W., Panzner, S. and Rudolph, R. (2002) ***In vitro* folding, functional characterization, and disulfide pattern of the extracellular domain of human GLP-1 receptor.** *Biophys. Chem.* 96, 305-318.

Beelman, C. A. and Parker, R. (1995) **Degradation of mRNA in eukaryotes.** *Cell* 81, 179-183.

Bennes, R., Calas, B., Chabrier, P. E., Demaille, J. and Heitz, F. (1990) **Evidence for aggregation of endothelin 1 in water.** *FEBS Lett.* 276, 21-24.

Bennes, R., Calas, B., Chabrier, P. E. and Heitz, F. (1991) **Micelle formation of endothelin-1.** *J. Cardiovasc. Pharmacol.* 17 Suppl 7, S44-46.

Berrier, C., Park, K. H., Abes, S., Bibonne, A., Betton, J. M. and Ghazi, A. (2004) **Cell-free synthesis of a functional ion channel in the absence of a membrane and in the presence of detergent.** *Biochemistry* 43, 12585-12591.

Bertin, B., Freissmuth, M., Breyer, R. M., Schutz, W., Strosberg, A. D. and Marullo, S. (1992) **Functional expression of the human serotonin 5-HT1A receptor in *Escherichia coli*. Ligand binding properties and interaction with recombinant G protein alpha-subunits.** *J. Biol. Chem.* 267, 8200-8206.

Boivin, S., Tessier, S., Aubin, J., Lampron, P., Detheux, M. and Fournier, A. (2004) **Identification of a binding domain of the endothelin-B receptor using a selective IRL-1620-derived photoprobe.** *Biochemistry* 43, 11516-11525.

Braasch, I., Volff, J. N. and Schartl, M. (2009) **The endothelin system: evolution of vertebrate-specific ligand-receptor interactions by three rounds of genome duplication.** *Mol. Biol. Evol.* 26, 783-799.

Brady, A. E. and Limbird, L. E. (2002) **G protein-coupled receptor interacting proteins: emerging roles in localization and signal transduction.** *Cell Signal.* 14, 297-309.

Brenzel, S., Kurpiers, T. and Mootz, H. D. (2006) **Engineering artificially split inteins for applications in protein chemistry: biochemical characterization of the split Ssp DnaB intein and comparison to the split Sce VMA intein.** *Biochemistry* 45, 1571-1578.

Buchan, K. W., Alldus, C., Christodoulou, C., Clark, K. L., Dykes, C. W., Sumner, M. J., Wallace, D. M., White, D. G. and Watts, I. S. (1994) **Characterization of three non-peptide endothelin receptor ligands using human cloned ETA and ETB receptors**. *Br. J. Pharmacol.* 112, 1251-1257.

Bui, H. T., Umakoshi, H., Ngo, K. X., Nishida, M., Shimanouchi, T. and Kuboi, R. (2008) **Liposome membrane itself can affect gene expression in the *Escherichia coli* cell-free translation system.** *Langmuir* 24, 10537-10542.

Bulenger, S., Marullo, S. and Bouvier, M. (2005) **Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation.** *Trends Pharmacol. Sci.* 26, 131-137.

Burger, K., Gimpl, G. and Fahrenholz, F. (2000) **Regulation of receptor function by cholesterol.** *Cell. Mol. Life Sci.* 57, 1577-1592.

Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R. and Hamm, H. E. (2003) **Insights into G protein structure, function, and regulation.** *Endocr. Rev.* 24, 765-781.

Caffrey, M. and Cherezov, V. (2009) **Crystallizing membrane proteins using lipidic mesophases.** *Nat. Protoc.* 4, 706-731.

Calhoun, K. A. and Swartz, J. R. (2005) **An economical method for cell-free protein synthesis using glucose and nucleoside monophosphates.** *Biotechnol. Prog.* 21, 1146-1153.

Cansell, M., Gouygou, J. P., Jozefonvicz, J. and Letourneur, D. (1997) **Lipid composition of cultured endothelial cells in relation to their growth.** *Lipids* 32, 39-44.

Cappuccio, J. A., Blanchette, C. D., Sulchek, T. A., Arroyo, E. S., Kralj, J. M., Hinz, A. K., Kuhn, E. A., Chromy, B. A., Segelke, B. W., Rothschild, K. J., Fletcher, J. E., Katzen, F., Peterson, T. C., Kudlicki, W. A., Bench, G., Hoeprich, P. D. and Coleman, M. A. (2008) **Cell-free co-expression of functional membrane proteins and apolipoprotein, forming soluble nanolipoprotein particles.** *Mol. Cell. Proteomics* 7, 2246-2253.

Carroll, F. Y., Stolle, A., Beart, P. M., Voerste, A., Brabet, I., Mauler, F., Joly, C., Antonicek, H., Bockaert, J., Muller, T., Pin, J. P. and Prezeau, L. (2001) **BAY36-7620: a potent non-**

competitive mGlu1 receptor antagonist with inverse agonist activity. *Mol. Pharmacol.* 59, 965-973.

Chabre, M. and Le Maire, M. (2005) **Monomeric G-protein-coupled receptor as a functional unit.** *Biochemistry* 44, 9395-9403.

Chelikani, P., Reeves, P. J., Rajbhandary, U. L. and Khorana, H. G. (2006) **The synthesis and high-level expression of a β_2-adrenergic recepter gene in a tertracycline-inducible stable mammalian cell line.** *Protein Sci.* 15, 1433-1440.

Chen, Y. H., Yang, J. T. and Chau, K. H. (1974) **Determination of the helix and beta form of proteins in aqueous solution by circular dichroism.** *Biochemistry* 13, 3350-3359.

Chen, Y. J., Pornillos, O., Lieu, S., Ma, C., Chen, A. P. and Chang, G. (2007) **X-ray structure of EmrE supports dual topology model.** *Proc. Natl. Acad. Sci. U S A* 104, 18999-19004.

Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., Choi, H. J., Kuhn, P., Weis, W. I., Kobilka, B. K. and Stevens, R. C. (2007) **High-Resolution Crystal Structure of an Engineered Human β_2-Adrenergic G Protein Coupled Receptor.** *Science* 318, 1258-1265.

Christie, W. W. (1985) **Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection.** *J. Lipid Res.* 26, 507-512.

Cid, G. M., Nugent, P. G., Davenport, A. P., Kuc, R. E. and Wallace, B. A. (2000) **Expression and characterization of the human endothelin-A-receptor in *Pichia pastoris*: influence of N-terminal epitope tags.** *J. Cardiovasc. Pharmacol.* 36, S55-57.

Couet, J., Sargiacomo, M. and Lisanti, M. P. (1997) **Interaction of a receptor tyrosine kinase, EGF-R, with caveolins. Caveolin binding negatively regulates tyrosine and serine/threonine kinase activities.** *J. Biol. Chem.* 272, 30429-30438.

Craig, D., Howell, M. T., Gibbs, C. L., Hunt, T. and Jackson, R. J. (1992) **Plasmid cDNA-directed protein synthesis in a coupled eukaryotic *in vitro* transcription-translation system.** *Nucleic Acids Res.* 20, 4987-4995.

Dalbey, R. E. and Chen, M. (2004) **Sec-translocase mediated membrane protein biogenesis.** *Biochim. Biophys. Acta* 1694, 37-53.

Daley, D. O., Rapp, M., Granseth, E., Melen, K., Drew, D. and Von Heijne, G. (2005) **Global topology analysis of the *Escherichia coli* inner membrane proteome.** *Science* 308, 1321-1323.

Dahmane, T., Damian, M., Mary, S., Popot, J. L. and Baneres, J. L. (2009) **Amphipol-assisted in vitro folding of G protein-coupled receptors.** *Biochemistry* 48, 6516-6521.

Davanloo, P., Rosenberg, A. H., Dunn, J. J. and Studier, F. W. (1984) **Cloning and expression of the gene for bacteriophage T7 RNA polymerase.** *Proc. Natl. Acad. Sci. U S A* 81, 2035-2039.

Davenport, A. P. and Battistini, B. (2002) **Classification of endothelin receptors and antagonists in clinical development.** *Clin. Sci. (Lond).* 103 Suppl 48, 1S-3S.

Davenport, A. P. and Maguire, J. J. (2006) **Endothelin.** *Handb. Exp. Pharmacol.* 295-329.

Davies, A., Gowen, B. E., Krebs, A. M., Schertler, G. F. and Saibil, H. R. (2001) **Three-dimensional structure of an invertebrate rhodopsin and basis for ordered alignment in the photoreceptor membrane.** *J. Mol. Biol.* 314, 455-463.

De Leon, H. and Garcia, R. (1995) **Characterization of endothelin receptor subtypes in isolated rat renal preglomerular microvessels.** *Regu. Pept.* 60, 1-8.

Deniaud, A., Liguori, L., Blesneac, I., Lenormand, J. L. and Pebay-Peyroula, E. (2010) **Crystallization of the membrane protein hVDAC1 produced in cell-free system.** *Biochim. Biophys. Acta* 1798, 1540-1546.

De Nucci, G., Thomas, R., D'Orleans-Juste, P., Antunes, E., Walder, C., Warner, T. D. and Vane, J. R. (1988) **Pressor effects of circulating endothelin are limited by its removal in the**

pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U S A* 85, 9797-9800.

Desmarets, J., Gresser, O., Guedin, D. and Frelin, C. (1996) **Interaction of endothelin-1 with cloned bovine ETA receptors: biochemical parameters and functional consequences.** *Biochemistry.* 35, 14868-14875.

Desmarets, J. and Frelin, C. (1999) **High affinity interaction of endothelin-3 with recombinant ETA receptors.** *Biochem. Biophys. Res. Commun.* 256, 357-360.

Doherty, A. M., Cody, W. L., Leitz, N. L., Depue, P. L., Taylor, M. D., Rapundalo, S. T., Hingorani, G. P., Major, T. C., Panek, R. L. and Taylor, D. G. (1991) **Structure-activity studies of the C-terminal region of the endothelins and the sarafotoxins.** *J. Cardiovasc. Pharmacol.* 17 Suppl 7, S59-61.

Doi, T., Hiroaki, Y., Arimoto, I., Fujiyoshi, Y., Okamoto, T., Satoh, M. and Furuichi, Y. (1997) **Characterization of human endothelin B receptor and mutant receptors expressed in insect cells.** *Eur. J. Biochem.* 248, 139-148.

Doi, T., Sugimoto, H., Arimoto, I., Hiroaki, Y. and Fujiyoshi, Y. (1999) **Interactions of endothelin receptor subtypes A and B with Gi, Go, and Gq in reconstituted phospholipid vesicles.** *Biochemistry* 38, 3090-3099.

Douglas, S. A. and Ohlstein, E. H. (1997) **Signal transduction mechanisms mediating the vascular actions of endothelin.** *J. Vasc. Res.* 34, 152-164.

Drew, D., Froderberg, L., Baars, L. and De Gier, J. W. (2003) **Assembly and overexpression of membrane proteins in *Escherichia coli*.** *Biochim. Biophys. Acta* 1610, 3-10.

Eckart, M. R. and Bussineau, C. M. (1996) **Quality and authenticity of heterologous proteins synthesized in yeast.** *Curr. Opin. Biotechnol.* 7, 525-530.

Elbaz, Y., Steiner-Mordoch, S., Danieli, T. and Schuldiner, S. (2004) **In vitro synthesis of fully functional EmrE, a multidrug transporter, and study of its oligomeric state.** *Proc. Natl. Acad.Sci. U S A* 101, 1519-1524.

Elefsinioti, A. L., Bagos, P. G., Spyropoulos, I. C. and Hamodrakas, S. J. (2004) **A database for G proteins and their interaction with GPCRs.** *BMC Bioinformatics* 5, 208.

