The Molecular Basis of V2 Vasopressin Receptor/G Protein Coupling Selectivity

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To my parents in love and gratitude

Meinen Eltern in Liebe und Dankbarkeit gewidmet

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1. Introduction and Aim of the Thesis

1.1 G protein-coupled receptors

Every cell communicates with its environment in order to function properly and to respond to environmental changes. This interaction is achieved through receptors, which are macromolecules that are able to transmit extracellular regulatory signals to the cell interior, thus modulating cell activity and function. Three major types of plasma membrane receptors are known, a) receptors that interact with G proteins (G protein-coupled receptors), b) ligand-gated ion channels (e.g. nicotinic cholinergic receptor), and c) receptors with enzymatic activity (e.g. insulin receptor).

G protein-coupled receptors (GPCRs) form one of the largest protein families found in nature (Watson and Arkinstall, 1994). Sequence analysis suggests that the human genome contains about 600-700 distinct GPCR genes (Venter *et al.*, 2001). To date several hundred GPCRs have been cloned and sequenced (Watson and Arkinstall, 1994). Fig. 1.1 demonstrates the topology of a prototypical member of the GPCR superfamily, the V2 vasopressin receptor.

All GPCR signal transduction pathways follow the same general scheme (Fig. 1.2). Binding of extracellular ligands to the cell surface GPCR induces conformational changes in the receptor protein, which enable the receptor to interact with G proteins that are localized intracellularly at the plasma membrane. G proteins are heterotrimers consisting of a guanine nucleotide (GTP/GDP) binding α-subunit and a $\beta\gamma$-complex. Activation of the G protein through ligand-occupied receptors promotes the release of GDP and subsequent exchange for GTP on the α-subunit, which then dissociates from the $\beta\gamma$ subunit. Both GTP-α and free $\beta\gamma$ are able to modulate the activity of distinct effector systems such as adenylyl and guanylyl cyclases, phosphodiesterases, phospholipases A and C, as well as K^+ and Ca^{2+} ion channels. This activity is terminated by hydrolysis of bound GTP to GDP by the GTPase ability intrinsic to the α-subunit. Hydrolysis of bound GTP can be accelerated by RGS (regulator of G protein signaling) proteins, which act allosterically on the Gα-subunit, increasing the rate at which the α-subunit hydrolyzes GTP (Ross and Wilkie, 2000). Subsequent reassociation of Gα with

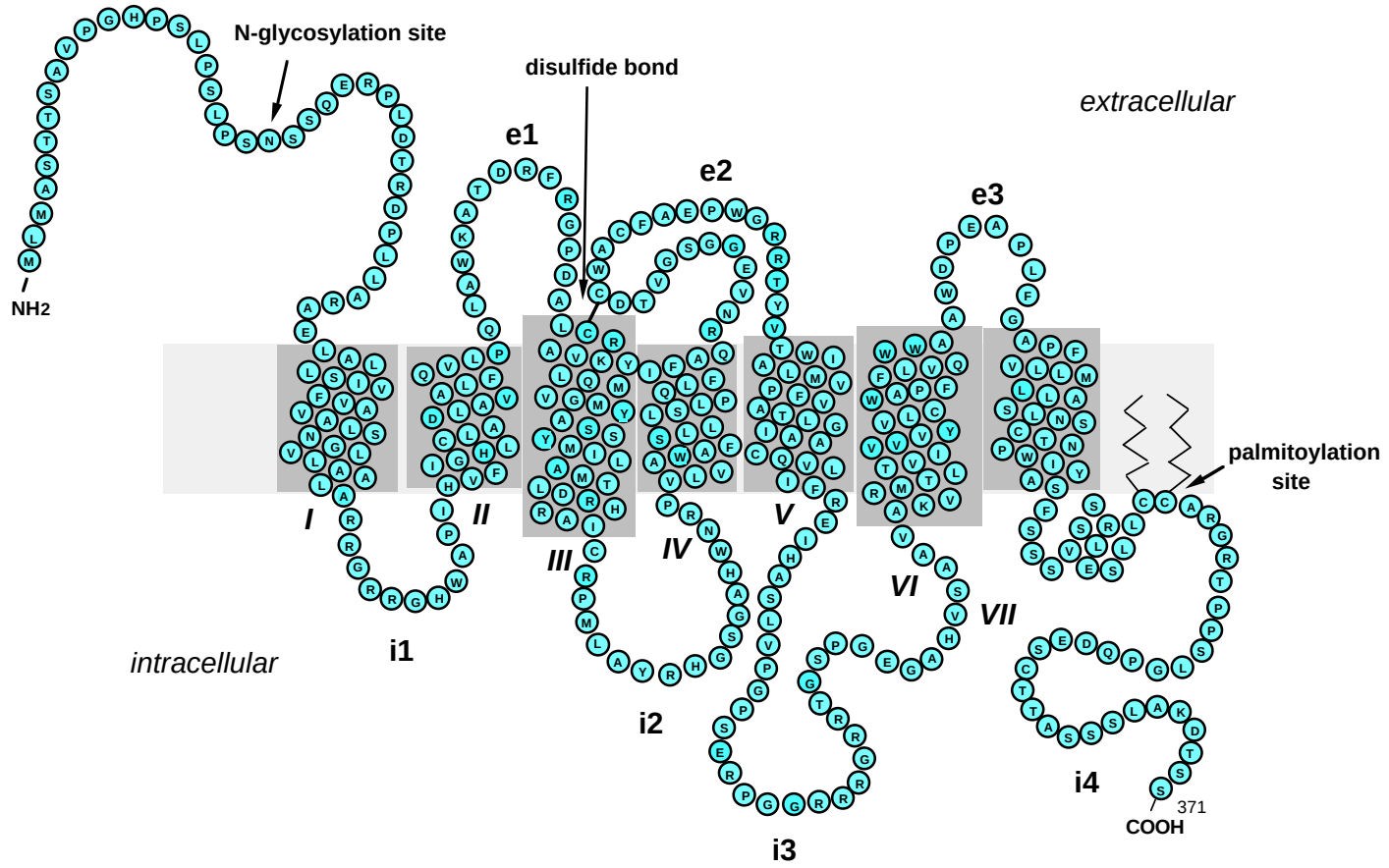


Fig. 1.1 Amino acid sequence and proposed transmembrane disposition of the human V2 vasopressin receptor. The V2 receptor, like all other G protein-coupled receptors, are predicted to be composed of seven hydrophobic transmembrane helices (*TM I-VII*), that are joined by three extracellular (e1-e3) and three intracellular (i1-i3) loops, with an extracellular N-terminal domain and an intracellular C-terminal tail (i4). According to the crystal structure of bovine rhodopsin (Palczewski *et al.*, 2000) the N-terminal domain of the cytoplasmic tail is α-helically arranged and runs parallel to the plane of the membrane. The putative i4 α-helical domain is shown in this scheme. Also, the N-glycosylation site, the putative disulfide bond formed by cysteines at the e1/TM III junction and e2 loop, and the palmitoylation site in the C-terminal tail are indicated. The single letter amino acid code is used.

Gβγ allows the system to return to the resting state (for reviews, see Ross, 1989; Conklin and Bourne, 1993). GPCRs are involved in many fundamental physiological processes and represent attractive therapeutic targets. Estimates are that about 50% of drugs in current clinical use act on specific GPCRs or GPCR-mediated signaling pathways (Hardman *et al*., 1996). Understanding how GPCRs function at a molecular level is therefore of great potential therapeutic interest. Structural analysis of the ligand/GPCR/G protein complex should eventually lead to the design of more selective therapeutic agents targeted at specific GPCRs or GPCR-dependent signal transduction pathways.

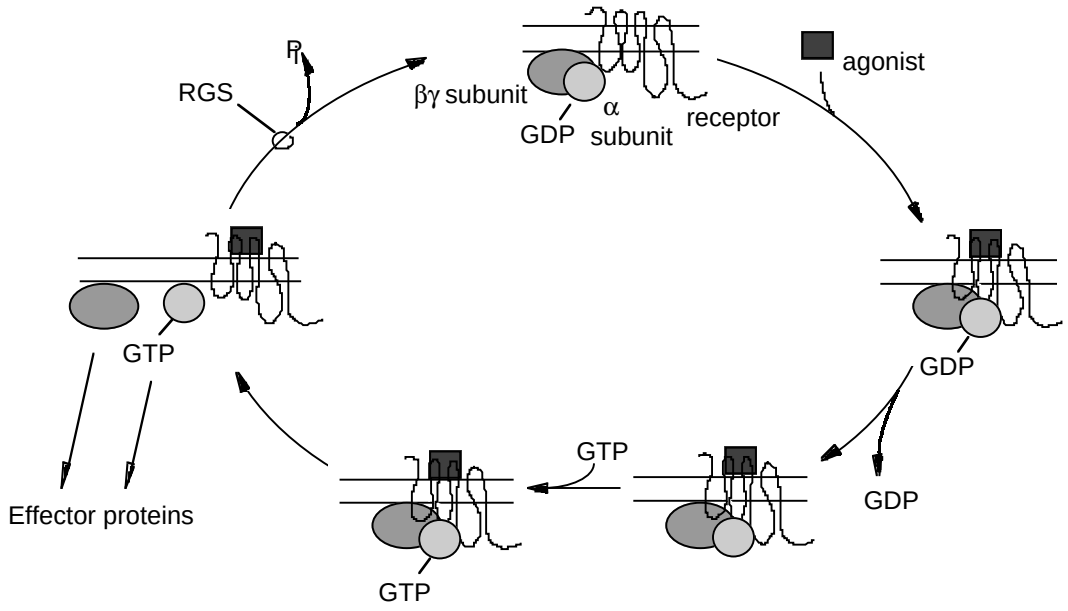


Fig. 1.2 The receptor-G protein activation cycle. The diagram briefly shows the events that occur during ligand binding and G protein activation. The heterotrimeric G protein is made up of the three subunits α, β, and γ. The α-subunit binds guanine nucleotides, the β- and γ-subunits are always associated. In the inactive state of the G protein, all three subunits are associated and GDP is bound to the α-subunit. Agonist binding to the receptor causes a conformational change, which increases heterotrimeric G protein binding to the receptors. Once the G protein binds to the receptor/agonist complex, GDP is exchanged for GTP on the α-subunit and the G protein dissociates into the α- and βγ-subunits, which both in turn can activate downstream effector proteins. The activation cycle is terminated by hydrolysis of GTP into GDP and P by the endogenous GTPase activity of the α-subunit. Hydrolysis of bound GTP can be accelerated by RGS (regulator of G protein signaling) proteins, which act allosterically on the Gα-subunit, increasing the rate at which the α-subunit hydrolyzes GTP. Subsequent reassociation of Gα with Gβγ allows the system to return to the resting state (see text for details).

1.1.1 Classification and diversity of GPCRs

Hydrophobicity analysis has shown that all GPCRs contain seven stretches of about 20-28 predominantly hydrophobic residues which are thought to form α helical membrane spanning domains (Trumpp-Kallmeyer *et al.*, 1992). These transmembrane domains (TMs) are connected by alternating intracellular (i1-i3) and extracellular loops (e1-e3), with an extracellular amino-terminus and an intracellular carboxy-terminus (Wang *et al*., 1989) (Fig. 1.1).

The ligands that act on GPCRs are highly diverse, comprising a large range of agents varying greatly in structure, size and physiological function. GPCR ligands can act as neurotransmitters, hormones, local mediator substances, or can convey sensory information. Such ligands include, for example, the small biogenic amines such as acetycholine, catecholamines, serotonin and histamine, as well as the large glycoprotein hormones follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and luteinizing hormone/choriogonadotropin (LH/CG). Peptide ligands, which are particularly abundant, can act as neurotransmitters as well as hormones. Other GPCR activators encompass stimulants as diverse as light, odorants, nucleotides (e.g. ATP/UTP), calcium ions, prostanoids, the amino acid L-glutamate, and lipids (e.g. platelet-activating factor).

Based on nucleotide and amino acid sequence similarity, the superfamily of GPCRs can be subdivided into four major groups of receptors (Bockaert and Pin, 1999; Wess, 1998). Class I type receptors share key structural features with the light receptor rhodopsin and are therefore referred to as rhodopsin-type receptors. This class comprises the majority of GPCRs that have been identified to date and has been studied in great detail (Baldwin, 1994; Strader *et al.*, 1994; Dohlman *et al.*, 1991). Class I type receptors are activated by a large variety of stimuli, including photons, odorants, hormones, and neurotransmitters, ranging in size from small biogenic amines to peptides and large glycoproteins (Nathans and Hogness, 1983; Pace *et al.*, 1985; Dixon *et al.*, 1986; Loosfelt *et al.*, 1989; Kaiser *et al.*, 1992, Karkar *et al.*, 1992; Vassart *et al.*, 1995). The rhodopsin-type receptors are characterized by a set of about 20 amino acids that are highly

conserved only in this GPCR subfamily (for more details, see chapter 1.6). Most of these residues are located in the transmembrane receptor core and are involved in protein folding and stability and relaying conformational changes during the receptor activation process (Wess *et al.*, 1993; Baldwin, 1994; Van Rhee and Jacobson, 1996). Unlike the transmembrane regions, both the N- and C-terminal domains can vary remarkably in length among Class I receptors. A reason for this structural variability may be the great structural diversity of activating agents.

The secretin/glucagon receptor family (Class II) comprises a relatively small group of receptors that bind neuropeptides and peptide hormones. Class II receptors do not exhibit the fingerprint residues that are characteristic of the rhodopsin-type receptors, with the exception of the putative disulfide bridge between the e1 and e2 loops (Ishigara *et al.*, 1991; Lin *et al.*, 1991; Ishigara *et al.*, 1992). However, secretin/glucagon-type receptors share about 20 identical amino acids and are characterized by a large N-terminal extracellular domain with at least six highly conserved cysteine residues that are thought to play an important role in ligand binding (Strader, 1994).

Class III receptors comprise the metabotropic glutamate receptors (mGluR) (Conn and Pin, 1997), the calcium sensor (Brown *et al.*, 1993), the γ-aminobutyric acid $(GABA)_B$ receptors (Kaupmann *et al.*, 1997), and a group of about 100 putative pheromone receptors (Herrada and Dulac, 1997; Matsunami and Buck, 1997). The group of the mGluRs encompasses eight subtypes that bind L-glutamate, the major excitatory neurotransmitter in the central nervous system. Class III receptors are characterized by a long N-terminal extracellular domain; the C-terminal tail is of variable length (Nakanishi, 1992, Wess, 1998).

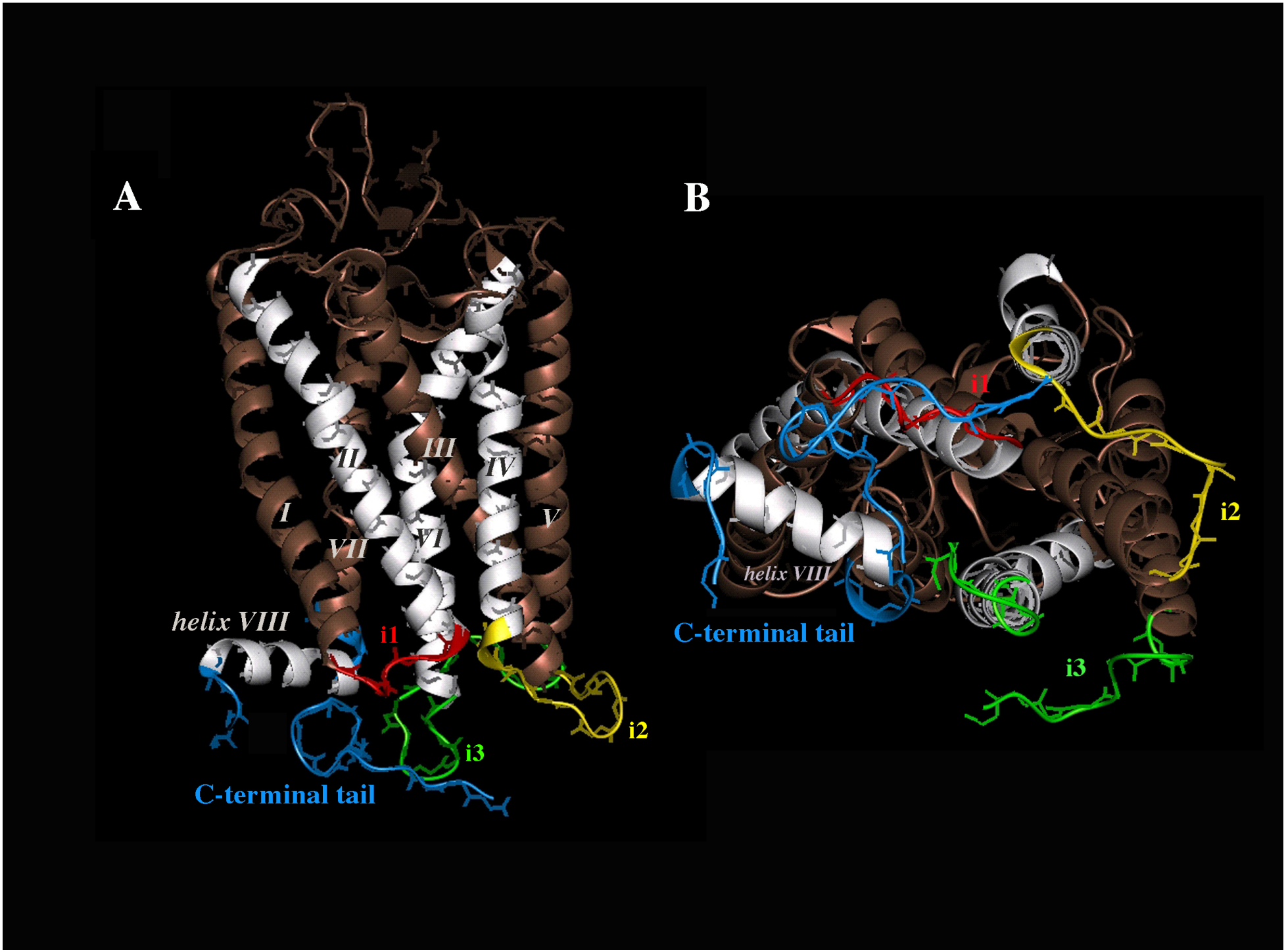
Class IV receptors are unrelated to any other group of GPCRs and are made up of pheromone receptors that are exclusively expressed in apical neurons of the vomeronasal organ (Dulac and Axel, 1995). These receptors share between 47-87% sequence identity and contain a relatively short N-terminal extracellular domain.

1.1.2 The three dimensional structure of rhodopsin-like receptors

The recent elucidation of the first high resolution X-ray structure of a GPCR represents a major breakthrough in GPCR research. Palczewski *et al.* (2000) generated bovine rhodopsin crystals from mixed micelles and obtained structural information for rhodopsin in the ground state by employing multiwavelength anomalous diffraction (MAD) methods. This yielded information of the three-dimensional crystal structure of rhodopsin at a high resolution (2.8 Å) (Fig. 1.3A, B).

Before the first GPCR X-ray was available, most structure information was derived from biochemical and theoretical studies and extrapolation from the structure of bacteriorhodopsin, a serpentine receptor protein from *Halobacterium halobium,* which is an ion channel and not coupled to G proteins. A low resolution image of bovine rhodopsin (Schertler *et al.*, 1993) and sequence analysis of 200 different GPCRs (Baldwin, 1993) led to the first structural model of the transmembrane receptor core, which was in good agreement with a great number of mutagenesis and biochemical studies (for reviews, see Baldwin, 1994; Bourne, 1997; Wess, 1997). In 1997, Unger *et al.* presented an improved low-resolution electron density map of frog rhodopsin. Combining this new structural information with the sequence analysis of about 500 GPCRs, Baldwin *et al* (1997) proposed a refined model for rhodopsin-like receptors. This model proved to be highly useful to interpret the results of a large number of GPCR structure-function studies.

As shown in Fig. 1.3, the seven transmembrane helices (TM I-VII) form a barrel-shaped tightly packed bundle, oriented roughly perpendicular to the plane of the membrane in a clockwise fashion (when viewed from the cytoplasm) (Palczeswki *et al.*, 2000). Most of the primary sequence homology among the different rhodopsin-like receptors is contained within the hydrophobic transmembrane domain. Interestingly, the various TMs differ in their hydrophobicity: TMs I, IV, and VII contain only one hydrophilic residue and are therefore more hydrophobic than TMs II, III, V, and VI which harbor several ionic and/or hydrophilic amino acids.


A
I
II
III
IV
V
VI
VII
helix VIII
i1
i2
i3
C-terminal tail
B
i1
i2
helix VIII
C-terminal tail
i3

According to the Unger model (1997), the helical bundle appeared to be packed much more tightly on the intracellular side of the membrane, resulting in an asymmetric protein with different sizes at either end. However, this prediction proved to be incorrect, since the high-resolution X-ray structure of rhodopsin indicate that the intra- and extracellular surface of the protein have about the same spatial dimensions.

Rhodopsin-type GPCRs harbor a highly conserved Pro residue in TMs IV, V, VI, and VII. Pro often causes a kink within a helix but according to the high resolution X-ray structure of rhodopsin, only TMs VI and VII are significantly bent (Fig. 1.3A). TM II is bent at Gly89 and Gly90, which brings this region in close contact to TM I. Apart from the helical structure, the axes of the different TMs also vary significantly. TMs IV, VI and VII are oriented almost perpendicular to the plane of the membrane, whereas the axes of TMs I, II, III, and V are tilted with TM III being the most inclined TM, deeply buried in the protein core. This causes the extracellular ends of TM III, I and II to be near each other, whereas with TM III moving between TMs II and IV toward the intracellular side of the membrane, the cytoplasmic end of TMs III lies near TM V (Fig. 1.3A, B). Likewise, TMs II and IV are in close proximity at the intracellular side of the membrane but diverge towards the extracellular side. This causes the relative positions of TM III and IV to be reversed on the intracellular side of the membrane (Fig. 1.3B). Both the Balwin model and the high-resolution X-ray data indicate that the seven TM helices vary in length, with TMs III and VI being the longest and TMs IV and VII being the shortest. The high-resolution X-ray structure indicates that TMs III and VI protrude into the cytoplasm by about two to three helical turns (Palczewski *et al.*, 2000).

Fig. 1.3 Model of the three dimensional structure of bovine rhodopsin (adapted from Palczewski *et al.*, 2000). *A,* Ribbon drawing of rhodopsin, viewed parallel to the plane of the membrane and *B,* viewed onto the membrane plane from the cytoplasmic side of the membrane. TMs I-VII and helix VIII are indicated: *copper*: TMs I, III, V, and VII; *white*: TMs II, IV, VI, and helix VIII. The intracellular loops are highlighted in different colors: *red*: first intracellular (i1) loop, *yellow*: second intracellular (i2) loop, *green*: third intracellular (i3) loop, *blue*: cytoplasmic tail (i4). Note that some few amino acids in the i3 and C-terminal domain are missing in the current model (Palczewski *et al.*, 2000).

The cytoplasmic tail of class I GPCRs contains one or two highly conserved cysteine residues, which are thought to be linked to the plasma membrane via fatty acid modifications (e.g. myristoylation or palmitoylation; note, however, that the GPCR structure was resolved using proteins devoid of lipid modifications), thus forming a fourth intracellular (i4) loop. Interestingly, the high resolution rhodopsin structure indicates that the "i4 loop" adopts an amphiphilic helical structure (helix VIII), which runs almost parallel to the plane of the membrane, with hydrophobic residues facing the receptor protein and charged/polar groups oriented towards the hydrophilic environment. Helix VIII is distinct from TM VII and connected with TM VII via a stretch of non-helically arranged amino acids (Palczeswski *et al.*, 2000). The existence of helix VIII has also been predicted based on NMR studies with a synthetic peptide derived from the corresponding β-adrenergic receptor region (Jung *et al.*, 1996).

The high-resolution structure of bovine rhodopsin also provides the first direct view of the three-dimensional architecture of the intra- and extracellular loops. The N-terminal extracelluar domain forms a compact structure consisting of five antiparallel strands forming β-sheets that run at times in close proximity to other extracellular loops, like the e1 and e3 loops. The e1 and e3 loops run along the periphery of the molecule, whereas parts of e2 project into the receptor core. Class I GPCRs contain two highly conserved cysteines, one is located at the extracellular end of TM III and the other one within e2. These cysteines form a disulfide bond that draws e2 into the receptor core. As a result, parts of the e2 loop are in close contact with other extracellular domains, while other parts are in proximity to regions deep within the receptor protein (e.g. the chromophore retinal in the case of rhodopsin). Mutagenesis studies have demonstrated that this disulfide bond is essential for receptor structure and function (Van Rhee and Jacobson, 1996; Cook and Eidne, 1997; Zeng *et al.*, 1999a, b). In the case of rhodopsin, it is required for formation of the light-activated metarhodopsin II state (Davidson *et al.*, 1994).

Analogous to the extracellular receptor region, the intracellular receptor surface also adopts a rather compact structure. Parts of the i1 loop run almost parallel to the C-terminal tail and seem to be in contact especially with its N-terminal portion (Fig. 1.3A,

B). The i2 loop exhibits a rigid, almost right-angled structure pointing away from the receptor protein. The tip of the loop is kinked and traces back to helix IV almost perpendicularly (Fig. 1.3A, B). The most flexible loop seems to be the i3 loop, which varies extensively in size among GPCRs. According to the high-resolution X-ray structure, the i3 loop takes on an S-shaped structure which runs almost parallel along the surface of the membrane (Fig. 1.3A, B). Parts of the C-terminal portion of the i3 loop also appear to be in contact with the C-terminal tail via hydrogen bonding. Even though a few amino acids in the central portion of the i3 loop and the C-terminal tail are missing in the current model, the X-ray data suggest that the intracellular loops do not fold over the TM bundle (Palczewski *et al.*, 2000) (Fig. 1.3B). The helical structure of the "i4 loop" has already been discussed earlier. The distal segment of the C-terminal tail seems to be the most exposed part of the receptor molecule (Palczewski *et al.*, 2000).

As discussed in chapter 1.3 (also see Fig. 1.6), Class I receptors share a set of highly conserved amino acids that are thought to be important for receptor folding, ligand binding and other important aspects of receptor function. The high-resolution X-ray structure of rhodopsin indicates that the receptor core is highly organized by interhelical constraints mediated by several of these conserved amino acids thus keeping the receptor in an inactive conformation (Palczewski *et al.*, 2000). Upon ligand binding, some of the conformational constraints are likely to be broken leading to a re-arrangement and movement of helices, notably TM III and TM VI (Farrens *et al.*, 1996), and allowing the receptor to adopt its active conformation. In its active conformation, the receptor is then able to productively interact with heterotrimeric G proteins.

1.1.3 Heterotrimeric G proteins and their effector systems

Heterotrimeric G proteins are members of the guanosine 5' triphosphatase (GTPase) superfamily and are made up of α-, β-, and γ-subunits (for reviews, see Hamm, 1998; Sprang, 1997). At least 20 α-, 6 β-, and 12 γ-subunits are expressed by mammalian cells, allowing the formation of a large number of different G protein heterotrimers. On the basis of amino acid similarity of the Gα-subunits, G proteins can be subdivided into

four major classes: G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$. G_s class G proteins comprise several splice variants of G_s and G_{olf} and have a *s*timulatory effect on adenylyl cyclase. Members of the $G_{i/o}$ family are α_i (with subtypes α_{i1}, α_{i2}, and α_{i3}) and α_o (there are two splice variants, α_{o1} and α_{o2}), α_{gust}, α_z and α_t (α_{t1} and α_{t2}, these subunits mediate rhodopsin-relayed stimulation of cyclic guanosine 5' monophosphate (cGMP) phosphodiesterase). G_i proteins have an *i*nhibitory effect on adenylyl cyclase; thus G_s and G_i proteins can be regarded as positive and negative regulators of intracellular cAMP production (Fig. 1.4). $G_{q/11}$ proteins encompass several members that are able to activate phospholipase Cβ: α_q, α_{11}, α_{14}, α_{15}, and α_{16}. α_{15} and α_{16}, which represent the murine and human version of the same gene, have been shown to be the most promiscuous G proteins to date (Offermanns and Simon, 1995). The function of the members of the fourth class of G proteins, G_{12} and G_{13}, is not well understood at present.

Like the activated α-subunits, free βγ-complexes can also interact with certain adenylyl cyclase and phospholipase Cβ isoforms and other effector proteins. In many cases, α- and βγ-subunits can act individually but also synergistically, thus amplifying certain effects (Sunahara *et al.*, 1996). Since βγ can act on downstream effectors only when released from the α-subunit, Gα can be viewed as negative regulator of βγ function. This is especially apparent in the pheromone signaling pathway of *S. cerevisiae* where the βγ-subunit appears to be the sole transducer of GPCR-relayed signaling (Leberer *et al.*, 1997; Dowell *et al.*, 1998).

Recently, two heterotrimeric G proteins, $G\alpha_{i1}\beta_1\gamma_2$ (Wall *et al.*, 1995) and $G\alpha_t\beta_1\gamma_1$ (Lambright *et al.*, 1996) have been crystallized to yield high-resolution structural information and insight into how G proteins operate. The α-subunit is characterized by two domains, which shield and keep the guanine nucleotide in its proper position. One domain is involved in GTP binding and its subsequent hydrolysis. This so-called ras-like GTPase domain (due to its structural similarity to members of the GTPase superfamily (Kjeldgaard *et al.*, 1996)) consists of a central six-stranded β-sheet surrounded by six α-helices. The other domain is a helical domain that holds the GTP/GDP within its binding site (Noel *et al.*, 1993; Coleman *et al.*, 1994). The β-subunit has a "propeller"-like structure (Sondek *et al.*, 1996) made up of seven β-sheets (which resemble "blades") with

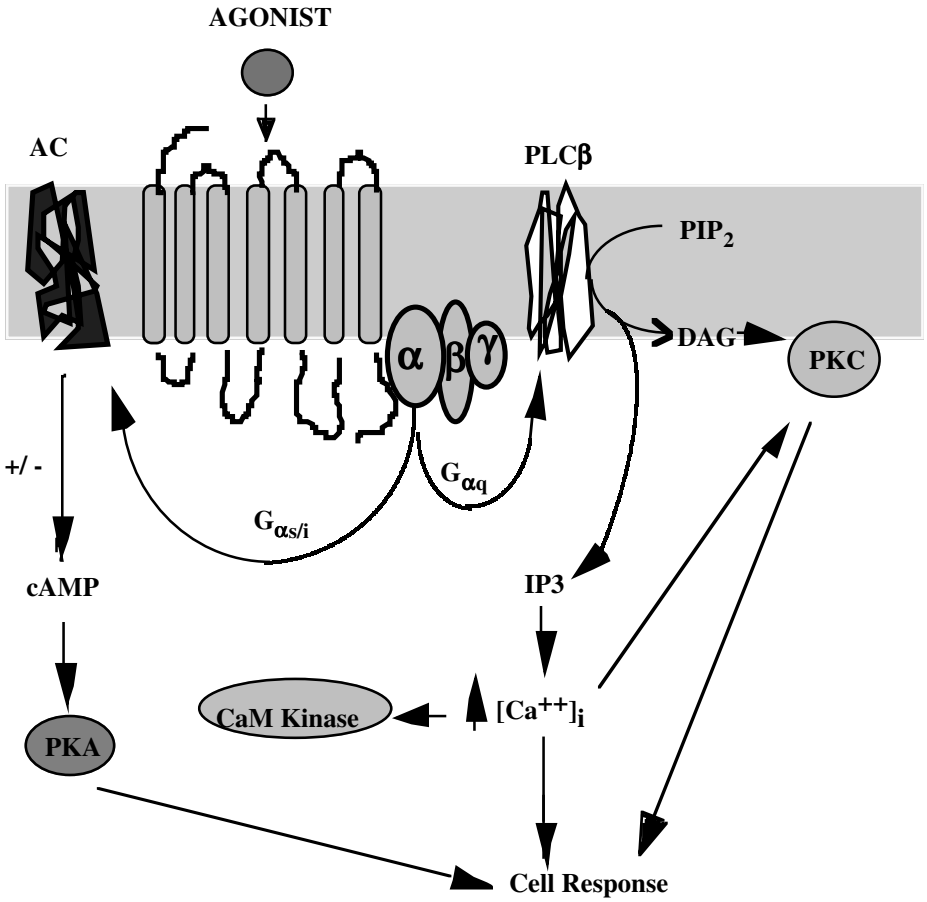


Fig. 1.4 Signaling pathways of GPCRs. Examples of major classical signaling events induced by agonist activation of a GPCR. Agonist binding results in the activation of heterotrimeric G proteins, which in turn regulate the activity of cellular effectors (*e.g.* AC: adenylyl cyclase, PLC_β: phospholipase Cβ). Other abbreviations: PKC: protein kinase C, PKA: protein kinase A, CaM: calmodulin, IP_3: inositol-1,4,5-trisphosphate, PIP_2: phosphatidylinositol-4,5-bisphosphate, DAG: diacylglycerol (see text for details).

contact points at opposite sides for the α- and γ-subunits (Bourne, 1997). The γ-subunit is relatively small, with two helical structures at either end, which both interact with the β-subunit. The N-terminal helical domain forms a "coiled-coil" with the N-terminus of Gβ, whereas the C-terminal helix interacts with one side of the β-subunit propeller. Free and bound βγ seem to have virtually the same three-dimensional structure (Wall *et al.*, 1995;

Lambright *et al.*, 1996; Sondek *et al.*, 1996)). Even though β- and γ-subunits are not covalently attached to each other, they can be regarded as one functional unit.