Elshourbagy, N. A., Korman, D. R., Wu, H. L., Sylvester, D. R., Lee, J. A., Nuthalaganti, P., Bergsma, D. J., Kumar, C. S. and Nambi, P. (1993) **Molecular characterization and regulation of the human endothelin receptors.** *J. Biol. Chem.* 268, 3873-3879.

Endo, Y. and Sawasaki, T. (2003) **High-throughput, genome-scale protein production method based on the wheat germ cell-free expression system.** *Biotechnol. Adv.* 21, 695-713.

Endo, Y. and Sawasaki, T. (2005) **Advances in genome-wide protein expression using the wheat germ cell-free system.** *Methods Mol. Biol.* 310, 145-167.

Erickson, A. H. and Blobel, G. (1983) **Cell-free translation of messenger RNA in a wheat germ system.** *Methods Enzymol.* 96, 38-50.

Ericsson, U. B., Hallberg, B. M., Detitta, G. T., Dekker, N. and Nordlund, P. (2006) **Thermofluor-based high-throughput stability optimization of proteins for structural studies.** *Anal. Biochem.* 357, 289-298.

Evans, N. J. and Walker, J. W. (2008) **Endothelin receptor dimers evaluated by FRET, ligand binding, and calcium mobilization.** *Biophys. J.* 95, 483-492.

Ezure, T., Suzuki, T., Higashide, S., Shintani, E., Endo, K., Kobayashi, S., Shikata, M., Ito, M., Tanimizu, K. and Nishimura, O. (2006) **Cell-free protein synthesis system prepared from insect cells by freeze-thawing.** *Biotechnol. Prog.* 22, 1570-1577.

Facey, S. J. and Kuhn, A. (2004) **Membrane integration of *E. coli* model membrane proteins.** *Biochim. Biophys. Acta* 1694, 55-66.

Fitzsimons, C. P., Monczor, F., Fernandez, N., Shayo, C. and Davio, C. (2004) **Mepyramine, a histamine H1 receptor inverse agonist, binds preferentially to a G protein-coupled form of the receptor and sequesters G protein.** *J. Biol. Chem.* 279, 34431-34439.

Fredriksson, R., Lagerstrom, M. C., Lundin, L. G. and Schioth, H. B. (2003) **The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints.** *Mol. Pharmacol.* 63, 1256-1272.

Fritze, O., Filipek, S., Kuksa, V., Palczewski, K., Hofmann, K. P. and Ernst, O. P. (2003) **Role of the conserved NPxxY(x)5,6F motif in the rhodopsin ground state and during activation.** *Proc. Natl. Acad. Sci. U S A* 100, 2290-2295.

Gallie, D. R., Sleat, D. E., Watts, J. W., Turner, P. C. and Wilson, T. M. (1987) **A comparison of eukaryotic viral 5'-leader sequences as enhancers of mRNA expression in vivo.** *Nucleic Acids Res.* 15, 8693-8711.

Garavito, R. M. and Ferguson-Miller, S. (2001) **Detergents as tools in membrane biochemistry.** *J. Biol. Chem.* 276, 32403-32406.

George, S. R., O'dowd, B. F. and Lee, S. P. (2002) **G-protein-coupled receptor oligomerization and its potential for drug discovery.** *Nat. Rev. Drug Discov.* 1, 808-820.

Gether, U., Ballesteros, J. A., Seifert, R., Sanders-Bush, E., Weinstein, H. and Kobilka, B. K. (1997) **Structural instability of a constitutively active G protein-coupled receptor. Agonist-independent activation due to conformational flexibility.** *J. Biol. Chem.* 272, 2587-2590.

Gimpl, G., Klein, U., Reilander, H. and Fahrenholz, F. (1995) **Expression of the human oxytocin receptor in baculovirus-infected insect cells: high-affinity binding is induced by a cholesterol-cyclodextrin complex.** *Biochemistry* 34, 13794-13801.

Gimpl, G., Burger, K. and Fahrenholz, F. (1997) **Cholesterol as modulator of receptor function.** *Biochemistry* 36, 10959-10974.

Goerke, A. R. and Swartz, J. R. (2008) **Development of cell-free protein synthesis platforms for disulfide bonded proteins.** *Biotechnol. Bioeng.* 99, 351-367.

Gonzalez De Valdivia, E. I. and Isaksson, L. A. (2004) **A codon window in mRNA downstream of the initiation codon where NGG codons give strongly reduced gene expression in *Escherichia coli*.** *Nucleic Acids Res.* 32, 5198-5205.

Gregan, B., Jurgensen, J., Papsdorf, G., Furkert, J., Schaefer, M., Beyermann, M., Rosenthal, W. and Oksche, A. (2004a) **Ligand-dependent differences in the internalization of endothelin A and endothelin B receptor heterodimers.** *J. Biol. Chem.* 279, 27679-27687.

Gregan, B., Schaefer, M., Rosenthal, W. and Oksche, A. (2004b) **Fluorescence resonance energy transfer analysis reveals the existence of endothelin-A and endothelin-B receptor homodimers.** *J. Cardiovasc. Pharmacol.* 44 Suppl 1., S30-33.

Grisshammer, R., Averbeck, P. and Sohal, A. K. (1999) **Improved purification of a rat neurotensin receptor expressed in *Escherichia coli*.** *Biochem. Soc. Trans.* 27, 899-903.

Guilvout, I., Chami, M., Berrier, C., Ghazi, A., Engel, A., Pugsley, A. P. and Bayan, N. (2008) ***In vitro* multimerization and membrane insertion of bacterial outer membrane secretin PulD.** *J. Mol. Biol.* 382, 13-23.

Haendler, B., Hechler, U., Becker, A. and Schleuning, W. D. (1993) **Expression of human endothelin receptor ETB by *Escherichia coli* transformants.** *Biochem. Biophys. Res. Commun.* 191, 633-638.

Hanahan, D. (1983) **Studies on transformation of *Escherichia coli* with plasmids.** *J. Mol. Biol.* 166, 557-580.

Hanson, M. A., Cherezov, V., Griffith, M. T., Roth, C. B., Jaakola, V. P., Chien, E. Y., Velasquez, J., Kuhn, P. and Stevens, R. C. (2008) **A specific cholesterol binding site is established by the 2.8 Å structure of the human β_2-adrenergic receptor.** *Structure* 16, 897-905.

Hara, M., Tozawa, F., Itazaki, K., Mihara, S. and Fujimoto, M. (1998) **Endothelin ET(B) receptors show different binding profiles in intact cells and cell membrane preparations.** *Eur. J. Pharmacol.* 345, 339-342.

Harada, N., Himeno, A., Shigematsu, K., Sumikawa, K. and Niwa, M. (2002) **Endothelin-1 binding to endothelin receptors in the rat anterior pituitary gland: possible formation of an ETA-ETB receptor heterodimer.** *Cell. Mol. Neurobiol.* 22, 207-226.

Harikumar, K. G., Puri, V., Singh, R. D., Hanada, K., Pagano, R. E. and Miller, L. J. (2005) **Differential effects of modification of membrane cholesterol and sphingolipids on the conformation, function, and trafficking of the G protein-coupled cholecystokinin receptor.** *J. Biol. Chem.* 280, 2176-2185.

Hassaine, G., Wagner, R., Kempf, J., Cherouati, N., Hassaine, N., Prual, C., Andre, N., Reinhart, C., Pattus, F. and Lundstrom, K. (2006) **Semliki Forest virus vectors for overexpression of 101 G protein-coupled receptors in mammalian host cells.** *Protein Expr. Purif.* 45, 343-351.

Hauser, H. (2000) **Short-chain phospholipids as detergents.** *Biochim. Biophys. Acta* 1508, 164-181.

Hay, D. L., Poyner, D. R. and Sexton, P. M. (2006) **GPCR modulation by RAMPs.** *Pharmacol. Ther.* 109, 173-197.

Haynes, W. G. and Webb, D. J. (1998) **Endothelin as a regulator of cardiovascular function in health and disease.** *J. Hypertens.* 16, 1081-1098.

Heginbotham, L., Kolmakova-Partensky, L. and Miller, C. (1998) **Functional reconstitution of a prokaryotic K+ channel.** *J. Gen Physiol.* 111, 741-749.

Hepler, J. R. and Gilman, A. G. (1992) **G proteins.** *Trends Biochem. Sci.* 17, 383-387.

Hebert, T.E. and Bouvier, M. (1998) **Structural and functional aspects of G protein-coupled receptor oligomerization.** *Biochem. Cell Biol.* 76, 1–10.

Hessa, T., Kim, H., Bihlmaier, K., Lundin, C., Boekel, J., Andersson, H., Nilsson, I., White, S. H. and Von Heijne, G. (2005) **Recognition of transmembrane helices by the endoplasmic reticulum translocon.** *Nature* 433, 377-381.

Hilairet, S., Bouaboula, M., Carriere, D., Le Fur, G. and Casellas, P. (2003) **Hypersensitization of the Orexin 1 receptor by the CB1 receptor: evidence for cross-talk blocked by the specific CB1 antagonist, SR141716.** *J. Biol. Chem.* 278, 23731-23737.

Hong, J., Wang, Y., Ye, X. and Zhang, Y. H. (2008) **Simple protein purification through affinity adsorption on regenerated amorphous cellulose followed by intein self-cleavage.** *J. Chromatogr. A* 1194, 150-154.

Hovijitra, N. T., Wuu, J. J., Peaker, B. and Swartz, J. R. (2009) **Cell-free synthesis of functional aquaporin Z in synthetic liposomes.** *Biotechnol. Bioeng.* 104, 40-49.

Howard, A. D., Mcallister, G., Feighner, S. D., Liu, Q., Nargund, R. P., Van Der Ploeg, L. H. and Patchett, A. A. (2001) **Orphan G-protein-coupled receptors and natural ligand discovery.** *Trends Pharmacol. Sci.* 22, 132-140.

Huggins, J. P., Trumpp-Kallmeyer, S., Hibert, M. F., Hoflack, J. M., Fanger, B. O. and Jones, C. R. (1993) **Modelling and modification of the binding site of endothelin and other receptors.** *Eur. J. Pharmacol.* 245, 203-214.

Hunte, C. (2005) **Specific protein-lipid interactions in membrane proteins.** *Biochem. Soc. Trans.* 33, 938-942.

Hunte, C. and Michel, H. (2002) **Crystallisation of membrane proteins mediated by antibody fragments.** *Curr. Opin. Struct. Biol.* 12, 503-508.

Hunte, C. and Michel, H. (2003) **Membrane Protein Crystallization.** In: *Membrane Protein Purification and Crystallization*, Hunte, C., von Jagow, G. and Schägger, H. (ed.), Academic Press, Elsevier Science, San Diego, California, USA.

Hunte, C. and Richers, S. (2008) **Lipids and membrane protein structures.** *Curr. Opin. Struct. Biol.* 18, 406-411.

Hunter, W. M. and Greenwood, F. C. (1962) **Preparation of iodine-131 labelled human growth hormone of high specific activity.** *Nature* 194, 495-496.

Huwiler, K. G., De Rosier, T., Hanson, B. and Vogel, K. W. (2010) **A fluorescence anisotropy assay for the muscarinic M1 G-protein-coupled receptor.** *Assay Drug Dev. Technol.* 8, 356-366.

Hyndman, K. A., Miyamoto, M. M. and Evans, D. H. (2009) **Phylogeny, taxonomy, and evolution of the endothelin receptor gene family.** *Mol. Phylogenet. Evol.* 52, 677-687.