Gα and Gβ are linked to each other at various points; the α-helix at the N-terminus of Gα contacts the Gβ-"propeller" at blade 1, and the Gα region that shows major mobility changes upon G protein activation (switch I and II) interacts with the face of the β-propeller that is opposite to that where the γ-subunit is bound (Wall *et al.*, 1995; Lambright *et al.*, 1996). Both Gα and Gγ contain lipid modifications. The N-terminus of the α-subunit is both myristoylated and/or palmitoylated, whereas the γ-subunit is prenylated at its C-terminus. Both α- and γ-subunits are attached to the plasma membrane via their lipid modifications, placing the N-terminus of Gα in close proximity to the C-terminus of Gγ and suggesting that there is a single tether point for both subunits.

1.1.4 The receptor activation process

The binding of activating agents (agonists) to GPCRs causes conformational changes in the receptor protein which lead to the stimulation of heterotrimeric G proteins that are located intracellularly on the plasma membrane. Many biochemical studies as well as the high-resolution rhodopsin structure (Palczewski *et al.*, 2000) indicate that the transmembrane core of GPCRs is tightly packed, involving many tertiary interactions of hydrophilic and hydrophobic nature as well as salt bridges. These interactions are thought to keep the receptor protein in its inactive conformation. Mutant GPCRs with constitutive active phenotypes have provided valuable insights into the processes that are involved in receptor activation (Scheer and Cotecchia, 1997b). For instance, single point mutations at Glu113 or Lys296 in rhodopsin led to constitutively active mutants (Robinson *et al.*, 1992), suggesting that these two amino acids are involved in forming a salt bridge that keeps the receptor in its inactive conformation. Constitutively active receptors have also been identified in many other classes of GPCRs including the α_{1b}–adrenergic (Porter *et al.*, 1996), the M_5 muscarinic (Spalding *et al.*, 1995, 1997), the M_1 muscarinic (Hulme and Lu, 1998) and the luteinizing hormone (LH) (Shenker *et al.*, 1993).

Wonerow *et al.* (1998) recently described a 9-amino-acid deletion mutation at the C-terminal portion of the i3 loop of the human TSH receptor, which leads to a constitutively active phenotype. A similar phenotype was observed with the LH receptor (Schulz *et al.*, 1999). More detailed mutational analysis indicated that an anionic amino acid (Asp564) was necessary to keep the receptor in the inactive state, probably by forming a salt bridge with other residues in adjacent receptor domains. The authors proposed that disruption of this interaction, either ligand- or mutation-induced, allows the receptor to take on its active conformation (Wonerow *et al.*, 1998; Schulz *et al.*, 1999).

A large body of evidence suggests that the highly conserved E/D-R-Y/W motif (DRY-motif), an amino acid triplet present at the cytosolic end of TM III in most class I GPCRs, takes on an important functional role in receptor activation. According to the high-resolution density map of bovine rhodopsin (Palczewski *et al.*, 2000), the carboxylate of Glu134 and the highly basic guanidium of Arg135 form a salt bridge. This interaction is thought to prevent the arginine from interacting with the G protein in the resting state. Several studies demonstrated that charge-neutralizing mutations of the glutamate (Glu->Gln) or aspartate (Asp->Asn) residues lead to receptors that display pronounced constitutive activity (Robinson *et al.*, 1992; Arnis *et al.*, 1994; Kim *et al.*, 1997; Alewijnse *et al.*, 2000). Therefore, it has been proposed that the highly conserved glutamate or aspartate residues might function as proton acceptors and that neutralization of these residues represents a key event in initiating receptor activation (Robinson *et al.*, 1992; Arnis *et al.*, 1994). Consistent with these findings, various other mutational studies showed that replacement of the conserved glutamate/aspartate residues with hydrophobic or neutral amino acids led to mutant receptors with constitutive phenotypes (Cohen *et al.*, 1993; Acharya and Karnik, 1996; Scheer *et al.*, 1996, 1997a). Also, the first constitutively active V2 receptor harboring an aspartate to alanine mutation (Asp136Ala) has been described recently (Morin *et al.*, 1998). However, several rhodopsin-like GPCRs are known in which the negatively charged amino acid is replaced by histidine, asparagine, valine, glycine, or cysteine (Schöneberg *et al.*, 1999). Also, mutation of the analogous glutamate in the LH receptor (Glu441) to glutamine did not affect coupling efficacy significantly (Wang *et al.*, 1993). Similar results were obtained in studies on the

M_1 muscarinic (Lu *et al.*, 1997), α_{1B}-adrenergic (Scheer *et al.*, 1997), and gonadotropin-releasing hormone receptors (Arora *et al.*, 1997). Interestingly, many of the resulting mutant receptors were expressed only poorly (Wang *et al.*, 1993; Lu *et al.*, 1997; Scheer *et al.*, 1997a), suggesting that the conserved glutamate/aspartate also plays an important role in receptor folding and trafficking.

Mutational analysis has demonstrated that the Tyr/Trp residue contained in the DRY-motif is not essential for G protein coupling but is required for receptor folding (Zhu *et al.*, 1994; Lu *et al.*, 1997). For instance, replacing the conserved tyrosine in the M_1 muscarinic receptor yielded mutant receptors that were only poorly expressed in the plasma membrane (Lu *et al.*, 1997).

The arginine residue within the DRY-motif represents one of the most highly conserved amino acids among rhodopsin-type receptors. The high-resolution rhodopsin structure indicates that this arginine (Arg135) is involved in hydrogen bond-interactions with its neighboring Glu134 (see above) and with Glu247 and Thr251 in TM VI. The arginine residue is considered to be the central trigger of GDP release from the G protein α-subunit (Acharaya and Karnik, 1996). This concept is supported by several mutagenesis studies: replacement of the conserved arginine with different amino acids virtually abolished G protein coupling in rhodopsin (Franke *et al.*, 1992), the α_{1b}-adrenergic receptor (Scheer *et al.*, 1996), the muscarinic receptors (Zhu *et al.*, 1994; Jones *et al.*, 1995), and several other GPCRs (Wess, 1998**).** Interestingly, Jones *et al.* (1995) demonstrated that a charge-conserving mutation (Arg->Lys) in the M_1 muscarinic receptor only slightly impaired receptor function suggesting that the positive charge in the arginine side chain is critical for G protein activation. Similarly, a recent study conducted on the β_2-adrenoceptor reported that mutation of the conserved arginine to histidine resulted in a mutant receptor with unchanged coupling efficacy (Seibold *et al.*, 1998).

Several studies have examined the conformational changes that accompany the activation of a GPCR. An early study on rhodopsin showed that the cytoplasmic end of TM III moves outwards during receptor activation accompanied by structural changes in the i2 loop (Farahbakhsh *et al.*, 1993). Farrens *et al.* (1996) employed SDSL (site-

directed spin labeling) technology using mutant rhodopsin receptors that contained one labeled cysteine in TM III and a second one in TM VI. This analysis showed that receptor activation is accompanied by a clock-wise rotation (about 30°C as viewed from the cytoplasm) and an outward movement of the cytoplasmic end of TM VI relative to TM III. Also, disulfide bonds generated between the cytoplasmic ends of TM III and TM VI prevented activation of transducin, the G protein linked to rhodopsin (Farrens *et al.*, 1996). In another study, histidine mutations were inserted into the cytoplasmic ends of TMs III and VI, resulting in the inhibition of receptor activation in the presence of zinc ions (Sheikh *et al.*, 1996). Apparently, the histidines created a "binding pocket" in which metal ions (e.g. zinc) were bound to prevent movement of the two helices. These findings suggest that major mobility changes occur in TMs III and VI during receptor activation, which eventually may allow the G protein to access functionally important residues (e.g. at the i3 loop/TM junctions) which had previously been buried. However, the exact molecular mechanisms and conformational changes accompanying receptor activation still remain elusive.

1.1.5 The heterotrimeric G protein activation cycle

As described above, the binding of agonist to GPCRs causes conformational changes in the receptor core that lead to the activation of heterotrimeric G proteins. A schematic overview displaying the receptor/G protein activation cycle is given in Fig. 1.2.

The G proteins, consisting of α-, β-, and γ-subunits, are linked to the plasma membrane via lipid modifications of the N-terminal domain of Gα and the C-terminal helix of Gγ. According to the X-ray structures of two G protein heterotrimers, the N- and C-termini of the α-subunit and the C-terminus of the γ-subunit are located in close proximity facing the plasma membrane (Wall *et al.*, 1995; Lambright *et al.*, 1996). Residues that are likely to define the G protein surface that is directly involved in receptor/G protein interactions have been mapped to the α4/β6 loop, the β6 strand, the α5 helix, and the C-terminal tail of Gα (Wall *et al.*, 1995; Lambright *et al.*, 1996).

Despite the high-resolution crystallographic data, detailed structural information on the dynamics of the receptor/G protein activation cycle is still limited. In its resting state, the Gα-subunit contains bound GDP and assembles with high affinity with Gβγ to form a trimer that is able to interact with GPCRs. Since the GDP/GTP binding pocket is approximately 30 Å away from the receptor, it is unlikely that the receptor is directly involved in the GDP/GTP exchange (Bourne, 1997). Three so-called switch regions on Gα (switch I, switch II, and switch III) are known to undergo major conformational changes upon G protein activation but the exact process by which GPCRs trigger these changes is yet unkown. Upon GTP-binding, the Gα-subunit undergoes conformational changes primarily in the three switch regions (see above), which results in a reduced affinity for Gβγ and the subsequent dissociation of Gα from Gβγ (Lambright *et al.*, 1994, 1996). Also, both G protein subunits dissociate from the receptor allowing them to interact with downstream effectors (reviewed by Sprang, 1997, and Hamm, 1998). The release of the G protein, in turn, causes the receptor to return to the inactive state. GTP is hydrolyzed to GDP by the GTPase ability intrinsic to Gα. The rate with which bound GTP is hydrolyzed is increased by RGS (regulator of G protein signaling) proteins, which act allosterically on the Gα-subunit (Ross and Wilkie, 2000). Hydrolysis of bound GTP to GDP causes the α-subunit to adopt its inactive conformation. This results in an increased affinity of Gα for the βγ-subunit and the reassociation of the trimer, returning the system to its resting state (Conklin and Bourne, 1993; Clapham and Neer, 1997).

Mutational and biochemical studies with synthetic peptides have delineated residues on the G protein which seem to form a surface that interacts with the activated receptor (Garcia *et al.*, 1995; Osawa and Weiss, 1995; Onrust *et al.*, 1997; Kallal and Kurjan, 1997; also see reviews by Neer, 1995; Bourne, 1997). The C-terminal domain of Gα harbors many key residues, with the most distal C-terminal 10 amino acids playing a major role in the receptor/G protein activation process. In particular, two conserved leucine residues at positions –2 and –7 are essential for receptor/G protein binding and subsequent G protein activation (Garcia *et al.*, 1995; Osawa and Weiss, 1995; Martin *et al.*, 1996; Onrust *et al.*, 1997). More detailed functional analysis has identified residues on various α-subunits that determine receptor/G protein coupling selectivity (see chapter

1.4 for a detailed discussion). Another region of the Gα-subunit involved in receptor/G protein binding seems to be the helical N-terminal domain. Studies with synthetic peptides showed that a peptide corresponding to the N-terminal portion of transducin could block rhodopsin/$G\alpha_t$ interactions (Hamm *et al.*, 1988). Likewise, peptides derived from receptor regions known to be essential for G protein activation (e.g. the i3 loop of the α_{2A}-adrenoceptor (Taylor *et al.*, 1994) or mastoparan, a receptor-mimetic peptide toxin (Wakamatsu *et al.*, 1992; Higashijima and Ross, 1994), could be cross-linked to the N-terminus of $G\alpha_o$. These findings support the notion that the N-terminus of Gα-subunits is in direct contact with the receptor protein during the receptor/G protein coupling process.

Despite the availability of high-resolution X-ray crystallography of both a GPCR (Palczewski *et al.*, 2000) and G proteins (Wall *et al.*, 1995; Lambright *et al*., 1996), the dynamic structural changes involved in the receptor/G protein activation process and the molecular architecture of the receptor/G protein complex still remain poorly understood. Therefore, molecular models derived from mutational and biochemical analyses still represent an indispensable tool to gain more insight into the molecular mechanisms involved in receptor/G protein coupling.

1.2 Vasopressin receptors

1.2.1 The neurohypophyseal hormones vasopressin and oxytocin

The V2 vasopressin receptor represents a prototypical GPCR and belongs to one of the largest GPCR subclasses, the peptide receptor subfamily. The physiological ligand that exerts its function via binding and activation of mammalian vasopressin receptors is the neurohypophyseal hormone 8-arginine-vasopressin (AVP). AVP is synthesized as a precursor protein linked to its carrier protein neurophysin II in the vasopressinergic neurons of the supraoptic and paraventricular nuclei of the hypothalamus (Schmale *et al.*, 1993). It is then packaged into vesicles and during its transport to the axon terminals, the biologically active form, AVP, is enzymatically released from its precusor. The vesicles

are stored in the neurohypophysis (posterior pituitary) where AVP is readily secreted into the blood stream upon stimulation (Mohr, 1995). The neurohypophysis is also the locus of release of oxytocin (OT), a hormone that is very similar in structure to AVP. Both hormones are cyclic nonapeptides that form a disulfide bond via two cysteines at positions 1 and 6, resulting in a cyclic part comprising six amino acids and a linear extension of three amino acids. AVP and OT differ only in the amino acids at positions 3 and 8. In some species, including pig, vasopressin contains a lysine at position 8 and is therefore called 8-lysine-vasopressin (LVP). AVP and OT have predominantly hormonal character, but may also exert neurotransmitter and neuromodulator functions in the brain (Dantzer and Bluthe, 1992).

Despite their structural similarity, AVP and OT exert very different biological activities. The main endocrine functions of OT are well defined and are confined to functions that are essential for mammalian reproduction such as uterine contraction, milk-ejecting activities and stimulation of the synthesis and release of prolactin in the anterior pituitary. Also, OT plays a role in the control of adaptive, social and sexual behavior and has been shown to strengthen partnership bonding in mammals (prairie vole) (Insel *et al*., 1993).

In contrast, the physiological activities of AVP are much more diverse. Its alternative name, antidiuretic hormone, reveals the primary role of AVP in the regulation of renal water reabsorption (Handler *et al*., 1981). However, AVP also mediates the contraction of smooth muscles (Hofbauer *et al*.,1984), stimulation of glycogenolysis in the liver (Hems *et al.*, 1976), aggregation of platelets (Haslem *et al.*, 1972), the modulation of insulin secretion by pancreatic β-cells (Dunning *et al*., 1984), and stimulation of adrenocorticotropic hormone (ACTH) release from anterior pituitary corticotrophs (Jard *et al.*, 1988). AVP also acts as a neuromodulator in the brain and is involved in temperature regulation, control of blood pressure, and learning and memory processes (Dantzer *et al*., 1992, Kovacs *et al*., 1994).

On the basis of pharmacological and molecular criteria, at least four receptor subtypes for neurohypophysial peptides can be distinguished: V1a, V1b (also referred to

as V3), V2 vasopressin receptors, and oxytocin receptors. The various vasopressin receptor subtypes will be discussed in the following chapter.

1.2.2 Localization and physiological functions of vasopressin receptor subtypes

Three different vasopressin receptor subtypes (V1a, V1b, and V2) can be distinguished on the basis of both the affinities they display for various vasopressin and oxytocin analogues and the second messenger systems that they are linked to. Molecular cloning studies have shown that the three receptors represent distinct molecular entities.

The vasopressin-1 receptors (further differentiated into V1a (Thibonnier *et al.*, 1994) and V1b (Sugimoto *et al.*, 1994) receptor subtypes) are selectively linked to G proteins of the $G_{q/11}$ class. Activation of the V1-type receptors leads to the stimulation of phosphatitylinositol (PI) turnover via activation of distinct phopholipase Cβ isoforms (Berridge, 1987, Thibonnier *et al.*, 1994). In contrast, the V2 vasopressin receptor preferentially couples to G proteins of the G_s class, which then stimulate adenylyl cyclase, elevating intracellular cAMP concentrations (Orloff and Handler, 1967) (Fig. 1.4, page 13).

The V1a receptor is widely distributed and is responsible for AVP-dependent contraction of vascular smooth muscle, the aggregation of platelets, and increased glycogenolysis in hepatocytes. Ostrowski *et al.* (1992, 1993) demonstrated the presence of V1a receptor mRNA in the kidney on glomerular mesangial cells, within the renal medulla and cortex. V1a receptors are also found in the brain (Tribollet *et al.*, 1988) and spinal cord (Sermasi *et al.*, 1993) and may be involved in central processes such as memory and learning.

The expression of the V1b receptor appeared to be restricted to the adenohypophysis (anterior pituitary) where AVP stimulation leads to the secretion of ACTH (Jard *et al.*, 1986). Lolait *et al.* (1995), however, demonstrated the presence of V1b receptor mRNA in many peripheral tissues and numerous other areas of the brain.

V2 receptors seem to have a rather limited tissue distribution and are primarily expressed in the collecting duct system of the kidney. However, the use of specific V2

receptor agonists also suggests the presence of extrarenal V2 receptors. Interestingly, the administration of desmopressin (1-deamino-[8-D-arginine]-vasopressin = dDAVP) leads to vasodilatory actions in the rat aorta through stimulation of nitric oxide release (Liard, 1994, Tamaki *et al.*, 1996). Also, administration of dDAVP to healthy subjects leads to facial flush, the release of von Willebrand factor, coagulation factors (factor VIIIc), and tissue plasminogen activator (Bichet *et al.*, 1988). In contrast, these effects cannot be observed in patients who suffer from X-linked nephrogenic diabetes insipidus, where the V2 vasopressin receptor is non-functional, suggesting that V2 vasopressin receptors mediate the observed responses (Bichet *et al.*, 1988; Moses *et al.*, 1995; van Lieburg *et al.*, 1995).

1.2.3 The renal physiology of the V2 vasopressin receptor

As described above, one of the main endocrine functions of AVP is the facilitation of water reabsorption by the kidneys. In humans, the kidneys process 180 L of plasma (glomerular filtrate) per day in order to eliminate toxic metabolites and to maintain proper water and electrolyte homeostasis (reviewed by Pocock, 1999).

A healthy human being produces between 1.5 and 2 L of urine a day. The reabsorption of 80% of the primary filtrate takes places in the proximal tubule of the kidney nephrons, mostly through active re-uptake of ions, glucose, and amino acids, which passively carries an obligatory amount of water. Another 15% of the glomerular filtrate are reabsorbed in the distal tubules. The remaining 5%, corresponding to about 9 L of hypotonic fluid, reach the collecting duct and are passively taken up under the control of AVP. AVP is released into the blood stream in response to stimuli such as increases in plasma osmolarity (as small as 1%) or volume depletion by >10%. It exerts its physiological role through binding to and activation of V2 vasopressin receptors, which are almost exclusively expressed at the basolateral surface of the principal cells of the collecting duct. Within one minute of AVP release, insertion of specific water channels, aquaporins (AQP), into the luminal side of the collecting duct cells can be observed which facilitates the reabsorption of water (review by Birnbaumer, 1999).

Activation of renal V2 receptors leads to the stimulation of G_s, which in turn interacts with and activates adenylyl cyclase. The resulting increase in cAMP and subsequent cAMP-dependent phosphorylation of protein kinase A (PKA) allows the fusion of AQP2-containing vesicles with the luminal membrane of cortical collecting duct cells (Snyder *et al.*, 1992; Nielsen *et al.*, 1993; Nishimoto *et al.*, 1999). PKA and cAMP also seem to influence the production and release of AQP2 in a more long-term manner by transcriptonal upregulation of AQP2 gene expression via a cAMP- responsive structural element in the AQP2 promoter (Hozawa, 1996; Yasui, 1997). Apparently, V2 receptor activation through AVP modulates both short-term and long-term mechanisms that assure proper water homeostasis (diGiovanni, 1994).

Insertion of AQP2 into the apical membrane of the collecting duct cells markedly increases their water permeability. As a result, water is passively drawn into the collecting duct cells due to the existing osmotic gradient between the interstitium and the filtrate. AQP3 (Ecelbarger *et al.*, 1995) and AQP4 (Terris *et al.*, 1995), which are constitutively expressed at the basolateral surface of the collecting duct cells allow the water to exit and re-enter the blood stream. Fig. 1.5 illustrates this process in a simplified scheme.

1.2.4 Nephrogenic Diabetes Insipidus (NDI) and treatments

1.2.4.1 NDI: general implications and clinical picture

Nephrogenic diabetes insipidus (NDI) is a disease that is characterized by the inability of the kidney to concentrate urine. NDI patients excrete large volumes of dilute urine (~9 L daily) (polyuria) and suffer from excessive thirst (polydypsia). NDI can either be acquired or inherited (congenital NDI). Acquired NDI is mostly caused by drugs (demeclocycline or lithium as standard treatment of bipolar psychosis), metabolic disorders (hypokalemia, hypercalcemia), or renal diseases. Two different forms of congenital NDI are known: autosomal NDI and X-linked NDI, which accounts for 95% of the cases. Congenital NDI is a rare disease (estimated prevalence: 1 per 250,000

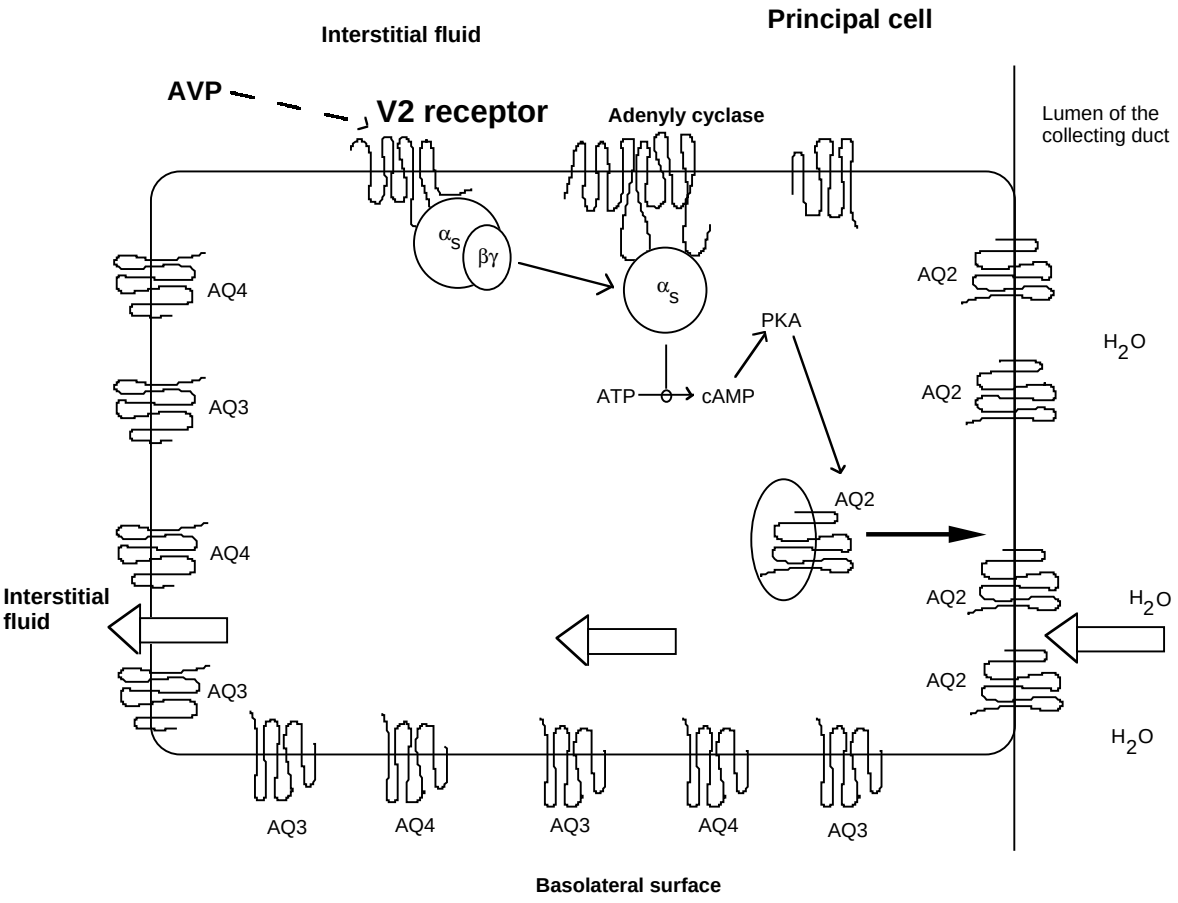


Fig. 1.5. Diagram of the principal cell of the kidney collecting duct. Aquaporins AQ3 and AQ4 are constitutively expressed and located on the basolateral surface of the principal cell. Stimulation of the V2 vasopressin receptor by AVP leads to activation of adenylyl cyclase and subsequent increases in intracellular cAMP concentration stimulate PKA, which in turn leads to the insertion of AQ2 into the apical surface of the principal cell (modified from Birnbaumer, 1999).

males) (Bichet *et al.*, 1992) but if not detected early in newborns, NDI may result in mental retardation or death due to severe hypernatremia (Knoers, 1992).

Autosomal NDI is caused by inactivating mutations in the AQP2 gene (Deen *et al.*, 1994). In contrast, as first shown by Rosenthal *et al.* (1992), X-linked nephrogenic diabetes insipidus (XNDI) is caused by inactivating V2 vasopressin receptor mutations.

To date, more than 150 different mutations have been identified (Yun *et al.*, 2000). The V2 receptor gene is located at the very end of the long arm (q28-qter) of the X chromosome (Seibold *et al.*, 1992), which seems to make it particularly susceptible for de novo mutations. Also, its location on the X chromosome accounts for the fact that XNDI mostly affects males.

Analysis of the different V2 receptor mutations, which encompass missense, nonsense, deletion, insertion, and frameshift mutations, revealed that genetic alterations occur throughout the coding region with no preference for so-called hot spots, even thought evidence exists that mutations occur preferably at CpG dinucleotides (Bichet 1998, 1999). Functional analysis of NDI-causing V2 mutant receptors has shown that the inactivating mutations can interfere with proper receptor function at various different steps. These include incorrect receptor folding (Sadeghi *et al.*, 1997c), defective transport (Tsukaguchi *et al.*, 1995), reduced cell surface expression (Birnbaumer *et al.*, 1994; Sadeghi *et al.*, 1997a), reduced or abolished binding of AVP (Pan *et al.*, 1994), or loss of the ability to stimulate G_s (Rosenthal *et al.*, 1993; Birnbaumer *et al.*, 1994; Sadeghi *et al.*, 1997a). 90% of inactivating receptor mutations result in mutant V2 receptors that are retained within the endoplasmatic reticulum (Yun *et al.*, 2000). Analysis of NDI-causing mutant V2 receptors has provided valuable information about the synthesis, processing and regulation of the receptor.

Another form of diabetes insipidus is caused by a defect in the synthesis and/or processing of AVP and is therefore called central diabetes insipidus (CDI). Patients suffering from CDI display the same clinical phenotype as NDI patients but can be treated with the AVP analogue desmopressin (dDAVP), which is administered daily via nasal applications (Singer, 1997).

1.2.4.2 Treatment of NDI

The most straightforward treatment of NDI is the administration of large amounts of water to compensate for the increased loss of urine, combined with a diet that is low in sodium. Pharmacological therapies encompass the use of thiazide diuretics, which block

the active uptake of NaCl in the distal tubule. This loss of sodium leads to an increased passive uptake of NaCl in the proximal tubule, which is accompanied by an obligatory volume of water (Schnermann, 1998). The volume of urine can thus be reduced up to 50%. Often thiazides are administered in combination with amiloride, a potassium-sparing diuretic, to prevent hypokalemia, which is a typical side effect of thiazides (Alon *et al.*, 1985). Another class of drugs that are being used in the treatment of NDI and drug-induced polyuria are cyclo-oxygenase inhibitors such as indomethacin. Prostaglandin (PG) E2 abates the formation of cAMP in collecting duct cells. Thus, inhibition of PGE2 production leads to more pronounced increases in cAMP following activation of the AVP/V2 receptor signaling cascade.

Future therapies of XNDI may include gene therapy strategies. In COS7-cells, co-expression of NDI-causing mutant V2 receptors with peptides comprising V2 domains that complement truncated or mutated V2 receptors yielded functional receptor units that were able to stimulate adenylyl cyclase in the presence of AVP (Schöneberg *et al.*, 1996). It remains to be seen whether this approach is also feasible in vivo.

1.3 Structural features of vasopressin receptors

The three vasopressin receptor subtypes have been cloned from many species, with the earliest being the rat and human the V1a (Morel *et al.*, 1992; Thibonnier *et al.*, 1994), the rat and human V1b (Lolait *et al.*, 1995; Sugimoto *et al.*, 1994; de Keyzer *et al.*, 1994), and the rat and human V2 receptors (Lolait *et al.*, 1992; Birnbaumer *et al.*, 1992). The genes encoding the various subtypes are interrupted by either one (V1a and V1b) or two introns (V2), with one intron being located at the same locus in all three members, interrupting the coding sequence immediately after the region encoding TM VI. The V2 receptor has a second intron situated in the region coding for the N-terminal domain. The resulting receptor proteins vary in length, with 371 amino acids for the human V2 (Birnbaumer *et al.*, 1992), 418 amino acids for the human V1a (Thibonnier *et al.*, 1994), and 424 for the human V1b receptors (Sugimoto *et al.*, 1994; de Keyzer *et al.*, 1994).