Inoue, H., Nojima, H. and Okayama, H. (1990) **High efficiency transformation of *Escherichia coli* with plasmids.** *Gene* 96, 23-28.

Ishihara, G., Goto, M., Saeki, M., Ito, K., Hori, T., Kigawa, T., Shirouzu, M. and Yokoyama, S. (2005) **Expression of G protein coupled receptors in a cell-free translational system using detergents and thioredoxin-fusion vectors.** *Protein Expr. Purif.* 41, 27-37.

Jaakola, V. P., Griffith, M. T., Hanson, M. A., Cherezov, V., Chien, E. Y., Lane, J. R., Ijzerman, A. P. and Stevens, R. C. (2008) **The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist.** *Science* 322, 1211-1217.

Jacobs, S., Chang, K. J. and Cuatrecasas, P. (1975) **Estimation of hormone receptor affinity by competitive displacement of labeled ligand: effect of concentration of receptor and of labeled ligand.** *Biochem. Biophys. Res. Commun.* 66, 687-692.

Janes, R. W., Peapus, D. H. and Wallace, B. A. (1994) **The crystal structure of human endothelin.** *Nat. Struct. Biol.* 1, 311-319.

Jastrzebska, B., Maeda, T., Zhu, L., Fotiadis, D., Filipek, S., Engel, A., Stenkamp, R. E. and Palczewski, K. (2004) **Functional characterization of rhodopsin monomers and dimers in detergents.** *J. Biol. Chem.* 279, 54663-54675.

Jastrzebska, B., Fotiadis, D., Jang, G. F., Stenkamp, R. E., Engel, A. and Palczewski, K. (2006) **Functional and structural characterization of rhodopsin oligomers.** *J. Biol. Chem.* 281, 11917-11922.

Jewett, M. C. and Swartz, J. R. (2004) **Substrate replenishment extends protein synthesis with an *in vitro* translation system designed to mimic the cytoplasm.** *Biotechnol. Bioeng.* 87, 465-472.

Ji, T. H., Grossmann, M. and Ji, I. (1998) **G protein-coupled receptors. I. Diversity of receptor-ligand interactions.** *J. Biol. Chem.* 273, 17299-17302.

Jiang, X., Ookubo, Y., Fujii, I., Nakano, H. and Yamane, T. (2002) **Expression of Fab fragment of catalytic antibody 6D9 in an *Escherichia coli in vitro* coupled transcription/translation system.** *FEBS Lett.* 514, 290-294.

Joseph, S. K., Boehning, D., Pierson, S. and Nicchitta, C. V. (1997) **Membrane insertion, glycosylation, and oligomerization of inositol trisphosphate receptors in a cell-free translation system.** *J. Biol. Chem.* 272, 1579-1588.

Jung, H., Tebbe, S., Schmid, R. and Jung, K. (1998) **Unidirectional reconstitution and characterization of purified Na+/proline transporter of *Escherichia coli*.** *Biochemistry* 37, 11083-11088.

Junge, F., Schneider, B., Reckel, S., Schwarz, D., Dötsch, V. and Bernhard, F. (2008) **Large-scale production of functional membrane proteins.** *Cell. Mol. Life Sci.* 65, 1729-1755.

Junge, F., Haberstock, S., Roos, C., Stefer, S., Proverbio, D., Dötsch, V. and Bernhard, F. (2010a) **Advances in cell-free protein synthesis for the functional and structural analysis of membrane proteins.** *N. Biotechnol*. doi:10.1016/j.nbt.2010.07.002

Junge, F., Luh, L. M., Proverbio, D., Schäfer, B., Abele, R., Beyermann, M., Dötsch, V. and Bernhard, F. (2010b) **Modulation of G-protein coupled receptor sample quality by modified cell-free expression protocols: A case study of the human endothelin A receptor.** *J. Struct. Biol*. 172, 94-106.

Kainosho, M., Torizawa, T., Iwashita, Y., Terauchi, T., Mei Ono, A. and Güntert, P. (2006) **Optimal isotope labelling for NMR protein structure determinations.** *Nature* 440, 52-57.

Kalmbach, R., Chizhov, I., Schumacher, M. C., Friedrich, T., Bamberg, E. and Engelhard, M. (2007) **Functional cell-free synthesis of a seven helix membrane protein: *in situ* insertion of bacteriorhodopsin into liposomes.** *J. Mol. Biol.* 371, 639-648.

Kamonchanok, S., Balog, C. I., Van Der Does, A. M., Booth, R., De Grip, W. J., Deelder, A. M., Bakker, R. A., Leurs, R. and Hensbergen, P. J. (2008) **GPCR proteomics: mass spectrometric and functional analysis of histamine H1 receptor after baculovirus-driven and *in vitro* cell free expression.** *J. Proteome Res.* 7, 621-629.

Kanda, T., Takai, K., Hohsaka, T., Sisido, M. and Takaku, H. (2000) **Sense codon-dependent introduction of unnatural amino acids into multiple sites of a protein.** *Biochem. Biophys. Res. Commun.* 270, 1136-1139.

Kasher, M. S., Wakulchik, M., Cook, J. A. and Smith, M. C. (1993) **One-step purification of recombinant human papillomavirus type 16 E7 oncoprotein and its binding to the retinoblastoma gene product.** *Biotechniques* 14, 630-641.

Katzen, F. and Kudlicki, W. (2006) **Efficient generation of insect-based cell-free translation extracts active in glycosylation and signal sequence processing.** *J. Biotechnol.* 125, 194-197.

Katzen, F., Fletcher, J. E., Yang, J. P., Vasu, S., Peterson, T. and Kudlicki, W. (2008) **Cell-free protein expression of membrane proteins using nanolipoprotein particles.** *Biotechniques* 45, 469.

Kaupmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., Beck, P., Mosbacher, J., Bischoff, S., Kulik, A., Shigemoto, R., Karschin, A. and Bettler, B. (1998) **GABA(B)-receptor subtypes assemble into functional heteromeric complexes.** *Nature* 396, 683-687.

Kedzierski, R. M. and Yanagisawa, M. (2001) **Endothelin system: the double-edged sword in health and disease.** *Annu Rev. Pharmacol. Toxicol.* 41, 851-876.

Keller, T., Schwarz, D., Bernhard, F., Dötsch, V., Hunte, C., Gorboulev, V. and Koepsell, H. (2008) **Cell free expression and functional reconstitution of eukaryotic drug transporters.** *Biochemistry* 47, 4552-4564.

Khan, S. M., Bolen, W., Hargrave, P. A., Santoro, M. M. and Mcdowell, J. H. (1991) **Differential scanning calorimetry of bovine rhodopsin in rod-outer-segment disk membranes.** *Eur. J. Biochem.* 200, 53-59.

Kiefer, H., Krieger, J., Olszewski, J. D., Von Heijne, G., Prestwich, G. D. and Breer, H. (1996) **Expression of an olfactory receptor in *Escherichia coli*: purification, reconstitution, and ligand binding.** *Biochemistry* 35, 16077-16084.

Kiefer, H. (2003) ***In vitro* folding of alpha-helical membrane proteins.** *Biochim. Biophys. Acta* 1610, 57-62.

Kiga, D., Sakamoto, K., Kodama, K., Kigawa, T., Matsuda, T., Yabuki, T., Shirouzu, M., Harada, Y., Nakayama, H., Takio, K., Hasegawa, Y., Endo, Y., Hirao, I. and Yokoyama, S. (2002) **An engineered *Escherichia coli* tyrosyl-tRNA synthetase for site-specific incorporation of an unnatural amino acid into proteins in eukaryotic translation and its application in a wheat germ cell-free system.** *Proc. Natl. Acad. Sci. U S A* 99, 9715-9720.

Kigawa, T. and Yokoyama, S. (1991) **A continuous cell-free protein synthesis system for coupled transcription-translation.** *J. Biochem.* 110, 166-168.

Kigawa, T., Yabuki, T., Yoshida, Y., Tsutsui, M., Ito, Y., Shibata, T. and Yokoyama, S. (1999) **Cell-free production and stable-isotope labeling of milligram quantities of proteins.** *FEBS Lett.* 442, 15-19.

Kigawa, T., Yamaguchi-Nunokawa, E., Kodama, K., Matsuda, T., Yabuki, T., Matsuda, N., Ishitani, R., Nureki, O. and Yokoyama, S. (2002) **Selenomethionine incorporation into a protein by cell-free synthesis.** *J. Struct. Funct. Genomics* 2, 29-35.

Kim, D. M. and Swartz, J. R. (2001) **Regeneration of adenosine triphosphate from glycolytic intermediates for cell-free protein synthesis.** *Biotechnol. Bioeng.* 74, 309-316.

Kim, T. W., Keum, J. W., Oh, I. S., Choi, C. Y., Park, C. G. and Kim, D. M. (2006) **Simple procedures for the construction of a robust and cost-effective cell-free protein synthesis system.** *J. Biotechnol.* 126, 554-561.

Klabunde, T. and Hessler, G. (2002) **Drug design strategies for targeting G-protein-coupled receptors.** *Chembiochem.* 3, 928-944.

Klammt, C., Löhr, F., Schäfer, B., Haase, W., Dötsch, V., Rüterjans, H., Glaubitz, C. and Bernhard, F. (2004) **High level cell-free expression and specific labeling of integral membrane proteins.** *Eur. J. Biochem.* 271, 568-580.

Klammt, C., Schwarz, D., Fendler, K., Haase, W., Dötsch, V. and Bernhard, F. (2005) **Evaluation of detergents for the soluble expression of alpha-helical and beta-barrel-type integral membrane proteins by a preparative scale individual cell-free expression system.** *FEBS J.* 272, 6024-6038.

Klammt, C., Schwarz, D., Löhr, F., Schneider, B., Dötsch, V. and Bernhard, F. (2006) **Cell-free expression as an emerging technique for the large scale production of integral membrane protein.** *FEBS J.* 273, 4141-4153.

Klammt, C., Schwarz, D., Dötsch, V. and Bernhard, F. (2007a) **Cell-free production of integral membrane proteins on a preparative scale.** *Methods Mol. Biol.* 375, 57-78.

Klammt, C., Schwarz, D., Eifler, N., Engel, A., Piehler, J., Haase, W., Hahn, S., Dötsch, V. and Bernhard, F. (2007b) **Cell-free production of G protein-coupled receptors for functional and structural studies.** *J. Struct. Biol.* 158, 482-493.

Klammt, C., Srivastava, A., Eifler, N., Junge, F., Beyermann, M., Schwarz, D., Michel, H., Dötsch, V. and Bernhard, F. (2007c) **Functional analysis of cell-free-produced human endothelin B receptor reveals transmembrane segment 1 as an essential area for ET-1 binding and homodimer formation.** *FEBS J.* 274, 3257-3269.

Knoflach, F., Mutel, V., Jolidon, S., Kew, J. N., Malherbe, P., Vieira, E., Wichmann, J. and Kemp, J. A. (2001) **Positive allosteric modulators of metabotropic glutamate 1 receptor: characterization, mechanism of action, and binding site.** *Proc. Natl. Acad. Sci. U S A* 98, 13402-13407.

Knol, J., Sjollema, K. and Poolman, B. (1998) **Detergent-mediated reconstitution of membrane proteins.** *Biochemistry* 37, 16410-16415.

Kobilka, B. K. (2007) **G protein coupled receptor structure and activation.** *Biochim. Biophys. Acta* 1768, 794-807.

Koglin, A., Klammt, C., Trbovic, N., Schwarz, D., Schneider, B., Schäfer, B., Löhr, F., Bernhard, F. and Dötsch, V. (2006) **Combination of cell-free expression and NMR spectroscopy as a new approach for structural investigation of membrane proteins**. *Magn. Reson. Chem.* 44 Spec No, S17-23.