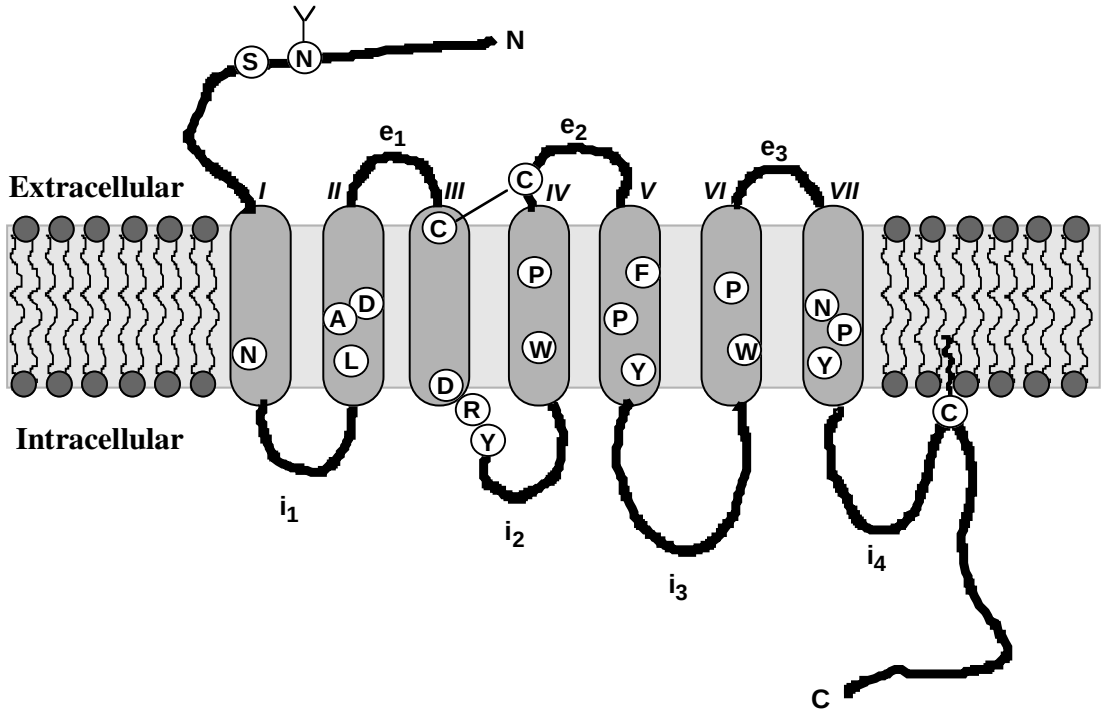


Fig. 1.6 Schematic two-dimensional structural representation of a class I GPCR. The characteristic seven transmembrane domains (*I-VII*) are joined by three extracellular (e1-e3) and three intracellular loops (i1-i3). The N-terminus is extracellular while the C-terminus is intracellular. Palmitoylation of one or more cysteine residues located within the carboxyl terminus leads to the formation of a fourth intracellular loop (i_4). Encircled amino acid residues are conserved in most rhodopsin-like GPCRs and are important for receptor folding and function. The two invariant cysteine residues at the e1 loop/TM III junction and the e2 loop are linked via a disulfide bond (solid line), an interaction that is crucial for receptor function and surface localization.

The three vasopressin receptor subtypes display the typical structural hallmarks characteristic of most rhodopsin-like GPCRs (see Fig. 1.6, also see Fig. 1.7): consensus sites for N-glycosylation (N-X-S/T) in the extracellular domain, an asparagine in TM I, a leucine, alanine, and aspartate (L-X_2-A-D) in TM II, and the DRY-motif at the junction of TM III/i2 loop. Additional conserved residues encompass two cysteines at the e1 loop/TM III junction and in the e2 loop forming a disulfide bond, one or more cysteine

residues in the C-terminal tail, and additional conserved motifs in TM IV (W-$X_{8/9}$-P), TM V (F-X_2-P-X_7-Y), and TM VI (W-X-P).

The conserved arginine residue within the DRY-motif in TM III has been shown to be crucial for G protein coupling for many GPCRs (Moro *et al.*, 1993; Scheer *et al.*, 1996; Van Rhee and Jacobson, 1996). Interestingly, it has been demonstrated that the NDI-causing Arg137His mutation in the V2 receptor (which is part of the DRY-motif) does not completely abolish G protein activation as previously assumed (Rosenthal *et al.*, 1993) but leads to ligand-independent receptor phosphorylation and constitutive arrestin-mediated desensitization (Barak *et al.*, 2001). The glutamate preceding the highly conserved arginine is thought to play a role in constraining the adjacent arginine side chain, thus preventing the arginine from interacting with the G protein in the resting state (Arnis *et al.*, 1994; Palczewski *et al.*, 2000). Consistent with this concept, a constitutively active V2 receptor has been described by Morin *et al.* (1998) which harbors an Asp136Ala mutation. The highly conserved N-P-X_2-Y motif in TM VII appears to be involved in receptor activation, sequestration and signaling (Barak *et al.*, 1994; Mitchell *et al.*, 1998). These highly conserved residues and motifs seem to be critical for proper receptor folding and function of rhodopsin-like GPCRs (Wess, 1998; Palczewski *et al.*, 2000). Amino acids that are conserved only in specific GPCR subfamilies account for structural features that are unique to only this subclass of receptors, such as ligand binding and signaling (Attwood *et al.*, 1991). Residues forming the ligand binding domain as well as the structural features regulating vasopressin receptor expression and function will be discussed in the following chapter.

1.3.1 Ligand binding domains

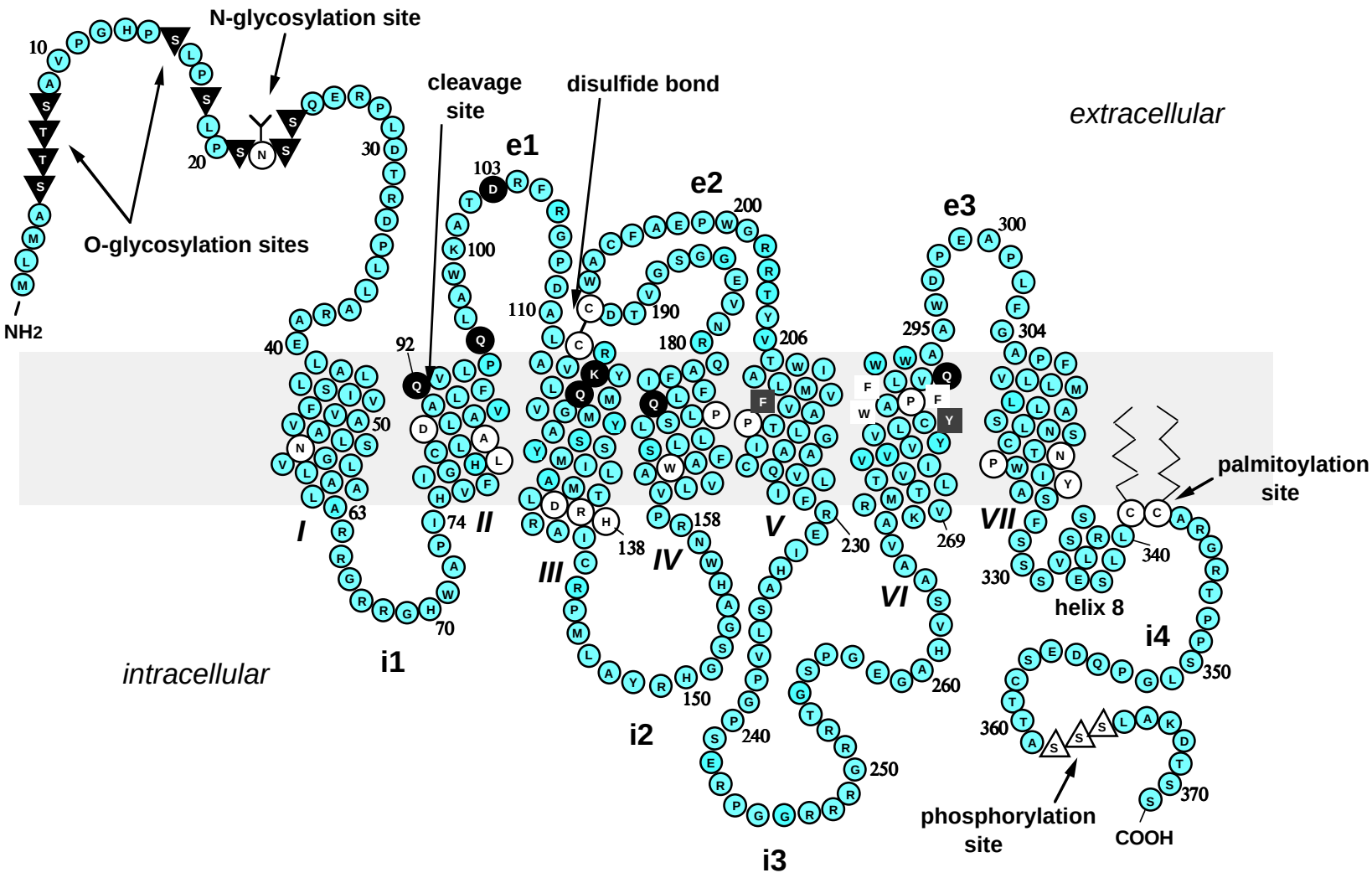
1.3.1.1 Agonist binding domain

The ligand binding domains of rhodopsin (Henderson *et al.*, 1990), adrenoceptors (Dohlman *et al.*, 1991) and muscarinic receptors (Wess, 1996) were the first to be studied extensively. Recently, a detailed three-dimensional model of the rat V1a receptor/AVP

complex was developed and validated by site-directed mutagenesis studies (Mouillac *et al.*, 1995) (Figs. 1.7, 1.8). In analogy to the agonist binding domain of biogenic amine neurotransmitter receptors (Strader *et al.*, 1987), the hydrophobic pocket forming the AVP binding site is deeply buried in the TM bundle at 15-20 Å from the extracellular surface, involving several TMs (mainly TM II to VII). AVP binding seems to involve an intricate network of hydrogen bond interactions between many different receptor residues and the peptide ligand. Interestingly, the residues thought to be involved in AVP binding are highly conserved among all vasopressin receptor subtypes, suggesting that there is a common agonist-binding site for the different members of the AVP/OT receptor family. Indeed, molecular modeling of the V2 (Czaplewski *et al.*, 1998) and the vasotocin (Hausmann *et al*, 1996) receptors and identification of their hormone binding domain confirmed that the key residues involved in hormone binding correspond to the ones already identified in the V1a receptor (Fig. 1.7). Specifically, a series of conserved Gln residues in TM II, III, IV, and VI, and a Lys in TM III have been demonstrated to be intimately involved in agonist binding by the V1a receptor (Mouillac *et al.*, 1995) (Figs. 1.7, 1.8). Interestingly, mutation of these residues affected the affinity of structurally diverse agonists in a very similar fashion, whereas antagonist binding affinities remained unaltered (Barberis *et al.*, 1998).

In addition, two aromatic residues, Phe225 (TM V) and Tyr300 (TM VI), at the bottom of the binding pocket, have been reported to modulate agonist efficacy at the V1a receptor (Chini *et al.*, 1996). Molecular modeling studies suggest that residues 2 and 3 of AVP interact with these aromatic amino acids (Hibert *et al.*, 1999) (Fig. 1.7). Indeed, mutations of the analogous residues in the oxytocin receptor, at which AVP acts only as a partial agonist, to either phenylalanine or tyrosine restored full AVP activity of the vasopressin hormone (Hibert *et al.*, 1999).

Furthermore, studies with synthetic peptides (Howl and Wheatley, 1996; Mendre *et al.*, 1997), photoaffinity labeling experiments (Kojro *et al.*, 1993), hybrid receptor approaches (Postina *et al.*, 1998), and mutagenesis studies have shown that the extracellular domains of the vasopressin receptors also contribute to AVP binding. For instance, synthetic peptides corresponding to the e1 loop (Howl and Wheatley, 1996) and


N-glycosylation site
O-glycosylation sites
cleavage site
disulfide bond
extracellular
intracellular
NH2
e1
e2
e3
i1
i2
i3
i4
I
II
III
IV
V
VI
VII
helix 8
palmitoylation site
phosphorylation site
COOH

the e2 loop of the V1a receptor (Mendre *et al.*, 1997) inhibited [^{3}H]AVP binding to the V1a receptor. In photoaffinity labeling studies, Kojro *et al.* (1993) identified the e1 loop of the V2 receptor to be important for agonist-binding. Interestingly, Hawtin *et al.* (2000) recently reported that an 11 amino acid subdomain of the N-terminus of the V1a receptor was required for high affinity agonist binding but not for antagonist binding. Substitution of this subdomain by homologous oxytocin receptor sequence recovered high affinity agonist binding, indicating that this receptor domain is required for agonist binding per se but does not influence subtype specific agonist recognition (Hawtin *et al.*, 2000). Site-directed mutagenesis has identified a series of single residues that directly modulate agonist binding selectivity. For instance, substitution of threonine in position 204 (e2 loop) in the V2 receptor with asparagine affects agonist binding selectively, resulting in AVP being 50 fold more potent than the selective V2 agonist dDAVP (the ratio for the wild type (wt) receptor is 2.3) (Postina *et al.*, 2000). Also, replacement of Tyr115 present in the e1 loop of the V1a receptor with aspartate (the analogous residue in the V2 receptor) or phenylalanine (the corresponding amino acid in the oxytocin receptor) leads to a pronounced increase in dDAVP or oxytocin agonist-binding affinities (Chini *et al.*, 1995). Likewise, site-directed mutagenesis of bovine and porcine V2

Fig. 1.8 Transmembrane topology of the V2 vasopressin receptor showing functionally important residues. Encircled amino acid residues on white background are conserved among most rhodopsin-like receptors (also see Fig. 1.6): *N-terminus*: Asn22, *TM I*: Asn55, *TM II*: Leu81, Ala 84 Asp85, *TM III*: Cys112, Asp136, Arg137, His178, *TM IV*: Trp164, Pro173, *e2*: Cys192, *TM V*: Phe214, Pro217, *TM VI*: Trp284, Pro286, *TM VII*: Asn321, Pro322, Tyr325, *C-terminal tail*: Cys341, Cys342. Amino acids highlighted in black circles correspond to the V1a receptor residues that have been shown to be critically involved in agonist binding (Mouillac *et al.*, 1995); those include: *TM II*: Gln92, Gln96, *e1*: Asp103, TM *III*: Lys116, Gln119, *TM IV*: Gln174, *TM VI*: Gln291. Asp103 is responsible for high affinity V2 selective agonist (dDAVP) binding (Ufer *et al.*, 1995). Amino acids in grey squares correspond to V1a receptor residues that interact with residue 2 and 3 of AVP (Chini *et al.*, 1996; Hibert *et al.*, 1999), those in white squares correspond to the V1a receptor residues that are involved in vasopressin antagonist binding (Phalipou *et al.*, 1999; Cotte *et al.*, 2000). Ligand-induced proteolytic cleavage occurs within the FQVLPQL-consensus sequence (TM II) between Gln92 and Val93 (Kojro *et al.*, 1999). Amino acids in black triangles indicate potential O-glycosylation sites (Sadeghi *et al.*, 1997), those in white triangles represent phosphorylation sites (Innamorati *et al.*, 1998a; Oakley *et al.*, 1999). The N-glycosylation site (Asn22), the putative disulfide bond formed by cysteines at the e1/TM III junction (Cys112) and e2 loop (Cys192), and the palmitoylation sites in the C-terminal tail (Cys341, Cys342) are indicated. The single letter amino acid code is used, amino acids and numbers refer to positions in the human V2 vasopressin receptor (Birnbaumer *et al.*, 1992).

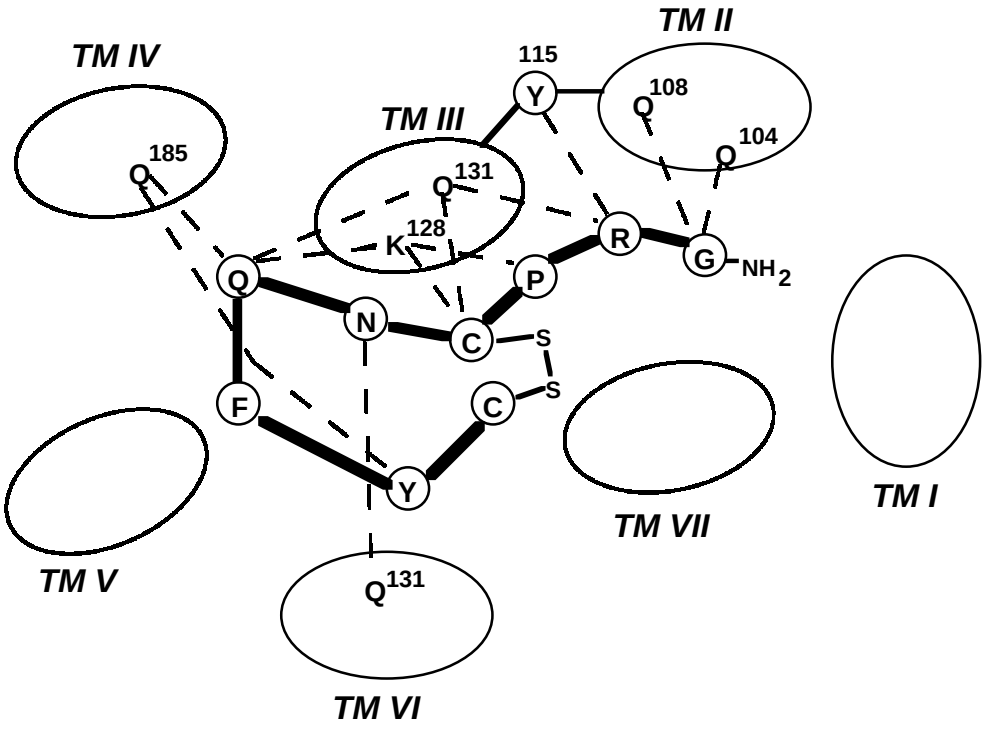


Fig. 1.8 Schematic representation of the interactions of vasopressin with the membrane spanning domains of the vasopressin V1a receptor. Helical wheel model of the V1a receptor, as viewed from the surface of the plasma membrane. Amino acids predicted to be involved in the interaction between AVP and the V1a receptor are indicated (Mouillac *et al.*, 1995) (see text for details). Modified from Barberis *et al.* (1998).

receptors identified Asp103 in the e1 loop of V2 receptors to be responsible for high affinity binding of dDAVP (Ufer *et al.*, 1995) (Fig. 1.7). Furthermore, mutagenesis studies suggested that Tyr115 of the V1a receptor is in direct contact with the side chain of Arg-8 in AVP (Chini *et al.*, 1995). Recently, a series of residues (Arg202, Gly304, Leu100) has been reported to be responsible for the differences in agonist binding

properties between the rat and human V2 receptors (Cotte *et al.*, 1998). Interestingly, these amino acids do not contribute to the binding of non-selective ligands (Cotte *et al.*, 1998). Moreoever, two of these residues (Arg202 and Gly304) were shown to fully account for the species selectivity of certain peptide antagonists.