Kolb, V. A., Makeyev, E. V. and Spirin, A. S. (2000) **Co-translational folding of an eukaryotic multidomain protein in a prokaryotic translation system.** *J. Biol. Chem.* 275, 16597-16601.

Koszelak-Rosenblum, M., Krol, A., Mozumdar, N., Wunsch, K., Ferin, A., Cook, E., Veatch, C. K., Nagel, R., Luft, J. R., Detitta, G. T. and Malkowski, M. G. (2009) **Determination and application of empirically derived detergent phase boundaries to effectively crystallize membrane proteins.** *Protein Sci.* 18, 1828-1839.

Krafft, M. P. and Riess, J. G. (1998) **Highly fluorinated amphiphiles and colloidal systems, and their applications in the biomedical field. A contribution.** *Biochimie* 80, 489-514.

Kristiansen, K. (2004) **Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function.** *Pharmacol. Ther.* 103, 21-80.

Krueger-Koplin, R. D., Sorgen, P. L., Krueger-Koplin, S. T., Rivera-Torres, I. O., Cahill, S. M., Hicks, D. B., Grinius, L., Krulwich, T. A. and Girvin, M. E. (2004) **An evaluation of**

detergents for NMR structural studies of membrane proteins. *J. Biomol. NMR* 28, 43-57.

Kuner, R., Kohr, G., Grunewald, S., Eisenhardt, G., Bach, A. and Kornau, H. C. (1999) **Role of heteromer formation in GABAB receptor function.** *Science* 283, 74-77.

Kurland, C. and Gallant, J. (1996) **Errors of heterologous protein expression.** *Curr. Opin. Biotechnol.* 7, 489-493.

Kuwabara, P. E. and Labouesse, M. (2002) **The sterol-sensing domain: multiple families, a unique role?** *Trends Genet.* 18, 193-201.

Laemmli, U. K., Beguin, F. and Gujer-Kellenberger, G. (1970) **A factor preventing the major head protein of bacteriophage T4 from random aggregation.** *J. Mol. Biol.* 47, 69-85.

Lambright, D. G., Noel, J. P., Hamm, H. E. and Sigler, P. B. (1994) **Structural determinants for activation of the alpha-subunit of a heterotrimeric G protein.** *Nature* 369, 621-628.

Lancaster, C. R., Kroger, A., Auer, M. and Michel, H. (1999) **Structure of fumarate reductase from *Wolinella succinogenes* at 2.2 Å resolution.** *Nature* 402, 377-385.

Lee, A. G. (2004) **How lipids affect the activities of integral membrane proteins.** *Biochim. Biophys. Acta* 1666, 62-87.

Lee, S. P., So, C. H., Rashid, A. J., Varghese, G., Cheng, R., Lanca, A. J., O'dowd, B. F. and George, S. R. (2004) **Dopamine D1 and D2 receptor Co-activation generates a novel phospholipase C-mediated calcium signal.** *J. Biol. Chem.* 279, 35671-35678.

Lefkowitz, R. J. and Shenoy, S. K. (2005) **Transduction of receptor signals by beta-arrestins.** *Science* 308, 512-517.

Le Maire, M., Champeil, P. and Moller, J. V. (2000) **Interaction of membrane proteins and lipids with solubilizing detergents.** *Biochim. Biophys. Acta* 1508, 86-111.

Lemmon, M. A., Flanagan, J. M., Hunt, J. F., Adair, B. D., Bormann, B. J., Dempsey, C. E. and Engelman, D. M. (1992) **Glycophorin A dimerization is driven by specific interactions between transmembrane alpha-helices.** *J. Biol. Chem.* 267, 7683-7689.

Li, H. and Papadopoulos, V. (1998) **Peripheral-type benzodiazepine receptor function in cholesterol transport. Identification of a putative cholesterol recognition/interaction amino acid sequence and consensus pattern.** *Endocrinology* 139, 4991-4997.

Li, Y., Wang, E. and Wang, Y. (1999) **A modified procedure for fast purification of T7 RNA polymerase.** *Protein Expr. Purif.* 16, 355-358.

Liang, Y., Fotiadis, D., Filipek, S., Saperstein, D. A., Palczewski, K. and Engel, A. (2003) **Organization of the G protein-coupled receptors rhodopsin and opsin in native membranes.** *J. Biol. Chem.* 278, 21655-21662.

Liguori, L., Marques, B., Villegas-Mendez, A., Rothe, R. and Lenormand, J. L. (2007) **Production of membrane proteins using cell-free expression systems.** *Expert Rev. Proteomics* 4, 79-90.

Lindstrom, J., Anholt, R., Einarson, B., Engel, A., Osame, M. and Montal, M. (1980) **Purification of acetylcholine receptors, reconstitution into lipid vesicles, and study of agonist-induced cation channel regulation.** *J. Biol. Chem.* 255, 8340-8350.

Lingappa, J. R., Hill, R. L., Wong, M. L. and Hegde, R. S. (1997) **A multistep, ATP-dependent pathway for assembly of human immunodeficiency virus capsids in a cell-free system.** *J. Cell Biol.* 136, 567-581.

Linke, D. (2009) **Detergents: an overview.** *Methods Enzymol.* 463, 603-617.

Long, S. B., Campbell, E. B. and Mackinnon, R. (2005) **Crystal structure of a mammalian voltage-dependent Shaker family K+ channel.** *Science* 309, 897-903.

Luecke, H., Schobert, B., Lanyi, J. K., Spudich, E. N. and Spudich, J. L. (2001) **Crystal structure of sensory rhodopsin II at 2.4 angstroms: insights into color tuning and transducer interaction.** *Science* 293, 1499-1503.

Lundstrom, K. (2006) **Structural genomics for membrane proteins.** *Cell. Mol. Life Sci.* 63, 2597-2607.

Luttrell, L. M. and Lefkowitz, R. J. (2002) **The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals.** *J. Cell Sci.* 115, 455-465.

Lyford, L. K. and Rosenberg, R. L. (1999) **Cell-free expression and functional reconstitution of homo-oligomeric alpha7 nicotinic acetylcholine receptors into planar lipid bilayers.** *J. Biol. Chem.* 274, 25675-25681.

Madin, K., Sawasaki, T., Ogasawara, T. and Endo, Y. (2000) **A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes.** *Proc. Natl. Acad. Sci. U S A* 97, 559-564.

Magnani, F., Shibata, Y., Serrano-Vega, M. J. and Tate, C. G. (2008) **Co-evolving stability and conformational homogeneity of the human adenosine A2a receptor.** *Proc. Natl. Acad. Sci. U. S. A.* 105, 10744-10749.

Magnin, T., Fiez-Vandal, C., Potier, N., Coquard, A., Leray, I., Steffan, T., Logez, C., Alkhalfioui, F., Pattus, F. and Wagner, R. (2009) **A novel, generic and effective method for the rapid purification of G protein-coupled receptors.** *Protein Expr. Purif.* 64, 1-7.

Marques, B., Liguori, L., Paclet, M. H., Villegas-Mendez, A., Rothe, R., Morel, F. and Lenormand, J. L. (2007) **Liposome-mediated cellular delivery of active gp91(phox).** *PLoS One* 2, e856.

Maslennikov, I., Klammt, C., Hwang, E., Kefala, G., Okamura, M., Esquivies, L., Mörs, K., Glaubitz, C., Kwiatkowski, W., Jeon, Y. H. and Choe, S. (2010) **Membrane domain structures of three classes of histidine kinase receptors by cell-free expression and rapid NMR analysis.** *Proc. Natl. Acad. Sci. U S A* 107, 10902-10907.

Massotte, D., Baroche, L., Simonin, F., Yu, L., Kieffer, B. and Pattus, F. (1997) **Characterization of δ, κ and μ Human Opioid Receptors Overexpressed in Baculovirus-infected Insect Cells.** *J. Biol. Chem.* 272, 19987-19992.

Massotte, D., Pereira, C. A., Pouliquen, Y. and Pattus, F. (1999) **Parameters influencing human μ opioid receptor over-expression in baculovirus-infected insect cells.** *J. Biotechnol.* 69, 39-45.

Mathys, S., Evans, T. C., Chute, I. C., Wu, H., Chong, S., Benner, J., Liu, X. Q. and Xu, M. Q. (1999) **Characterization of a self-splicing mini-intein and its conversion into autocatalytic N- and C-terminal cleavage elements: facile production of protein building blocks for protein ligation.** *Gene* 231, 1-13.

Matveev, S. V., Vinokurov, L. M., Shaloiko, L. A., Davies, C., Matveeva, E. A. and Alakhov Yu, B. (1996) **Effect of the ATP level on the overall protein biosynthesis rate in a wheat germ cell-free system.** *Biochim. Biophys. Acta* 1293, 207-212.

McCusker, E. C., Bane, S. E., O'Malley, M. A. and Robinson, A. S. (2007) **Heterologous GPCR expression: a bottleneck to obtaining crystal structures.** *Biotechnol. Prog.* 23, 540-547.

Michalke, K., Graviere, M. E., Huyghe, C., Vincentelli, R., Wagner, R., Pattus, F., Schroeder, K., Oschmann, J., Rudolph, R., Cambillau, C. and Desmyter, A. (2009) **Mammalian G-protein-coupled receptor expression in *Escherichia coli*: I. High-throughput large-scale production as inclusion bodies.** *Anal. Biochem.* 386, 147-155.

Mignone, F., Gissi, C., Liuni, S. and Pesole, G. (2002) **Untranslated regions of mRNAs.** *Genome Biol.* 3, reviews 0004.0001-0004.0010.

Milligan, G. (2004) **G protein-coupled receptor dimerization: function and ligand pharmacology.** *Mol. Pharmacol.* 66, 1-7.

Milligan, G. (2007) **G protein-coupled receptor dimerisation: molecular basis and relevance to function.** *Biochim. Biophys. Acta* 1768, 825-835.

Mirzadegan, T., Benko, G., Filipek, S. and Palczewski, K. (2003) **Sequence analyses of G-protein-coupled receptors: similarities to rhodopsin.** *Biochemistry* 42, 2759-2767.

Miyauchi, T., Yorikane, R., Sakai, S., Sakurai, T., Okada, M., Nishikibe, M., Yano, M., Yamaguchi, I., Sugishita, Y. and Goto, K. (1993) **Contribution of endogenous endothelin-1 to the progression of cardiopulmonary alterations in rats with monocrotaline-induced pulmonary hypertension.** *Circ. Res.* 73, 887-897.

Molenaar, P., Kuc, R. E. and Davenport, A. P. (1992) **Characterization of two new ETB selective radioligands, [125I]-BQ3020 and [125I]-[Ala1,3,11,15]ET-1 in human heart.** *Br. J. Pharmacol.* 107, 637-639.

Mondon, F., Anouar, A. and Ferre, F. (1998) **Endothelin receptor subtypes in the microvillous trophoblastic membrane of early gestation and term human placentas.** *Eur. J. Endocrinol.* 139, 231-237.

Mullis, K. B. and Faloona, F. A. (1987) **Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction.** *Methods Enzymol.* 155, 335-350.

Murakami, M. and Kouyama, T. (2008) **Crystal structure of squid rhodopsin.** *Nature* 453, 363-367.

Mureev, S., Kovtun, O., Nguyen, U. T. and Alexandrov, K. (2009) **Species-independent translational leaders facilitate cell-free expression.** *Nat. Biotechnol.* 27, 747-752.

Murthy, T. V., Wu, W., Qiu, Q. Q., Shi, Z., Labaer, J. and Brizuela, L. (2004) **Bacterial cell-free system for high-throughput protein expression and a comparative analysis of *Escherichia coli* cell-free and whole cell expression systems.** *Protein Expr. Purif.* 36, 217-225.

Nelson, J., Bagnato, A., Battistini, B. and Nisen, P. (2003) **The endothelin axis: emerging role in cancer.** *Nat. Rev. Cancer* 3, 110-116.

Nguyen, T. A., Lieu, S. S. and Chang, G. (2010) **An *Escherichia coli*-Based Cell-Free System for Large-Scale Production of Functional Mammalian Membrane Proteins Suitable for X-Ray Crystallography.** *J. Mol. Microbiol. Biotechnol.* 18, 85-91.

Nirenberg, M. W. and Matthaei, J. H. (1961) **The dependence of cell-free protein synthesis in *E. coli* upon naturally occurring or synthetic polyribonucleotides.** *Proc. Natl. Acad. Sci. U S A* 47, 1588-1602.

Nishiyama, K., Ikegami, A., Moser, M., Schiltz, E., Tokuda, H. and Muller, M. (2006) **A derivative of lipid A is involved in signal recognition particle/SecYEG-dependent and -independent membrane integrations.** *J. Biol. Chem.* 281, 35667-35676.

Nozawa, A., Nanamiya, H., Miyata, T., Linka, N., Endo, Y., Weber, A. P. and Tozawa, Y. (2007) **A cell-free translation and proteoliposome reconstitution system for functional analysis of plant solute transporters.** *Plant Cell Physiol.* 48, 1815-1820.

O' Malley, M. A., Lazarova, T., Britton, Z. T. and Robinson, A. S. (2007) **High-level expression in *Saccharomyces cerevisiae* enables isolation and spectroscopic characterization of functional human adenosine A2a receptor.** *J. Struct. Biol.* 159, 166-178.

Ohashi, H., Kanamori, T., Shimizu, Y. and Ueda, T. (2010) **A highly controllable reconstituted cell-free system-a breakthrough in protein synthesis research.** *Curr. Pharm. Biotechnol.* 11, 267-271.

Okada, T., Sugihara, M., Bondar, A. N., Elstner, M., Entel, P. and Buss, V. (2004) **The retinal conformation and its environment in rhodopsin in light of a new 2.2 Å crystal structure.** *J. Mol. Biol.* 342, 571-583.

Okamoto, T., Sekiyama, N., Otsu, M., Shimada, Y., Sato, A., Nakanishi, S. and Jingami, H. (1998) **Expression and purification of the extracellular ligand binding region of metabotropic glutamate receptor subtype 1.** *J. Biol. Chem.* 273, 13089-13096.

Opekarova, M. and Tanner, W. (2003) **Specific lipid requirements of membrane proteins--a putative bottleneck in heterologous expression.** *Biochim. Biophys. Acta* 1610, 11-22.

Orry, A. J. and Wallace, B. A. (2000) **Modeling and docking the endothelin G-protein-coupled receptor.** *Biophys. J.* 79, 3083-3094.

Ottensmeyer, F. P., Beniac, D. R., Luo, R. Z. and Yip, C. C. (2000) **Mechanism of transmembrane signaling: insulin binding and the insulin receptor.** *Biochemistry* 39, 12103-12112.

Overton, M. C. and Blumer, K. J. (2002) **Use of fluorescence resonance energy transfer to analyze oligomerization of G-protein-coupled receptors expressed in yeast.** *Methods* 27, 324-332.

Ozaki, S., Ohwaki, K., Ihara, M., Ishikawa, K. and Yano, M. (1997) **Coexpression studies with endothelin receptor subtypes indicate the existence of intracellular cross-talk between ET(A) and ET(B) receptors.** *J. Biochem.* 121, 440-447.

Ozawa, K., Headlam, M. J., Mouradov, D., Watt, S. J., Beck, J. L., Rodgers, K. J., Dean, R. T., Huber, T., Otting, G. and Dixon, N. E. (2005) **Translational incorporation of L-3,4-dihydroxyphenylalanine into proteins.** *FEBS J.* 272, 3162-3171.

Padan, E. (2008) **The enlightening encounter between structure and function in the NhaA Na+-H+ antiporter.** *Trends Biochem. Sci.* 33, 435-443.

Paila, Y. D. and Chattopadhyay, A. (2009) **The function of G-protein coupled receptors and membrane cholesterol: specific or general interaction?** *Glycoconj. J.* 26, 711-720.

Pak, Y., Pham, N. and Rotin, D. (2002) **Direct binding of the beta1 adrenergic receptor to the cyclic AMP-dependent guanine nucleotide exchange factor CNrasGEF leads to Ras activation.** *Mol. Cell Biol.* 22, 7942-7952.

Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Trong, I. L., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M. and Miyano, M. (2000) **Crystal Structure of Rhodopsin: A G Protein-Coupled Receptor.** *Science* 289, 739-745.

Pantoliano, M. W., Petrella, E. C., Kwasnoski, J. D., Lobanov, V. S., Myslik, J., Graf, E., Carver, T., Asel, E., Springer, B. A., Lane, P. and Salemme, F. R. (2001) **High-density miniaturized thermal shift assays as a general strategy for drug discovery.** *J. Biomol. Screen.* 6, 429-440.

Park, J. H., Scheerer, P., Hofmann, K. P., Choe, H. W. and Ernst, O. P. (2008) **Crystal structure of the ligand-free G-protein-coupled receptor opsin.** *Nature* 454, 183-187.

Park, K. H., Berrier, C., Lebaupain, F., Pucci, B., Popot, J. L., Ghazi, A. and Zito, F. (2007) **Fluorinated and hemifluorinated surfactants as alternatives to detergents for membrane protein cell-free synthesis.** *Biochem. J.* 403, 183-187.

Patel, R. C., Lange, D. C. and Patel, Y. C. (2002) **Photobleaching fluorescence resonance energy transfer reveals ligand-induced oligomer formation of human somatostatin receptor subtypes.** *Methods* 27, 340-348.

Pin, J. P., Kniazeff, J., Liu, J., Binet, V., Goudet, C., Rondard, P. and Prezeau, L. (2005) **Allosteric functioning of dimeric class C G-protein-coupled receptors.** *Febs. J.* 272, 2947-2955.

Pla, P. and Larue, L. (2003) **Involvement of endothelin receptors in normal and pathological development of neural crest cells.** *Int. J. Dev. Biol.* 47, 315-325.

Pocanschi, C. L., Dahmane, T., Gohon, Y., Rappaport, F., Apell, H. J., Kleinschmidt, J. H. and Popot, J. L. (2006) **Amphipathic polymers: tools to fold integral membrane proteins to their active form.** *Biochemistry* 45, 13954-13961.

Poklar, N., Lah, J., Salobir, M., Macek, P. and Vesnaver, G. (1997) **pH and temperature-induced molten globule-like denatured states of equinatoxin II: a study by UV-melting, DSC, far- and near-UV CD spectroscopy, and ANS fluorescence.** *Biochemistry* 36, 14345-14352.

Polidori, A., Presset, M., Lebaupain, F., Ameduri, B., Popot, J. L., Breyton, C. and Pucci, B. (2006) **Fluorinated and hemifluorinated surfactants derived from maltose: synthesis and application to handling membrane proteins in aqueous solution.** *Bioorg. Med. Chem. Lett.* 16, 5827-5831.

Popot, J. L. (2010) **Amphipols, Nanodiscs, and Fluorinated Surfactants: Three Nonconventional Approaches to Studying Membrane Proteins in Aqueous Solutions.** *Annu. Rev. Biochem.* 9, 737-775.

Porath, J. (1988) **High-performance immobilized-metal-ion affinity chromatography of peptides and proteins.** *J. Chromatogr.* 443, 3-11.

Portoghese, P. S. (1989) **Bivalent ligands and the message-address concept in the design of selective opioid receptor antagonists.** *Trends Pharmacol. Sci.* 10, 230-235.

Rasmussen, S. G., Choi, H. J., Rosenbaum, D. M., Kobilka, T. S., Thian, F. S., Edwards, P. C., Burghammer, M., Ratnala, V. R., Sanishvili, R., Fischetti, R. F., Schertler, G. F., Weis, W. I. and Kobilka, B. K. (2007) **Crystal structure of the human beta2 adrenergic G-protein-coupled receptor.** *Nature* 450, 383-387.

Reckel, S., Sobhanifar, S., Schneider, B., Junge, F., Schwarz, D., Durst, F., Löhr, F., Güntert, P., Bernhard, F. and Dötsch, V. (2008) **Transmembrane segment enhanced labeling as a tool for the backbone assignment of alpha-helical membrane proteins.** *Proc. Natl. Acad. Sci. U S A* 105, 8262-8267.

Reiländer, H. and Weiss, H. M. (1998) **Production of G-protein-coupled receptors in yeast.** *Curr. Opin. Biotechnol.* 9, 510-517.

Ren, J. M., Marshall, B. A., Gulve, E. A., Gao, J., Johnson, D. W., Holloszy, J. O. and Mueckler, M. (1993) **Evidence from transgenic mice that glucose transport is rate-limiting for glycogen deposition and glycolysis in skeletal muscle.** *J. Biol. Chem.* 268, 16113-16115.

Rigaud, J. L., Pitard, B. and Levy, D. (1995) **Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins.** *Biochim. Biophys. Acta* 1231, 223-246.

Rigaud, J. L., Levy, D., Mosser, G. and Lambert, O. (1998) **Detergent removal by non-polar polystyrene beads - Applications to membrane protein reconstitution and two-dimensional crystallization.** *Eur. Biophys. J.* 27, 305-319.

Rios, C. D., Jordan, B. A., Gomes, I. and Devi, L. A. (2001) **G-protein-coupled receptor dimerization: modulation of receptor function.** *Pharmacol. Ther.* 92, 71-87.

Rosenbaum, D. M., Rasmussen, S. G. and Kobilka, B. K. (2009) **The structure and function of G-protein-coupled receptors.** *Nature.* 459, 356-363.

Rosenbusch, J. P. (2001) **Stability of membrane proteins: relevance for the selection of appropriate methods for high-resolution structure determinations.** *J .Struct. Biol.* 136, 144-157.

Rovati, G. E., Capra, V. and Neubig, R. R. (2007) **The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state.** *Mol. Pharmacol.* 71, 959-964.

Russell, F. D., Skepper, J. N. and Davenport, A. P. (1998) **Human endothelial cell storage granules: a novel intracellular site for isoforms of the endothelin-converting enzyme.** *Circ. Res.* 83, 314-321.

Ryabova, L. A., Vinokurov, L. M., Shekhovtsova, E. A., Alakhov, Y. B. and Spirin, A. S. (1995) **Acetyl phosphate as an energy source for bacterial cell-free translation systems.** *Anal. Biochem.* 226, 184-186.

Ryabova, L. A., Desplancq, D., Spirin, A. S. and Pluckthun, A. (1997) **Functional antibody production using cell-free translation: effects of protein disulfide isomerase and chaperones.** *Nat. Biotechnol.* 15, 79-84.

Saeki, T., Ihara, M., Fukuroda, T., Yamagiwa, M. and Yano, M. (1991) **[Ala1,3,11,15]endothelin-1 analogs with ETB agonistic activity.** *Biochem. Biophys. Res. Commun.* 179, 286-292.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. (1988) **Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase.** *Science* 239, 487-491.

Sakamoto, A., Yanagisawa, M., Sawamura, T., Enoki, T., Ohtani, T., Sakurai, T., Nakao, K., Toyo-Oka, T. and Masaki, T. (1993) **Distinct subdomains of human endothelin receptors determine their selectivity to endothelinA-selective antagonist and endothelinB-selective agonists.** *J. Biol. Chem.* 268, 8547-8553.