Natural occurring mutations in the V2 receptor also uncovered single point mutations that affect agonist binding, such as an Arg113Trp mutation. Arg113 is located right next to the conserved cysteine at the e1 loop/TM III junction and mutation to tryptophane results in a mutant vasopressin receptor with reduced agonist-binding affinity (Bichet *et al.*, 1993; Birnbaumer *et al.*, 1994). Likewise, deletion of Arg202 located in the e2 loop critically affects high affinity AVP binding (Ala *et al.*, 1998).

1.3.1.2 Antagonist binding domain

Molecular modeling and mutagenesis studies have provided considerable insight into the structural features forming the agonist binding pocket of the neurohypophyseal hormone receptors (the AVP/OT receptor family), yet the structural determinants defining the antagonist binding domains remain rather elusive.

Mutagenesis studies initially showed that antagonist binding remained largely unaffected by mutations that altered agonist binding affinities (Mouillac *et al.*, 1995). However, a recent study combining photoaffinity labeling, molecular modeling and site-directed mutagenesis suggested that the agonist- and antagonist-binding pocket might overlap (Phalipou *et al.*, 1999). The development of iodinated photosensitive antagonist ligands with high affinity for the V1a receptor subtype (Carnazzi *et al.*, 1994, 1997) allowed the covalent labeling of specific receptor subdomains. Employing two different linear peptide V1a receptor antagonists that were labeled at different positions allowed the identification of a restricted region spanning the e1 loop (Phalipou *et al.*, 1999) and domain spanning TM VII and the membrane-proximal portion of the C-terminal tail (Ni4) (Phalipou *et al.*, 1997). Mutagenesis studies also identified a number of aromatic residues in TM VI of the V1a receptor (Trp304, Phe307, Phe308) that when mutated led

to a reduction in antagonist binding affinities (Phalipou *et al.*, 1997; Cotte *et al.*, 2000) (Fig. 1.7).

Molecular modeling and site-directed mutagenesis studies of the V1a receptor also identified a series of amino acids that are also involved in high affinity agonist binding, such as Leu128 (TM III) and Gln185 (TM IV) (Phalipou *et al.*, 1999; Cotte *et al.*, 2000). Moreover, Tyr115 in the e1 loop that plays a key role in modulating agonist selectivity also appears to be involved in antagonist binding (Phalipou *et al.*, 1999). Interestingly, modeling studies suggest that the linear peptide antagonists, which differ from AVP in five out of nine amino acids, adopt a pseudocyclic conformation similar to that of the cyclic AVP (Phalipou *et al.*, 1999). These antagonists could enter the transmembrane core and occupy a similar binding pocket, establishing their own network of interactions (Phalipou *et al.*, 1999).

Taken together, these data suggest that the vasopressin antagonist binding site significantly overlaps with that of AVP. The concept that agonists and antagonists occupy similar binding pockets has also been proposed for other peptide receptors, such as the cholecystokinin (CCK)-A receptor (Dong *et al.*, 1999).

1.3.2 Regulation of receptor expression and function

1.3.2.1 Receptor glycosylation

A large body of evidence suggests that the N-terminal domain of most GPCRs is glycosylated. The N-terminus of the V2 receptor harbors one site for N-linked glycosylation (Asn22)(consensus sequence: Asn-X-Ser/Thr) (Innamorati *et al.*, 1996) and several sites for O-linked glycosylation (Sadeghi *et al.*, 1999)(Fig. 1.7). The mature V2 receptor protein has an apparent molecular mass of around 45 kDa as determined by Western blot analysis (Zhu and Wess, 1998). To study the importance of N-linked glycosylation, Innamorati *et al.* (1996a) eliminated the glycosylation site by replacing Asn22 with Gln, and investigated the functional properties of the resulting V2(Asn22Glu) receptor. Interestingly, the ligand binding affinities (K_D), receptor expression levels

(B_{max}), and G protein coupling properties (E_{max} and EC_{50}) of the non-glycosylated receptor were virtually identical to those of the wt V2 receptor (Innamorati *et al.*, 1996a). The N-terminal domain also contains several serines and threonines that could serve as potential sites for O-glycosylation (Fig. 1.7). A site-directed mutagenesis study confirmed that the V2 receptor is indeed subjected to O-glycosylation (Sadeghi *et al.*, 1999). Removal of all serines and threonines in the N-terminal domain yielded a mutant V2 receptor that was devoid of O-glycosylation. Interestingly, the lack of O-glycosylation had no effect on V2 receptor function (Sadeghi *et al.*, 1999).

The V1a receptor contains four potential N-glycosylation sites based on the Asn-X-Ser/Thr consensus sequence: two in the N-terminal region, one in the e2 and one in the e3 loop. Hawtin *et al.* (1997) demonstrated that three out of these four potential sites indeed undergo glycosylation (the e3 loop is not glycosylated). Removal of the glycosylation site in the e2 loop by a mutation of the asparagine to glutamine did not alter ligand affinity, expression levels or functional coupling of the resulting mutant receptor, as compared to the wt protein (Hawtin *et al.*, 1997). The role of N-terminal glycosylation in V1a receptor function has not been investigated yet.

It has been speculated that GPCR glycosylation may increase protein stability and prolong the life time of cell surface receptors. However, the potential role of V1a and V2 receptor glycosylation remains elusive at present.

1.3.2.2 Disulfide-linkage of extracellular domains and receptor palmitoylation

Class I GPCRs contain highly conserved cysteine residues at the e1 loop/TM III junction, within the e2 loop and within the C-terminal tail. Affinity labeling and mutagenesis studies (Curtis *et al.*, 1989; Cook and Eidne, 1997; Ehrlich *et al.*, 1998; Hoffmann *et al.*, 1999; Scholl and Wells, 2000; Elling *et al.*, 2000) have suggested that the two cysteines at the e1 loop/TM III junction and e2 loop are engaged in a disulfide bridge, which has now been confirmed by the X-ray analysis of bovine rhodopsin (Palczewski *et al.*, 2000). Likewise, all members of the vasopressin receptor family harbor the two conserved cysteines, which are predicted to form a disulfide bond

(Gopalakrishnan *et al.*, 1988; Schülein *et al.*, 2000; Schulz *et al.*, 2000) (Fig. 1.7). Interestingly, some GPCRs appear to contain an additional disulfide bond. For instance, the β_2-adrenergic receptor seems to have a second disulfide bond between two cysteines in the e2 loop next to the highly conserved one between TM III and the e2 loop (Noda *et al.*, 1994). Also, mutagenesis studies predict the presence of a second disulfide bond formed between two cysteines at the N-terminal domain and the e3 loop in the P2Y1 receptor (Hoffmann *et al.*, 1999).

The conserved disulfide bond is predicted to be important for both protein processing/folding and trafficking to the plasma membrane as well as for ligand binding and functional activity. For instance, the lack of the disulfide bond in the GnRH receptor does not impair correct insertion into the plasma membrane but abolishes ligand binding and second messenger production (Cook and Eidne, 1997). Davidson *et al.* (1994) demonstrated a pivotal role of the disulfide bond in formation of the light-activated metarhodopsin II state. Two recent studies examined the functional role of the conserved disulfide linkage in the V2 vasopressin receptor (Schülein *et al.*, 2000; Schulz *et al.*, 2000). Exchanging either one of the two cysteines against alanine or serine strongly impaired receptor transport to the cell surface. Both Elisa studies (Schulz *et al.*, 2000) and [^{3}H]AVP (Schülein *et al.*, 2000) binding studies demonstrated drastically reduced numbers of cell surface receptors, even though total cellular expression levels remained unaffected. Loss of the disulfide bridge did not abolish the ability of the mutant V2 receptors to activate G_s, albeit the resulting AVP EC_{50} values were significantly shifted to higher concentrations.

The role of the two conserved cysteines in the C-terminal tail of the V2 receptor has been studied in detail (Sadeghi *et al.*, 1997b; Schülein *et al.*, 1996). Both cysteines are thought to act as acceptor sites for fatty acid modification and to be linked to the plasma membrane via palmitoylation (Fig. 1.7). To study the importance of these two residues, single point mutations (Cys341Ser and Cys342Ser) as well as a double mutation were introduced into the wt V2 receptor and the three resulting mutant receptors were characterized in functional assays (Schülein *et al.*, 1996). Interestingly, the single point mutations failed to produce alterations in receptor expression levels, receptor affinity for

AVP, and receptor function. The double mutant displayed a reduction in protein expression levels, but showed wt-like ligand binding and coupling properties. These observations were confirmed by Sadeghi *et al.* (1997b) who also demonstrated that both C-terminal cysteines can be palmitoylated independently of each other and that one palmitoylation site is sufficient to result in normal levels of receptor expression. Elimination of both cysteines led to a reduction of AVP binding sites on the cell surface by about 50%, suggesting that palmitoylation promotes retention of the properly targeted receptors in the plasma membrane (Sadeghi *et al.*, 1997b).

1.3.2.3 Receptor phosphorylation and desensitization

GPCR-mediated responses are subject to regulatory mechanisms in order to prevent excessive stimulation in the presence of ligand. Physiologically, this phenomenon can be seen in a gradual attenuation of the intensity of receptor signaling despite the continuous presence of agonist. This process of cellular desensitization involves several molecular mechanisms. Depending on the amount of time a GPCR is exposed to agonist, the effect of desensitization can be divided into a short-term component (receptor internalization/sequestration, which is the disappearance of receptors from the cell surface without a change in total receptor number) and a long-term (receptor down-regulation) one (Hardman *et al.*, 1996; Böhm *et al.*, 1997).

The first step in receptor desensitization is the uncoupling of the receptor from its G protein, which occurs within seconds to minutes after receptor activation due to agonist stimulation. Phosphorylation by various protein kinases has been shown to be important in causing the uncoupling response (see below). The ligand-occupied and phosphorylated receptor is removed from the plasma membrane and internalized into endocytic vesicles that provide the correct pH to allow removal of the ligand and dephosphorylation so that the receptor eventually can be recycled to the plasma membrane (Pitcher *et al.*, 1998; Krupnick and Benovic, 1998). Many GPCRs undergo internalization in an arrestin-mediated fashion (Barak *et al.*, 1997; Zhang *et al.*, 1999; Barak *et al.*, 1999; Oakley *et al.*,

2000), but arrestin-independent pathways also play a role in receptor desensitization (Lee *et al.*, 1998).

When GPCRs are exposed to agonist for a prolonged time (minutes to hours), a reduction in total receptor number can be observed. This phenomenon is called down-regulation and involves the degradation of receptors (Klein *et al.*, 1979; Doss *et al.*, 1981). Moreover, it was shown for the M_1 muscarinic receptor that prolonged agonist exposure leads to a decreased receptor mRNA production (Lee *et al.*, 1994). Whether receptor internalization is a prerequisite for receptor down-regulation is still a subject of controversy.

As already indicated above, GPCR phosphorylation represents a major mechanism in the receptor desensitization response. Most GPCRs have been shown to contain sites that are recognized by both second messenger dependent kinases (for instance, protein kinase A (PKA) or protein kinase C (PKC)) and specific serine/threonine kinases (GPCR kinases: GRK1-GRK6) (Pitcher et al., 1998; Krupnick and Benovic, 1998). PKA and PKC seem to phosphorylate GPCRs in an agonist independent manner and are thought to be involved in heterologous receptor desensitization, whereas GRKs strictly phosphorylate the activated receptor protein (Pitcher et al., 1998; Krupnick and Benovic, 1998). Generally, phosphorylation occurs in the i3 loop and C-terminal tail, which are rich in serines and threonines (Watson and Arkinstall, 1994; Pitcher et al., 1998; Krupnick and Benovic, 1998).

The V1a receptor undergoes homologous as well as heterologous desensitization (Innamorati *et al.*, 1998b; Ancellin *et al.*, 1999) and harbors various PKC consensus sites for phosphorylation (Morel *et al.*, 1992). Upon ligand stimulation, the V1a receptor is phosphorylated and internalized very rapidly. Following removal of the agonist, it is then quickly recycled to the cell surface (Innamorati *et al.*, 1998b). In contrast, the V2 receptor undergoes solely homologous desensitization catalyzed by GRKs, despite the presence of a putative PKC phosphoylation site in the i3 loop (Kennelly *et al.*, 1991). The PKC site appears not to be accessible because stimulation of PKC either indirectly by $G_{q/11}$-coupled receptors or directly by phorbol esters did not lead to phosphorylation of the V2 receptor (Innamorati *et al.*, 1997). Studies conducted to elucidate mechanisms involving

homologous desensitization of the V2 receptor have revealed clusters of serines and threonines at the C-terminus as acceptor sites for phosphorylation (Innamorati *et al.*, 1997, 1998a) (Fig. 1.7). Removal of the last 14 (Innamorati *et al.*, 1997) or 10 (Oakley *et al.*, 1999) amino acids of the V2 receptor was sufficient to abolish ligand-induced phosphorylation without affecting receptor expression or coupling. In particular, a cluster of three serines (Ser362, Ser363, Ser364) present at the C-terminus of the V2 receptor could be identified as acceptor sites for phosphorylation (Fig. 1.7) (Innamorati *et al.*, 1998a, Oakley *et al.*, 1999). In contrast to the V1a receptor, the wt V2 receptor does not recycle to the plasma membrane for hours but is retained in endocytic vesicles despite removal of the agonist (Innamorati *et al.*, 1997). Interestingly, a cluster of serines at the C-terminus seems to play a key role in preventing the V2 receptor from recycling to the cell surface (Fig. 1.7). Substitution of individual or multiple serines by alanines prevented phosphorylation and allowed recycling of the full-length V2 receptor to the plasma membrane (Innamorati *et al.*, 1998a). It has also been shown that mutant V2 receptors, which could no longer be phosphorylated, failed to associate with arrestins (Oakley *et al.*, 1999). Moreover, the phosphorylation-defective mutant receptors were able to undergo ligand-induced internalization, albeit at much slower rate than the wt V2 receptor (Innamorati *et al.*, 1996a).

Interestingly, Kojro *et al.* (1995) reported evidence for a metalloproteinase-catalyzed proteolytic cleavage of the V2 receptor upon agonist stimulation, which could represent an additional mechanism to regulate receptor function. The structural requirements for this phenomenon were investigated further recently (Kojro *et al.*, 1999). The vasopressin and oxytocin receptors contain the FQVLPQL-consensus sequence at the junction of TM II/e1 loop (Fig. 1.8). However, only members of the vasopressin family are subject to ligand-induced proteolytic cleavage, whereas the oxytocin receptor is not sensitive (Kojro *et al.*, 1999). This observation prompted Kojro *et al.* (1999) to create hybrid V2/oxytocin receptors and to study the agonist-dependent cleavage of the various chimeric receptor constructs in photoaffinity labeling experiments. This analysis revealed that structural elements in the N-terminal domain and the e1 loop of the V2 receptor play a key role in defining the conformation that is necessary to allow access of the

metalloproteinase to the cleavage site. Moreover, Asp103 in the e1 loop, which had formerly been identified as a site that critically influences agonist specificity (Ufer *et al.*, 1995) (Fig. 1.7), is of pivotal importance in regulating proteolytic cleavage of the V2 receptor. It seems likely that full agonist activity is needed to induce exposure of the cleavage site (Kojro *et al.*, 1999). Interestingly, the two receptor fragments that result from agonist-induced V2 receptor cleavage are not able to bind AVP.