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K. and Masaki, T. (1990) **Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor.** *Nature* 348, 732-735.

Saravanan, K., Paramasivam, M., Dey, S., Singh, T. P. and Srinivasan, A. (2004) **Biotinyl endothelin-1 binding to endothelin receptor and its applications.** *J. Cardiovasc. Pharmacol.* 44, 287-293.

Saravanan, K., Hariprasad, G., Jitesh, O., Das, U., Dey, S., Sharma, S., Kaur, P., Singh, T. P. and Srinivasan, A. (2007) **Endothelin and its receptor interactions: role of extracellular receptor domain and length of peptide ligands.** *Protein Pept. Lett.* 14, 779-783.

Sarramegna, V., Demange, P., Milon, A. and Talmont, F. (2002) **Optimizing functional versus total expression of the human mu-opioid receptor in *Pichia pastoris*.** *Protein Expr. Purif.* 24, 212-220.

Satoh, M., Miyamoto, C., Terashima, H., Tachibana, Y., Wada, K., Watanabe, T., Hayes, A. E., Gentz, R. and Furuichi, Y. (1997) **Human endothelin receptors ET(A) and ET(B) expressed in baculovirus-infected insect cells--direct application for signal transduction analysis.** *Eur. J. Biochem.* 249, 803-811.

Sawasaki, T., Ogasawara, T., Morishita, R. and Endo, Y. (2002) **A cell-free protein synthesis system for high-throughput proteomics.** *Proc. Natl. Acad. Sci. U S A* 99, 14652-14657.

Sawasaki, T., Gouda, M. D., Kawasaki, T., Tsuboi, T., Tozawa, Y., Takai, K. and Endo, Y. (2005) **The wheat germ cell-free expression system: methods for high-throughput materialization of genetic information.** *Methods Mol. Biol.* 310, 131-144.

Sawasaki, T. and Endo, Y. (2007) **The wheat germ cell-free protein synthesis system.** In: *Cell-free Protein Synthesis: Methods and Protocols*, Spirin, A. S. a. S., J.R. (ed.), John Wiley & Sons, Ltd., Weinheim, Germany.

Schägger, H. and Von Jagow, G. (1987) **Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa.** *Anal. Biochem.* 166, 368-379.

Schägger, H., Cramer, W. A. and Von Jagow, G. (1994) **Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis.** *Anal. Biochem.* 217, 220-230.

Scheerer, P., Park, J. H., Hildebrand, P. W., Kim, Y. J., Krauss, N., Choe, H. W., Hofmann, K. P. and Ernst, O. P. (2008) **Crystal structure of opsin in its G-protein-interacting conformation.** *Nature* 455, 497-502.

Schiller, H., Haase, W., Molsberger, E., Janssen, P., Michel, H. and Reiländer, H. (2000) **The human ET(B) endothelin receptor heterologously produced in the methylotrophic yeast *Pichia pastoris* shows high-affinity binding and induction of stacked membranes.** *Receptors Channels* 7, 93-107.

Schiller, H., Molsberger, E., Janssen, P., Michel, H. and Reiländer, H. (2001) **Solubilization and purification of the human ETB endothelin receptor produced by high-level fermentation in *Pichia pastoris*.** *Receptors Channels* 7, 453-469.

Schneider, B., Junge, F., Shirokov, V. A., Durst, F., Schwarz, D., Dötsch, V. and Bernhard, F. (2010) **Membrane protein expression in cell-free systems.** *Methods Mol. Biol.* 601, 165-186.

Schnell, D. J. and Hebert, D. N. (2003) **Protein translocons: multifunctional mediators of protein translocation across membranes.** *Cell* 112, 491-505.

Schöneberg, T., Schultz, G. and Gudermann, T. (1999) **Structural basis of G protein-coupled receptor function.** *Mol. Cell. Endocrinol.* 151, 181-193.

Schwarz, D., Junge, F., Durst, F., Frölich, N., Schneider, B., Reckel, S., Sobhanifar, S., Dötsch, V. and Bernhard, F. (2007a) **Preparative scale expression of membrane proteins in *Escherichia coli*-based continuous exchange cell-free systems.** *Nat. Protoc.* 2, 2945-2957.

Schwarz, D., Klammt, C., Koglin, A., Löhr, F., Schneider, B., Dötsch, V. and Bernhard, F. (2007b) **Preparative scale cell-free expression systems: new tools for the large scale preparation of integral membrane proteins for functional and structural studies.** *Methods* 41, 355-369.

Schwarz, D., Dötsch, V. and Bernhard, F. (2008) **Production of membrane proteins using cell-free expression systems.** *Proteomics* 8, 3933-3946.

Schwarz, D., Daley, D., Beckhaus, T., Dötsch, V. and Bernhard, F. (2010) **Cell-free expression profiling of *E. coli* inner membrane proteins.** *Proteomics* 10, 1762-1779.

Seddon, A. M., Curnow, P. and Booth, P. J. (2004) **Membrane proteins, lipids and detergents: not just a soap opera.** *Biochim. Biophys. Acta* 1666, 105-117.

Sengupta, K., Klammt, C., Bernhard, F. and Rüterjans, H. (2003) **Incorporation of fluorescent labels into cell-free produced proteins.** In: *Cell-Free Protein Expression* Swartz, J. (ed.), Springer, Berlin Heidelberg New York.

Senisterra, G. A., Ghanei, H., Khutoreskaya, G., Dobrovetsky, E., Edwards, A. M., Prive, G. G. and Vedadi, M. (2010) **Assessing the stability of membrane proteins to detect ligand binding using differential static light scattering.** *J. Biomol. Screen.* 15, 314-320.

Serrano-Vega, M. J., Magnani, F., Shibata, Y. and Tate, C. G. (2008) **Conformational thermostabilization of the beta1-adrenergic receptor in a detergent-resistant form.** *Proc. Natl. Acad. Sci. U. S. A.* 105, 877-882.

Shibata, Y., White, J. F., Serrano-Vega, M. J., Magnani, F., Aloia, A. L., Grisshammer, R. and Tate, C. G. (2009) **Thermostabilization of the neurotensin receptor NTS1.** *J. Mol. Biol.* 390, 262-277.

Shimada, Y., Wang, Z. Y., Mochizuki, Y., Kobayashi, M. and Nozawa, T. (2004) **Functional expression and characterization of a bacterial light-harvesting membrane protein in *Escherichia coli* and cell-free synthesis systems.** *Biosci. Biotechnol. Biochem.* 68, 1942-1948.

Shimamura, T., Hiraki, K., Takahashi, N., Hori, T., Ago, H., Masuda, K., Takio, K., Ishiguro, M. and Miyano, M. (2008) **Crystal structure of squid rhodopsin with intracellularly extended cytoplasmic region.** *J. Biol. Chem.* 283, 17753-17756.

Shimizu, Y., Inoue, A., Tomari, Y., Suzuki, T., Yokogawa, T., Nishikawa, K. and Ueda, T. (2001) **Cell-free translation reconstituted with purified components.** *Nat. Biotechnol.* 19, 751-755.

Shimono, K., Goto, M., Kikukawa, T., Miyauchi, S., Shirouzu, M., Kamo, N. and Yokoyama, S. (2009) **Production of functional bacteriorhodopsin by an *Escherichia coli* cell-free protein synthesis system supplemented with steroid detergent and lipid.** *Protein Sci.* 18, 2160-2171.

Shirokov, V. A., Kommer, A., Kolb, V. A. and Spirin, A. S. (2007) **Continuous-exchange protein-synthesizing systems.** *Methods Mol. Biol.* 375, 19-55.

Shraga-Levine, Z. and Sokolovsky, M. (1998) **Functional role for glycosylated subtypes of rat endothelin receptors.** *Biochem. Biophys. Res. Commun.* 246, 495-500.

Sitaraman, K., Esposito, D., Klarmann, G., Le Grice, S. F., Hartley, J. L. and Chatterjee, D. K. (2004) **A novel cell-free protein synthesis system.** *J. Biotechnol.* 110, 257-263.

Slotboom, D. J., Duurkens, R. H., Olieman, K. and Erkens, G. B. (2008) **Static light scattering to characterize membrane proteins in detergent solution.** *Methods* 46, 73-82.

Sobhanifar, S., Schneider, B., Löhr, F., Gottstein, D., Ikeya, T., Mlynarczyk, K., Pulawski, W., Ghoshdastider, U., Kolinski, M., Filipek, S., Güntert, P., Bernhard, F. and Dötsch, V. (2010) **Structural investigation of the C-terminal fragment of presenilin-1.** *Proc. Natl. Acad. Sci. U S A.* 107, 9644-9649.

Sohlemann, P., Soppa, J., Oesterhelt, D. and Lohse, M. J. (1997) **Expression of beta 2-adrenoceptors in halobacteria.** *Naunyn Schmiedebergs Arch. Pharmacol.* 355, 150-160.

Sorensen, H. P., Sperling-Petersen, H. U. and Mortensen, K. K. (2003) **Production of recombinant thermostable proteins expressed in *Escherichia coli*: completion of protein synthesis is the bottleneck.** *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 786, 207-214.

Sorensen, H. P. and Mortensen, K. K. (2005) **Advanced genetic strategies for recombinant protein expression in *Escherichia coli*.** *J. Biotechnol.* 115, 113-128.

Spirin, A. S., Baranov, V. I., Ryabova, L. A., Ovodov, S. Y. and Alakhov, Y. B. (1988) **A continuous cell-free translation system capable of producing polypeptides in high yield.** *Science* 242, 1162-1164.

Springael, J. Y., Urizar, E., Costagliola, S., Vassart, G. and Parmentier, M. (2007) **Allosteric properties of G protein-coupled receptor oligomers.** *Pharmacol. Ther.* 115, 410-418.

Standfuss, J., Xie, G., Edwards, P. C., Burghammer, M., Oprian, D. D. and Schertler, G. F. (2007) **Crystal structure of a thermally stable rhodopsin mutant.** *J. Mol. Biol.* 372, 1179-1188.

Starling, A. P., East, J. M. and Lee, A. G. (1996) **Separate effects of long-chain phosphatidylcholines on dephosphorylation of the Ca(2+)-ATPase and on Ca2+ binding.** *Biochem. J.* 318 (Pt 3), 785-788.

Stroebel, D., Choquet, Y., Popot, J. L. and Picot, D. (2003) **An atypical haem in the cytochrome b(6)f complex.** *Nature* 426, 413-418.

Studier, F. W. and Moffatt, B. A. (1986) **Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes.** *J. Mol. Biol.* 189, 113-130.

Sun, L., Rush, J., Ghosh, I., Maunus, J. R. and Xu, M. Q. (2004) **Producing peptide arrays for epitope mapping by intein-mediated protein ligation**. *Biotechniques* 37, 430-436.

Swartz, J. R., Jewett, M. C. and Woodrow, K. A. (2004) **Cell-free protein synthesis with prokaryotic combined transcription-translation.** *Methods Mol. Biol.* 267, 169-182.

Swartz, J. (2006) **Developing cell-free biology for industrial applications.** *J. Ind. Microbiol. Biotechnol.* 33, 476-485.

Takagi, Y., Ninomiya, H., Sakamoto, A., Miwa, S. and Masaki, T. (1995) **Structural basis of G protein specificity of human endothelin receptors. A study with endothelinA/B chimeras**. *J. Biol. Chem.* 270, 10072-10078.

Takai, K., Sawasaki, T. and Endo, Y. (2010) **The wheat-germ cell-free expression system.** *Curr. Pharm. Biotechnol.* 11, 272-278.

Takasuka, T., Akiyama, N., Horii, I., Furuichi, Y. and Watanabe, T. (1992) **Different stability of ligand-receptor complex formed with two endothelin receptor species, ETA and ETB.** *J. Biochem.* 111, 748-753.

Takasuka, T., Sakurai, T., Goto, K., Furuichi, Y. and Watanabe, T. (1994) **Human endothelin receptor ETB. Amino acid sequence requirements for super stable complex formation with its ligand.** *J. Biol. Chem.* 269, 7509-7513.

Tate, C. G. (2001) **Overexpression of mammalian integral membrane proteins for structural studies.** *FEBS Lett.* 504, 94-98.

Taylor, T. A., Gariepy, C. E., Pollock, D. M. and Pollock, J. S. (2003) **Unique endothelin receptor binding in kidneys of ETB receptor deficient rats.** *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 284, R674-681.

Tessier, S., Boivin, S., Aubin, J., Lampron, P., Detheux, M. and Fournier, A. (2005) **Transmembrane domain V of the endothelin-A receptor is a binding domain of ETA-selective TTA-386-derived photoprobes.** *Biochemistry* 44, 7844-7854.

Tie, J. K., Nicchitta, C., Von Heijne, G. and Stafford, D. W. (2005) **Membrane topology mapping of vitamin K epoxide reductase by in vitro translation/cotranslocation.** *J. Biol. Chem.* 280, 16410-16416.

Torizawa, T., Shimizu, M., Taoka, M., Miyano, H. and Kainosho, M. (2004) **Efficient production of isotopically labeled proteins by cell-free synthesis: a practical protocol.** *J. Biomol. NMR* 30, 311-325.

Trbovic, N., Klammt, C., Koglin, A., Löhr, F., Bernhard, F. and Dötsch, V. (2005) **Efficient strategy for the rapid backbone assignment of membrane proteins.** *J. Am. Chem. Soc.* 127, 13504-13505.

Tuan L. Q., Umakoshi, H., Shimanouchi, T. and Kuboi, R. (2008) **Liposome-recruited activity of oxidized and fragmented superoxide dismutase.** *Langmuir* 24, 350-354.

Tucker, J. and Grisshammer, R. (1996) **Purification of a rat neurotensin receptor expressed in *Escherichia coli*.** *Biochem. J.* 317 (Pt 3), 891-899.

Turner, G. J., Reusch, R., Winter-Vann, A. M., Martinez, L. and Betlach, M. C. (1999) **Heterologous gene expression in a membrane-protein-specific system.** *Protein Expr. Purif.* 17, 312-323.

Ulmschneider, M. B., Sansom, M. S. and Di Nola, A. (2005) **Properties of integral membrane protein structures: derivation of an implicit membrane potential.** *Proteins* 59, 252-265.

Umakoshi, H., Suga, K., Bui, H. T., Nishida, M., Shimanouchi, T. and Kuboi, R. (2009) **Charged liposome affects the translation and folding steps of *in vitro* expression of green fluorescent protein.** *J. Biosci. Bioeng.* 108, 450-454.

Vallejo, L. F. and Rinas, U. (2004) **Strategies for the recovery of active proteins through refolding of bacterial inclusion body proteins.** *Microb. Cell Fact.* 3, 11.

Van Dalen, A., Hegger, S., Killian, J. A. and De Kruijff, B. (2002) **Influence of lipids on membrane assembly and stability of the potassium channel KcsA.** *FEBS Lett.* 525, 33-38.

Vedadi, M., Niesen, F. H., Allali-Hassani, A., Fedorov, O. Y., Finerty, P. J., Jr., Wasney, G. A., Yeung, R., Arrowsmith, C., Ball, L. J., Berglund, H., Hui, R., Marsden, B. D., Nordlund, P., Sundstrom, M., Weigelt, J. and Edwards, A. M. (2006) **Chemical screening methods to identify ligands that promote protein stability, protein crystallization, and structure determination.** *Proc. Natl. Acad. Sci. U S A* 103, 15835-15840.

Veiksina, S., Kopanchuk, S. and Rinken, A. (2010) **Fluorescence anisotropy assay for pharmacological characterization of ligand binding dynamics to melanocortin 4 receptors.** *Anal. Biochem.* 402, 32-39.

Vinarov, D. A., Lytle, B. L., Peterson, F. C., Tyler, E. M., Volkman, B. F. and Markley, J. L. (2004) **Cell-free protein production and labeling protocol for NMR-based structural proteomics.** *Nat. Methods* 1, 149-153.

Von Heijne, G. and Gavel, Y. (1988) **Topogenic signals in integral membrane proteins.** *Eur. J. Biochem.* 174, 671-678.

Von Heijne, G. (1992) **Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule.** *J. Mol. Biol.* 225, 487-494.

Wagner, S., Bader, M. L., Drew, D. and De Gier, J. W. (2006) **Rationalizing membrane protein overexpression.** *Trends Biotechnol.* 24, 364-371.

Wallin, E. and Von Heijne, G. (1998) **Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms.** *Protein Sci.* 7, 1029-1038.

Wang, Y. and Jardetzky, O. (2002) **Probability-based protein secondary structure identification using combined NMR chemical-shift data.** *Protein Sci.* 11, 852-861.

Warne, T., Serrano-Vega, M. J., Baker, J. G., Moukhametzianov, R., Edwards, P. C., Henderson, R., Leslie, A. G., Tate, C. G. and Schertler, G. F. (2008) **Structure of a β_1-adrenergic G-protein-coupled receptor.** *Nature.* 454, 486-491.

Watts, S. W. (2009) **Endothelin Receptors: what's new and what do we need to know?** *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 298, R254-260.

Weiss, H. M. and Grisshammer, R. (2002) **Purification and characterization of the human adenosine A(2a) receptor functionally expressed in *Escherichia coli*.** *Eur. J. Biochem.* 269, 82-92.

White, J. F., Trinh, L. B., Shiloach, J. and Grisshammer, R. (2004) **Automated large-scale purification of a G protein-coupled receptor for neurotensin.** *FEBS Lett.* 564, 289-293.

White, J. F., Grodnitzky, J., Louis, J. M., Trinh, L. B., Shiloach, J., Gutierrez, J., Northup, J. K. and Grisshammer, R. (2007) **Dimerization of the class A G protein-coupled neurotensin receptor NTS1 alters G protein interaction.** *Proc. Natl. Acad. Sci. U S A* 104, 12199-12204.

White, J. H., Wise, A., Main, M. J., Green, A., Fraser, N. J., Disney, G. H., Barnes, A. A., Emson, P., Foord, S. M. and Marshall, F. H. (1998) **Heterodimerization is required for the formation of a functional GABA(B) receptor.** *Nature* 396, 679-682.

White, S. H. and Von Heijne, G. (2008) **How translocons select transmembrane helices.** *Annu. Rev. Biophys.* 37, 23-42.

Wiener, M. C. (2004) **A pedestrian guide to membrane protein crystallization.** *Methods* 34, 364-372.

Williams, K. A., Farrow, N. A., Deber, C. M. and Kay, L. E. (1996) **Structure and dynamics of bacteriophage IKe major coat protein in MPG micelles by solution NMR.** *Biochemistry* 35, 5145-5157.

Wishart, D. S. and Sykes, B. D. (1994) **Chemical shifts as a tool for structure determination.** *Methods Enzymol.* 239, 363-392.

Wittig, I., Braun, H. P. and Schägger, H. (2006) **Blue native PAGE.** *Nat. Protoc.* 1, 418-428.

Wu, H., Hu, Z. and Liu, X. Q. (1998) **Protein trans-splicing by a split intein encoded in a split DnaE gene of *Synechocystis sp*. PCC6803.** *Proc. Natl. Acad. Sci. U S A* 95, 9226-9231.

Wuu, J. J. and Swartz, J. R. (2008) **High yield cell-free production of integral membrane proteins without refolding or detergents.** *Biochim. Biophys. Acta.* 1778, 1237-1250.

Xie, J., Bogdanov, M., Heacock, P. and Dowhan, W. (2006) **Phosphatidylethanolamine and monoglucosyldiacylglycerol are interchangeable in supporting topogenesis and function of the polytopic membrane protein lactose permease.** *J. Biol. Chem.* 281, 19172-19178.

Xie, Z., Bhushan, R. G., Daniels, D. J. and Portoghese, P. S. (2005) **Interaction of bivalent ligand KDN21 with heterodimeric delta-kappa opioid receptors in human embryonic kidney 293 cells.** *Mol. Pharmacol.* 68, 1079-1086.

Yanagisawa, M., Kurihara, H., Kimura, S., Goto, K. and Masaki, T. (1988) **A novel peptide vasoconstrictor, endothelin, is produced by vascular endothelium and modulates smooth muscle Ca2+ channels.** *J. Hypertens. Suppl.* 6, S188-191.

Yao, Z. and Kobilka, B. (2005) **Using synthetic lipids to stabilize purified beta2 adrenoceptor in detergent micelles.** *Anal. Biochem.* 343, 344-346.

Yeagle, P. L. (1985) **Cholesterol and the cell membrane.** *Biochim. Biophys. Acta* 822, 267-287.

Yeagle, P. L. and Albert, A. D. (2007) **G-protein coupled receptor structure.** *Biochim. Biophys. Acta* 1768, 808-824.

Yeliseev, A. A., Wong, K. K., Soubias, O. and Gawrisch, K. (2005) **Expression of human peripheral cannabinoid receptor for structural studies.** *Protein Sci.* 14, 2638-2653.

Yokoyama, S. (2003) **Protein expression systems for structural genomics and proteomics.** *Curr. Opin. Chem. Biol.* 7, 39-43.

Yu, H., Kono, M., Mckee, T. D. and Oprian, D. D. (1995) **A general method for mapping tertiary contacts between amino acid residues in membrane-embedded proteins.** *Biochemistry* 34, 14963-14969.

Zhang, Z., Austin, S. C. and Smyth, E. M. (2001) **Glycosylation of the human prostacyclin receptor: role in ligand binding and signal transduction.** *Mol. Pharmacol.* 60, 480-487.

Zubay, G. (1973) ***In vitro* synthesis of protein in microbial systems.** *Annu. Rev. Genet.* 7, 267-287.

7. Appendix

7.1. Primer sequences

The respective restriction sites are indicated in bold letters.