To investigate the effect of metalloproteinase-catalyzed V2 receptor cleavage on desensitization, a chimeric V2/oxytocin receptor that was largely resistant to agonist-induced proteolytic cleavage was studied for its ability to activate adenylyl cyclase. Activation of this receptor led to significantly higher agonist-induced cAMP levels and impaired desensitization (Kojro *et al.*, 1999). The relative contributions of V2 receptor cleavage and phosphorylation to V2 receptor desensitization remain to be elucidated.

1.3.3 G protein coupling domains

As described earlier, the antidiuretic effects of AVP are caused by coupling of V2 receptors to the stimulatory G protein, G_s, which activates adenylyl cyclase, thus leading to an increase in intracellular cAMP concentrations (Birnbaumer, 1999). In contrast, the two V_1 receptor subtypes, V1a and V1b, mediate the activation of isoforms of phopholipase Cβ, which catalyze the breakdown of phosphatidylinositol-4,5-bisphophate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Michell *et al.*, 1979). These actions are mediated through receptor interactions with G proteins of the G_q family (Fig. 1.4). Single amino acids and distinct sequence motifs have been found to be critical for the recognition and activation of G proteins by both the V1a and V2 receptors. Mutational analysis confirmed an important role of the conserved Asp97 in TM II of the V1a receptor in G protein coupling (Mouillac *et al.*, 1995), as has been described for many other GPCRs (Savarese and Fraser, 1992). A natural occurring mutation of the analogous Asp in the V2 receptor (Asp85Asn) causes X-linked NDI (Sadeghi *et al.*, 1997a). Coupling of this mutant V2 receptor to G_s was characterized by a 20-fold decrease in AVP potency and a pronounced reduction in maximal response as compared

to the wt receptor protein (Sadeghi *et al.*, 1997a). Detailed studies with mutant V2 receptors that cause X-linked NDI have revealed three more single point mutations that lead to reduced coupling efficiency to G_s: a Tyr205Cys mutation in the e2 loop (Yokoyama *et al.*, 1996), two mutations of the conserved proline in TM VII, Pro322Ser(Ala *et al.*, 1998) or Pro322His (Tajima *et al.*, 1996), and a mutation in the highly conserved DRY-motif at the junction of TM III/i2 loop, Arg137His (Rosenthal *et al.*, 1993). The key role of the conserved TM VII proline residue in G protein coupling has also been shown for the M3 muscarinic receptor (Wess *et al.*, 1993; also see chapter

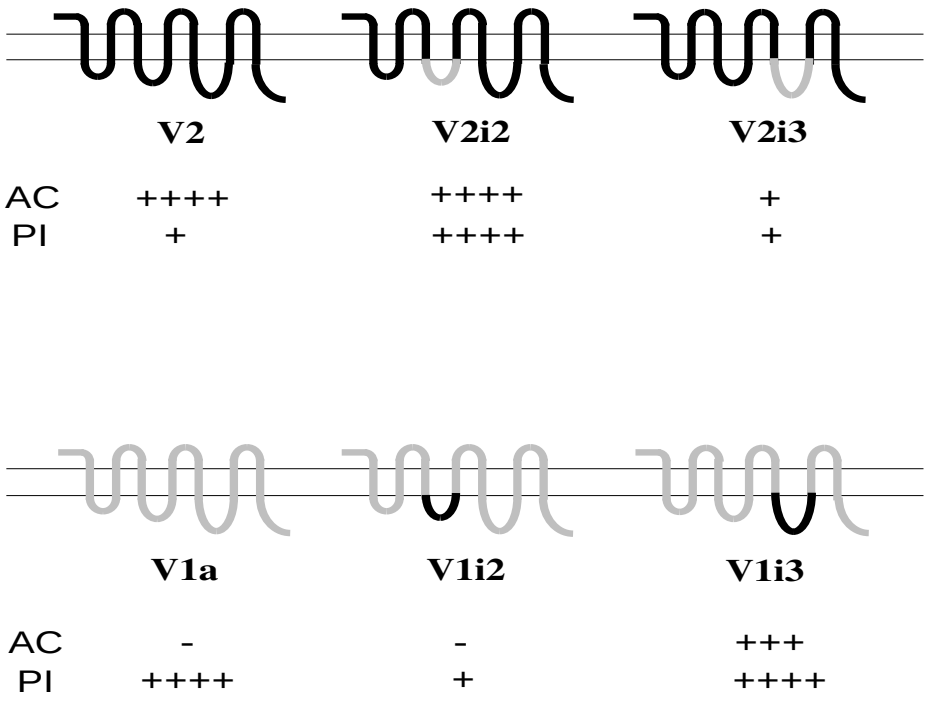


Fig. 1.9. Structure and functional profile of wt and mutant V1a/V2 vasopressin receptors. The functional properties of the various receptors are summarized underneath the receptor structures (*PI,* stimulation of PI hydrolysis; *AC,* stimulation of adenylyl cyclase). The symbols are defined as the percentage of maximum PI and cAMP responses induced by the wt V1a and V2 receptor, respectively: ++++, 90-100%; +++, 80-90%; +, 10-30%; -, no significant response. The following sequences were exchanged between the rat V1a (Morel *et al.*, 1992) and human V2 (Birnbaumer *et al.*, 1992) receptor (amino acid numbers in parentheses): *V2i2*, V2(138-160)->V1a(150-171); *V2i3*, V2(225-279)->V1a(237-305); *V1i2*, V1a(152-172)->V2(140-161); *V1i3*, V1a(237-303)->V2(225-277) (adapted and modified from Liu and Wess, 1996).

1.3). Also, the importance of the conserved arginine residue (part of the DRY-motif) for G protein coupling has been demonstrated for many other GPCRs (Savarese and Fraser, 1992).

To further investigate the structural elements that determine vasopressin receptor/G protein coupling selectivity, Liu and Wess (1996) created a series of V1a/V2 hybrid receptors in which individual intracellular loops were systematically exchanged between the two wt receptors. Biochemical and functional studies revealed that the i2 loop of the V1a receptor fully accounts for the $G_{q/11}$ coupling preference, whereas selective coupling of the V2 receptor to G_s largely determined by its i3 loop (Fig. 1.9). The chimeric receptors that contained the i2 loop of the V1a receptor and the i3 loop of the V2 receptor (V1i3 and V2i2) gained the ability to recognize both $G_{q/11}$ and G_s with high efficacy. These data demonstrated that, despite their structural similarity, the coupling preference of the V1a and V2 receptors is determined by different intracellular receptor domains (Liu and Wess, 1996).

1.4 Structural basis of receptor/G protein coupling selectivity

As mentioned earlier, most GPCRs can interact with and activate only a limited set of G proteins expressed by a typical mammalian cell (Savarese and Fraser, 1992; Gudermann *et al.*, 1996,1997; Wess, 1997). To elucidate the structural basis underlying this coupling selectivity has become a research focus of a great number of laboratories. Over the past decade, the G protein coupling profiles of many GPRCs have been studied in great detail. The different approaches that were applied to investigate receptor/G protein interactions encompass electrophysiological and/or biochemical studies, receptor/G protein co-expression experiments, reconstitution experiments, studies with synthetic peptides, hybrid receptor experiments, site-directed mutagenesis, gene knockout studies and several other approaches (reviewed by Wess, 1998).

Most GPCRs preferentially couple to G_s, $G_{q/11}$, or $G_{i/o}$-type G proteins. It should be noted though that coupling selectivity has to be understood more as a relative than an absolute term. Depending on different factors such as the choice of expression system,

expression levels of GPCRs and/or G proteins, and ligand concentration, many GPCRs can recognize more than one class of G proteins (reviewed by Gudermann *et al.*, 1996, 1997). For instance, various $G_{i/o}$-linked α_2-adrenergic receptors (Eason *et al.*, 1992) and the M_4 muscarinic (Jones *et al.*, 1991; Migeon and Nathanson, 1994) are also able to activate the stimulatory G_s protein, at least under certain experimental conditions, an effect that becomes more evident under conditions where $G_{i/o}$ is inactivated (e.g. by pretreatment with PTX). A recent study employed a β_2-adrenoceptor/Gα subunit fusion protein approach to study the interaction of the β_2-adrenergic receptor with various G proteins (Wenzel-Seifert and Seifert, 2000). In this system, the β_2-adrenoceptor did not only recognize G_s but also G_i and G_o. Likewise, Offermanns *et al.* (1994b) reported that the M_1 muscarinic receptor, which is a prototypical $G_{q/11}$-coupled receptor, can activate $G_{i/o}$ proteins under certain assay conditions (e.g. in the presence of high agonist concentrations). M_1 receptor coupling to G_s has also been reported (Migeon and Nathanson, 1994; Burford and Nahorski, 1996). It remains to be determined though whether this "promiscuous" coupling behavior is of physiological relevance. The most promiscuous GPCR seems to be the TSH receptor, which is capable of activating G proteins of all four classes (Laugwitz *et al.*, 1996). Interestingly no receptors have been identified that solely recognize G proteins of the $G_{12/13}$ class.

Usually, GPCRs are able to couple to all members within their preferred G protein family, although often with varying efficiencies (Milligan, 1995; Raymond, 1995). For instance, it appears that all G_q-coupled GPCRs efficiently interact with G_{11}, G_{14}, G_{15}, and G_{16} (Milligan, 1995; Gudermann, 1996). Amatruda *et al.* (1993) and Wu *et al.* (1993) reported that the $G_{i/o}$-linked C5a and IL-8 receptors, respectively, are able to discriminate between members of the $G_{q/11}$ class: both GPCRs do not interact with $G\alpha_q$ and $G\alpha_{11}$ but are capable of coupling to G_{14}, G_{15}, or G_{16}. Interestingly, G_{15} and G_{16}, which represent the murine and human forms, respectively, of the same G protein, have been shown to display coupling promiscuity, as indicated by their ability to be activated by most GPCRs (Offermanns and Simon, 1995). The molecular mechanism underlying this coupling profile still remains to be determined.

In recent years, the idea emerged that different active receptor conformations may exist for a given GPCR depending on the ligand that makes contact with the receptor protein. This hypothesis is supported by various findings. The α_{1b} adrenergic receptor, for instance, couples to both the stimulation of phospholipase C and phopholipase A2**.** A point mutation in TM III, however, leads to a mutant α_{1b} receptor which constitutively activates exclusively the phopholipase C pathway (Perez *et al.*, 1996). Likewise, the human TSH receptor that is known to couple to all four G protein families loses its ability to activate G_q in the presence of a single point mutation in TM V (Biebermann *et al.*, 1998). Similar results had also been obtained earlier by Suprenant *et al.* (1992) and Gilchrist *et al.* (1996). Recently, Stanasila *et al.* (2000) expressed human μ-opioid receptor (hMOR) fusion proteins (hMOR-$G\alpha_{o1}$ and hMOR-$G\alpha_{i2}$) in *E. coli*. In this system, various agonists displayed different affinities for the human μ-opioid receptor depending on the linked $G\alpha$ subunit supporting the hypothesis of the existence of multiple receptor conformational states. Other laboratories studying the β_2-adrenoceptor (Zuscik *et al.*, 1998; Wenzel-Seifert *et al.*, 2000) have reported similar findings (reviewed by Strange, 1999).

Physiologically, receptor/G protein coupling selectivity can also be achieved through compartmentalization (co-expression of certain receptors and G proteins within a cell or domains of the plasma membrane). Moreoever, actual receptor and/or G protein expression ratios may affect the observed coupling selectivity in specific cells or tissues (Neubig, 1994; Raymond, 1995; Gudermann *et al.*, 1996, 1997; Remmers *et al.*, 2000; Ostrom *et al.* 2000).

Mutagenesis studies have shown that residues that are critically involved in determining the coupling profile of a specific member of a given GPCR family are usually not well conserved among GPCRs that display similar G protein coupling preferences (Hedin *et al.*, 1993; Horn *et al.*, 2000). Likewise, different classes of GPCRs recognize the same array of G proteins despite the fact that they share little sequence homology. Therefore it is difficult to predict the G protein coupling profile of a GPCR simply based on its primary sequence. However, since the three-dimensional fold of all

GPCRs and all G proteins is thought to be similar, it is likely that all receptor/G protein complexes generally display the same overall geometry.

1.4.1 Identification of G protein coupling domains

Different experimental approaches such as functional analysis of hybrid receptors, site-directed mutagenesis, and studies with synthetic peptides have helped to identify receptor domains that play key roles in G protein recognition. Considerable insight into the structural basis of receptor/G protein coupling selectivity has been provided by detailed mutational analysis of biogenic amine receptors such as the muscarinic acetylcholine (Wess, 1996) and adrenergic receptors (Dohlman *et al.*, 1991; Strader *et al.*, 1994; Cotecchia *et al.*, 1998), which were among the first GPCRs to be cloned. These studies have shown that the most critical sites for G protein recognition are to be found in the i2 and i3 loops, in particular in their membrane-proximal portions (Wess, 1997). Many reports indicate that both receptor regions act in a concerted fashion to achieve optimal and efficient G protein activation (Wong *et al.*, 1990; Pin *et al.*, 1994; Blin *et al.*, 1995; Gomeza *et al.*, 1996a; Thompson *et al.*, 1998; Ulloa-Aguirre *et al.*, 1998; Cypress *et al.*, 1999). Several mutagenesis studies also suggest an involvement of Ni4 and residues within the i1 loop in modulating the efficiency of G protein coupling (O'Dowd *et al.*, 1988; Liggett *et al.*, 1991; Nussenzweig *et al.*, 1994; Gomeza *et al.*, 1996a; Wu *et al.*, 1997). Synthetic peptides corresponding to various intracellular receptor domains were able to either interfere with receptor/G protein interactions by uncoupling the receptor or activate G proteins in a receptor-like fashion (Koenig *et al.*, 1989; Palm *et al.*, 1989; Cheung *et al.*, 1991; Muench *et al.*, 1991; Voss *et al.*, 1993; Merkouris *et al.*, 1996; Abell and Segaloff, 1997; Thompson *et al.*, 1998; Ortiz *et al.*, 2000). Consistent with the mutagenesis studies described above, this approach has implicated the i2 loop, the membrane-proximal parts of the i3 loop, and the Ni4 segment as domains critical for G protein coupling.

1.4.2 Involvement of the intracellular loops of GPCRs in G protein coupling selectivity

1.4.2.1 First intracellular (i1) loop

To date, only relatively few studies examined the role of the i1 loop in G protein recognition and coupling. Nevertheless, recent data indicate that i1 loop sequences are able to modulate the selectivity of receptor/G protein interactions. For instance, experiments with the cholecystokinin (CCK)-A and –B receptors, which are both coupled to G proteins of the $G_{q/11}$ class, revealed that the ability of the CCK-A receptor to also stimulate G_s was critically depending on the presence of a 5-amino- acids sequence in the i1 loop (Wu *et al.*, 1997). Insertion of these residues into the CCK-B receptor enabled the resulting mutant CCK-B receptor to interact with G_s in a fashion similar to the CCK-A receptor. Similar results were obtained by Nussenzweig *et al.* (1994) examining two isofoms of the calcitonin receptor. Also, in the murine GnRH receptor, residues within the i1 loop are necessary for stimulating the cAMP pathway, whereas the i1 loop is not involved in activating the PI signaling cascade (Arora *et al.*, 1998). Interestingly, a naturally occurring single point mutation in the i1 loop of the melanocyte-stimulating hormone (MSH) receptor (Ser69Leu) leads to constitutive receptor activation (Robbins *et al.*, 1993). Several loss-of-function studies underline the importance of the structural integrity of the i1 loop for optimal receptor/G protein coupling (O'Dowd *et al.*, 1988 (β_2-adrenergic receptor); Moro *et al.*, 1994 (M_1 muscarinic receptor); Amatruda *et al.*, 1995 (fMLP receptor)). It remains to be elucidated, however, whether the i1 loop is in direct contact with the G protein trimer or whether mutations in the i1 loop can affect the conformations of other receptor regions which are known to interact directly with the G protein (such as the i2 and i3 loops).