7.1.1. Sequencing primers for pET-Vectors

Vector	Tag	Name	Sequence	T_M [°C]
pET	-	T7-prom	5' TAA TAC GAC TCA CTA TAG GG 3'	54
pET	-	T7-term	5' CTA GTT ATT GCT CAG CGG T 3'	55

7.1.2. Cloning primers for ETB without signal sequence and truncations

Vector	Tag	Name	Sequence	T_M [°C]
pET 21a(+)	T7 (N) His_{10} (C)	ETB1-bam_up	5' CGG **GGA TCC** GAG GAG AGA GGC TTC CCG CCT GAC AGG 3'	75
pET 21a(+)	T7 (N) His_{10} (C)	ETB93_up	5' CGG **GGA TCC** CCC ATC GAG ATC AAG GAG ACT TTC AAA 3'	68
pET 21a(+)	T7 (N) His_{10} (C)	ETB131_low	5' CGG **CTC GAG** GCA CTT GTT CTT GTA GAT AAT TCT CAG AAG 3'	67
pET 21a(+)	T7 (N) His_{10} (C)	ETB131_lowstop	5' CGG **CTC GAG** TTA CTT GTT CTT GTA GAT AAT TCT CAG AAG 3'	64
pET 21a(+)	T7 (N) His_{10} (C)	ETB_low_wo_ Cterm	5' CGG **CTC GAG** GCA TGA CTT AAA GCA GTT TTT GAA TCT 3'	64

7.1.3. Cloning primers for site-directed mutagenesis in the ETA full-length receptor

Vector	Tag	Name	Sequence	T_M [°C]
pET 21a(+)	T7 (N) His_{10} (C)	ETA C/A 158_up	5' GGC GTA TTT CTT GCC AGG CTG TTC CCC 3'	71
pET 21a(+)	T7 (N) His_{10} (C)	ETA C/A 158_low	5' GGG GAA CAG CTT GGC AAG AAA TAC GCC 3'	71
pET 21a(+)	T7 (N) strep (C)	ETA C/A 239_up	5' CAG CAT AAA ACC GCT ATG GGC 3'	68
pET 21a(+)	T7 (N) His_{10} (C)	ETA C/A239_low	5'GCC ATT GAG CAT AGC GGT TTT ATG CTG 3'	68

7.1.4. Cloning primers for ETA full-length and truncations

Vector	Tag	Name	Sequence	T_M [°C]
pET 21a(+)	T7 (N) His_{10} (C)	ETA_up	5' CCG **AGA TCT** ATG GAA ACC CTT TGC CTC AGG GCA TCC 3'	69
pET 21a(+)	T7 (N) His_{10} (C)	ETA_low	5' CCG **CTC GAG** GTT CAT GCT GTC CTT ATG GCT GCT CCG 3'	71
pET 21a(+)	T7 (N) strep (C)	ETAlow strep	5' CCG **CCA TGG** CAT GCT GTC CTT ATG GCT GCT CCG 3'	71
pET 21a(+)	T7 (N) His_{10} (C)	ETA107_low	5' CCG **CTC GAG** CTG GTA AAT GAT CCT GAG CAG AGT TGC 3'	69
pET 21a(+)	T7 (N) strep (C)	ETA107_low strep	5' CGA **CCA TGG** CTG GTA AAT GAT CCT GAG CAG AGT TGC 3'	66
pET 21a(+)	T7 (N) His_{10} (C)	ETA198_up	5' CGA **AGA TCT** ATT GGG ATT CCT TTG GTA ACT GCC 3'	63
pET 21a(+)	T7 (N) His_{10} (C)	ETA197_low	5' CCG **CTC GAG** TCC CTG AAC ACG ACT CCA GGA GGC 3'	73
pET 21a(+)	T7 (N) strep (C)	ETA197_low strep	5' CGA **CCA TGG** TCC CTG AAC ACG ACT CCA GGA GGC 3'	71

7.2. Vector map of pET21a(+) used for the CF expression of the ET receptom

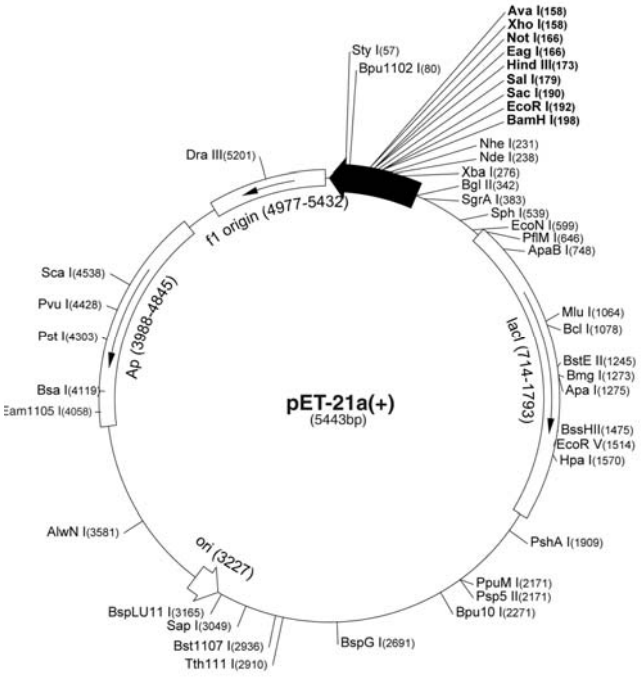


Fig. 60: Schematic vector table of pET21a(+). The pET21a(His)$_{10}$ plasmid used in present study is a modified pET21a(+) vector. It contains additional four histidine codons downstream of the present six histidine codons resulting in a C-terminal poly(His)$_{10}$-tag. The additional codons were inserted by site-directed mutagenesis by Dr. Christian Klammt. The vector carries a gene for ampicillin resistance. The black arrow represents the multiple cloning site.

7.3. Primary sequences and labels of peptide ligands used for the functional analysis of CF expressed ET receptors

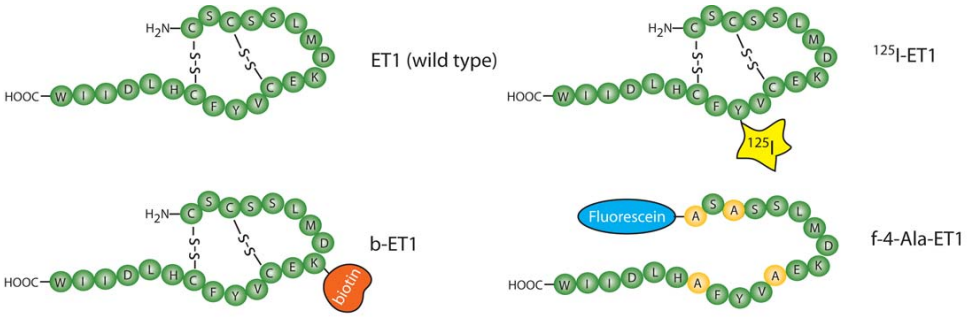


Fig. 61: ETA ligand overview. Schematic illustration of ET-1 derivatives successfully used in the functional analysis of detergent-solubilized and reconstituted ETA. Type and sites of the various labels are indicated. The circular ET-1 was used as WT variant without any label, as commercial radioligand or as biotinylated ligand. The linear ET-1 was used in its fluorescein labelled form. All ligands except for the ^{125}I-ET-1 were kindly provided by Dr. Michael Beyermann (FMP, Berlin).

7.4. Standard curves for the calibration of size exclusion chromatography columns for the analysis of homogeneity and MALS

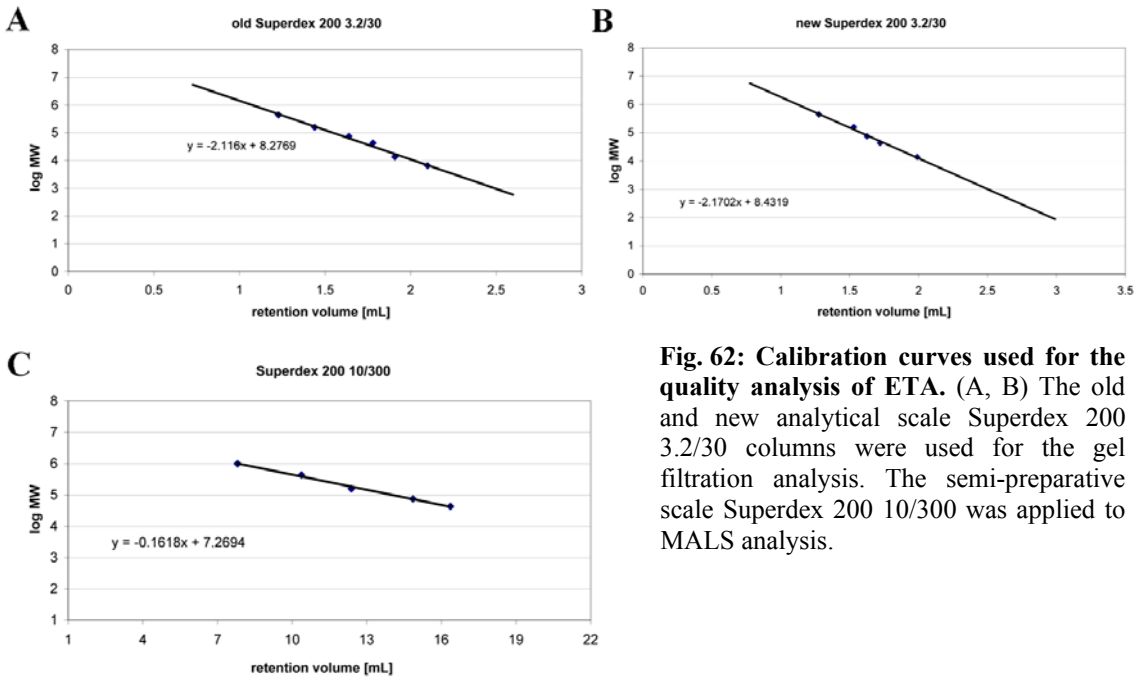


Fig. 62: Calibration curves used for the quality analysis of ETA. (A, B) The old and new analytical scale Superdex 200 3.2/30 columns were used for the gel filtration analysis. The semi-preparative scale Superdex 200 10/300 was applied to MALS analysis.

7.5. List of crystallization screens used in the CrystalMation automated robotic parameter screening of CF expressed ETA

7.5.1. Commercially available screens

The CubicPhase I Suite (Qiagen GmbH ,Germany), 22 °C

The CubicPhase II Suite (Qiagen GmbH ,Germany), 22 °C

The MbClass Suite (Qiagen GmbH ,Germany), 4 °C

The MbClass II Suite (Qiagen GmbH ,Germany), 4 °C

MemGold™ (Molecular Dimensions Ltd., UK), 4 and 18 °C

MemStart and MemPlus™ (Molecular Dimensions Ltd., UK), 4 and 18 °C

MemSys™ and Sigma Membrane Kit (Molecular Dimensions Ltd., UK and Sigma-Aldrich Chemie GmbH, Germany), 4 and 18 °C

PGA Screen™ (Molecular Dimensions Ltd., UK), 4 °C

JBScreen Pentaerythritol HTS (Jena Bioscience GmbH, Germany), 4 °C

7.5.2. Custom MPI screens

Those screens were partly set up after Koszelak-Rosenblum et al. 2009 for crystallization in the MPI of Biophysics, Frankfurt/Main, Germany.

Custom MPI pH Screen from 4 to 9.5, 4 °C and 18 °C

MPI Custom Screen pH 5.6 A, 4 °C

MPI Custom Screen pH 5.6 B, 4 °C

MPI Custom Screen pH 5.6 C, 4 °C

MPI Custom Screen pH 5.6 D, 4 °C

MPI Custom Screen pH 7.0 A, 4 °C

MPI Custom Screen pH 7.0 B, 4 °C

MPI Custom Screen pH 7.0 C, 4 °C

MPI Custom Screen pH 7.0 D, 4 °C

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I have long searched for proper words as to how to begin the “last but in no way least” of my tasks.
My dissertation would have been a lot harder and a lot less ‘fun’ if I had been a solitary worker without all my colleagues and the following people, who often lent a helping hand, encouraged my thinking, supported me in alternating phases of depression and exhilaration and enriched my life simply by getting to know them.
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The frequent meetings to play badminton with Horng Der Ou and Stephie, or Daniel, Birgit, Jakob Lopez and Susanne Stefer or the past and recent running groups with Peter Tufar, Alena Busche, Alexis Rozenknop, Christopher Hein and Nina Ripin (or the long and exhausting distance run with Horng in Breckenheim with a very, very steep hill) helped and still help me to release my stress and tension and I am really happy to have found people with whom I can share my enthusiasm for sports and nature and hiking!

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Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt, dass ich die vorgelegte Dissertation über „Biochemical and functional characterization of cell-free expressed G-protein coupled receptors of the human endothelin system" selbständig angefertigt und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe. Des Weiteren versichere ich, dass Entlehnungen, aus anderen Schriften soweit sie in der Dissertation nicht ausdrücklich als solche mit Angabe der betreffenden Schrift bezeichnet sind, nicht stattgefunden haben.
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Frankfurt am Main, den

Friederike Junge