1.4.2.2 Second intracellular (i2) loop

As mentioned in chapter 1.3 (see Fig. 1.6), the i2 loop contains sequences that play a major role in G protein activation and proper folding. The highly conserved triplet E/D-R-Y (corresponding to Asp136-Arg137-His138 in the wt V2 receptor (Fig. 1.8)) is located at the junction between TM III and the i2 loop (see chapter 1.3 for the role of the DRY-motif).

Many studies indicate that the i2 loop also plays a crucial role in regulating receptor/G protein coupling selectivity, either alone or in combination with other cytoplasmic domains such as the i3 loop or the i4 region. As discussed in chapter 1.3.3, the selective coupling of the V1a receptor to G proteins of the G_q family critically depends on the structural integrity of the i2 loop. Analogous results were obtained by Näsman *et al.* (1997) in the adrenergic receptor system. The α_2-adrenergic receptors generally couple to $G_{i/o}$ but under certain conditions the α_{2B} receptor subtype, but not the α_{2A}, is able to also activate G_s. A hybrid receptor approach was employed to study the molecular basis underlying this selectivity. This analysis revealed that a mutant α_{2A}-adrenergic receptor, in which the i2 loop was replaced by homologous α_{2B}-adrenergic receptor sequence, gained productive coupling to G_s (Näsman *et al.*, 1997). Moreover, a recent study described the functional properties of hybrid receptors made up of structurally dissimilar GPCRs such as the thrombin receptor (which activates several classes of G proteins including $G_{q/11}$ proteins) and the G_s-linked β_2-adrenergic and D2 dopamine receptors (Verrall *et al.*, 1997). Substitution of the i2 loop of the thrombin receptor into both G_s-coupled GPCRs conferred on the β_2-adrenergic and D2 dopamine receptors G_q-like coupling properties. Wang *et al.* (2000) described a series of naturally occurring splice variants of the 5-HT_{2C} receptor that are generated by mRNA editing. The various isoforms differ only in their i2 loops but display significantly altered G protein coupling efficacies, suggesting that mRNA editing can modulate the efficiency of receptor/G protein interactions. Studies with M_1 (or M_2) muscarinic/β_1-adrenergic (Wong *et al.*, 1990; Wong and Ross, 1994) and mGluR1/mGluR3 glutamate hybrid receptors (Pin *et al.*, 1994; Gomeza *et al.*, 1996b), AT_{1a} (Thompson *et al.*, 1998), GnRH receptors

(Ulloa-Aguirre *et al.*, 1998), and glucagon receptors (Cypress *et al.*, 1999) indicate that the i2 loop acts in concert with other intracellular domains, such as the i3 and i4 loops, to regulate receptor/G protein coupling selectivity.

A detailed analysis of functionally important i2 loop residues was conducted by Blin *et al.* (1995). Biochemical studies revealed that a set of four amino acids residing within the i2 loop of the $G_{q/11}$-coupled M_3 muscarinic receptor make a major contribution to the coupling preference of this receptor subtype. Substituting these four amino acids into the $G_{i/o}$-linked M_2 muscarinic receptor resulted in a mutant receptor that gained the ability to activate $G_{q/11}$ (Blin *et al.*, 1995). Another study investigated the ability of the metabotropic glutamate receptor 1α (mGluR1α) to couple to both G_q and G_s (Francesconi and Duvoisin, 1998). Introduction of single point mutations into the i2 loop identified three specific residues within the i2 loop that are selectively involved in the interaction with G_q class proteins, whereas a point mutation and deletion mutation at different sites within the i2 loop affected G_s coupling. In combination with residues in the i3 loop, these different sets of amino acids account for the ability of the mGlu1αR to couple to different sets of G proteins (Francesconi and Duvoisin, 1998).

1.4.2.3 Third intracellular (i3) loop

Mutational analysis has provided strong evidence that the i3 loop plays a key role in regulating the G protein coupling preference of the majority of GPCRs, as initially demonstrated in studies with biogenic amine receptors such as the muscarinic (Wess, 1996) and adrenergic receptors (reviewed by Dohlman *et al.*, 1991; Kobilka, 1992). More recent studies with many other classes of GPCRs have corroborated these findings. For instance, functional analysis of hybrid V1a/V2 vasopressin receptors showed that insertion of the i3 loop of the V2 receptor into the V1a receptor conferred on the resulting mutant receptor the ability to efficiently activate G_s (Liu and Wess, 1996). Likewise, Takagi *et al.* (1995) demonstrated that the ability of the endothelin ET_B receptor to interact with G_i is largely depending on sequences within its i3 loop. In another study, the i3 loop was exchanged between the monocyte chemoattractant protein-1 (MCP-1)

receptor (which couples to several G proteins including G_q) and the IL-8 chemokine receptor, which does not recognize G_q. Functional studies showed that the resulting mutant IL-8 receptor gained the ability to activate G_q (Arai and Charo, 1996). These findings are confirmed by studies employing synthetic peptides derived from different intracellular receptor domains to inhibit receptor/G protein coupling. Koenig *et al.* (1989) could demonstrate that synthetic peptides corresponding to the i2 and i3 loops and to the Ni4 segment of rhodopsin could effectively compete with metarhodopsin II for binding to G_t. Georgoussi *et al.* (1997) studied the effect of various peptides corresponding to specific μ-opioid receptor sequence on agonist-stimulated G protein activation and [^{3}H] agonist binding to the μ-opioid receptor. This study found that peptides derived from the N-terminal portion of the i3 loop and the C-terminal tail had the most pronounced inhibitory effect. Similarly, a study using synthetic peptides derived from the i3 loop of the G_q-coupled oxytocin receptor described peptide-induced inhibition of PI hydrolysis (Qian *et al.*, 1998). The i3 loop derived from the α_{1B}-adrenergic receptor, which also activates $G_{q/11}$, was also able to interfere with oxytocin-stimulated phosphoinositide breakdown, whereas co-expression of the i3 loop of the G_s-linked dopamine 1A receptor had no effect oxytocin receptor function (Qian *et al.*, 1998). Another study investigated the effect of different peptides derived from individual intracellular loops of the galanin type 1 receptor (Gal_1R) on [^{35}S]-GTPγS binding to $Gi_1\alpha_1\beta_2$ (Rezaei *et al.*, 2000). It could be demonstrated that peptides corresponding to the i1 and i2 loops had no effect on [^{35}S]-GTPγS binding, whereas i3 loop peptides, especially peptides derived from the N-terminus, were able to increase binding of [^{35}S]-GTPγS to $Gi_1\alpha_1\beta_2$.

Although exchange of the i3 loop between receptor subtypes with different G protein coupling profiles usually leads to altered G protein coupling properties, the resulting mutant receptors generally couple with reduced potency and/or efficiency compared to the wt receptor (Kobilka *et al.*, 1988; Wess *et al.*, 1989, 1990b; Olah, 1997). These observations are consistent with the concept that the i3 loop acts in cooperation with other receptor regions to achieve maximum coupling efficiency and selectivity.

In contrast to the i2 loop, the length of the i3 loop varies considerably among GPCRs and can range from 15 (N-formyl peptide receptor) to 240 amino acids (M_3

muscarinic receptor). Interestingly, deletion of major portions in the central part of the i3 loop of many GPCRs does not interfere with efficient coupling to G proteins (Maggio *et al.*, 1996; Buck *et al.*, 2000), confirming that the N- and C-terminal domains of the i3 loop (in the following referred to as Ni3 and Ci3, respectively) are most critical for G protein activation. Extensive mutagenesis studies revealed that the N- and C-terminal eight to 15 amino acids contain key structural information required for efficient interaction with G proteins (reviewed by Strader *et al.*, 1994; Wess, 1996). However, the relative contribution of the Ni3 and Ci3 regions to coupling selectivity can vary among different classes of GPCRs, even among structurally closely related receptor subtypes (Wess, 1998).

Examples for which the N-terminal portion of the i3 loop largely determines the G protein coupling selectivity include various muscarinic (Wess *et al.*, 1989, 1990b; Lechleiter *et al.*, 1990), adrenergic (Cotecchia *et al.*, 1992), opioid (Georgoussi *et al.*, 1997), adenosine (Olah, 1997), and galanin (Rezaei *et al.*, 2000) receptors. Biochemical analysis of the angiotensin II AT_1 (which couples to $G_{q/11}$) and AT_2 (which is linked to $G_{i/o}$) receptors revealed that the Ni3 region of the AT_1 receptor is of pivotal importance for activation of $G_{q/11}$ but that amino acids within the Ci3 segment enhance AT_1 receptor/$G_{q/11}$-coupling selectivity (Wang *et al.*, 1995). This cooperative mechanism of G protein activation was also described by Eason and Ligget (1996) who noted that the α_{2A}-adrenergic receptor requires the presence of both Ni3 and Ci3 sequences to efficiently stimulate G_s.

Many studies suggest that the i3 loop residues dictating receptor/G protein coupling selectivity are primarily hydrophobic or non-charged. This notion is supported by both gain- and loss-of-function studies with different muscarinic receptor subtypes (Blüml *et al.*, 1994a; Blin *et al.*, 1995; Liu *et al.*, 1995; Hill-Eubanks *et al.*, 1996) and various other GPCRs, including the β_2-adrenergic (Cheung *et al.*, 1992), thyrotropin (Kosugi *et al.*, 1993), thromboxane A_2 (D'Angelo *et al.*, 1996), rhodopsin (Yang *et al.*, 1996b), glucagon-like peptide-1 (Mathi *et al.*, 1997), and the GnRH receptors (Chung *et al.*, 1999). The critical residues predicted to be intimately involved in G protein recognition appear to be located on the hydrophobic face of amphiphilic helical domains.

Like the Ni3 segment, the Ci3 domain contributes to selective G protein recognition (Wess, 1998). The importance of the Ci3 domain in receptor activation is further underlined by the observation that single point mutations (Shenker, 1995, 1997; Zuscik *et al.*, 1998; Abadji *et al.*, 1999; Arseven *et al.*, 2000) or deletion mutations (Schulz *et al.*, 1999) within this region can lead to constitutive receptor activation. Interestingly, Malmberg and Strange (2000) reported a single point mutation in the Ci3 region of the 5-HT_{1a} receptor, Val344Glu, which alters the receptor/G protein coupling preference, allowing interaction with G_s in addition to $G_{i/o}$. Analogous mutations at this position have formerly been reported to frequently lead to constitutive activity.

Many mutagenesis studies have demonstrated that a set of charged amino acids in the Ci3 region are also important for the activation of G proteins (Kunkel and Peralta, 1993; Hoegger *et al.*, 1995; Lee *et al.*, 1996; Obosi *et al.*, 1997; Wang, 1997; Burstein *et al.*, 1998; Wade *et al.*, 1999; Buck *et al.*, 2000; Zhang *et al.*, 2000). Mutation of mostly basic amino acids to hydrophobic or non-charged residues significantly reduced or abolished G protein coupling. Many of these studies report that mutational modification of the most C-terminal residue in this stretch of amino acids had the most pronounced effect on receptor function (Hoegger *et al.*, 1995; Lee *et al.*, 1996; Obosi *et al.*, 1997).

To further elucidate the role of the Ci3 segment in G protein coupling selectivity, different muscarinic receptor subtypes were subjected to systematic mutagenesis studies (reviewed by Wess, 1996). Blin *et al.* (1995) has identified four hydrophobic amino acids at the C-terminus of the M_3 receptor, which act in a concerted fashion with residues present in the i2 loop and Ni3 domain in dictating $G_{q/11}$ coupling selectivity. The four corresponding residues present at the same position in the $G_{i/o}$-linked M_2 muscarinic receptor take on a similar role in determining selective activation of $G_{i/o}$ proteins (Liu *et al.*, 1995; Kostenis *et al.*, 1997a).

The functionally critical residues at both the N- and C-terminus of the i3 loop of the muscarinic receptor subtypes seem to form two hydrophobic surfaces, which play a key role in G protein recognition (Wess, 1998). In the inactive receptor state, these receptor domains are predicted to be deeply buried, projecting into the receptor protein core (Baldwin *et al.*, 1997). The current model of receptor activation suggests an outward

movement of the i3/TM VI junction accompanied by a clock-wise rotation (about 30°) (Farrens *et al.*, 1996; Abell and Segaloff, 1997), as part of the re-orientation of the transmembrane helices. It is conceivable that the re-arrangement of the helical bundle leads to an opening of the intracellular receptor surface, thus exposing functionally important receptor sites. This may allow the G protein to access functionally important residues at the membrane-proximal portions of the i3 loop, resulting in productive receptor/G protein coupling.

1.4.2.4 Fourth intracellular (i4) loop

The cytoplasmic tail of most GPCRs is characterized by a short membrane-proximal segment (Ni4), which is generally linked to the plasma membrane via palmitoylation or myristoylation of two highly conserved cysteine residues, and a C-terminal (Ci4) segment of varying lengths. The high-resolution crystal structure of rhodopsin (Palczewski *et al.*, 2000) indicates that the Ni4 segment is α-helically arranged. The notion that Ni4 contributes to the G protein activation process is also supported by biochemical studies employing short synthetic Ni4 peptides derived from many different GPCRs (Koenig *et al.*, 1989; Muench *et al.*, 1991; Schreiber *et al.*, 1994; Shirai *et al.*, 1995; Merkouris *et al.*, 1996; Georgoussi *et al.*, 1997) and various loss-of-function mutagenesis studies (Huang *et al.*, 1995; Sano *et al.*, 1997; Hoare *et al.*, 1999).

Different laboratories have studied the role of the Ni4 domain in regulating receptor/G protein coupling selectivity. Hybrid receptor approaches using α_{2A}/β_2-adrenergic (Liggett *et al.*, 1991) and $mGluR_1/mGluR_3$ (Pin *et al.*, 1994; Gomeza *et al.* 1996a) receptors have demonstrated that the Ni4 segment can contribute to regulating G protein coupling selectivity at least in certain GPCR subfamilies. However, this contribution became evident only in the presence of other receptor regions (such as the i2 or i3 loop) known to play key roles in regulating the selectivity of G protein recognition. Usually, exchange of the Ni4 region alone between functionally distinct receptor subtypes does not lead to hybrid mutant receptors with reversed G protein coupling

profiles (Wess *et al.*, 1990a; Takagi *et al.*, 1995; Wang *et al.*, 1995; Liu and Wess, 1996; Olah *et al.*, 1997).

Receptor truncation studies have shown that complete or partial removal of the C-terminal tail leads to inactive receptors that are retained intracellularly (Segaloff and Ascoli, 1993; Huang *et al.*, 1995; Sadeghi *et al.*, 1997c; Hukovic *et al.*, 1998; Schülein *et al.*, 1998). On the other hand, deletion of the Ci4 region alone usually has little effect on proper GPCR function (Lattion *et al.*, 1994; Alblas *et al.*, 1995; Hipkin *et al.*, 1995; Huang *et al.*, 1995; Hermans *et al.*, 1996; Unson *et al.*, 1995; Ancellin *et al.*, 1997; Innamorati *et al.*, 1997). In some cases, Ci4-truncated GPCRs (Parker and Ross, 1991; Matus-Leibovitch *et al.*, 1995; Hasegawa *et al.*, 1996) displayed elevated basal activities or improved coupling efficiency (Parker and Ross, 1991; Alblas *et al.*, 1995; Hipkin *et al.*, 1995; Iida-Klein *et al.*, 1995). The Ci4 region often contains sites for receptor phosphorylation required for receptor desensitization. Removal of such phosphorylation sites by receptor truncations may explain that the resulting mutant GPCRs often display increased basal activities and coupling efficiencies.

1.4.3 G protein domains determining coupling selectivity

As detailed in chapter 1.1.3, the C-terminal 45 amino acids of Gα harbor key residues that are essential for receptor/G protein activation in general. Mutational studies indicated that the last five amino acids of the Gα-subunits contain key structural information that determines the specificity of receptor/G protein coupling. For instance, Conklin *et al.* (1993) created a chimeric $G\alpha_q$-subunit, in which the last five amino acids were replaced by either homologous $G\alpha_i$ or $G\alpha_o$ sequence. In functional assays, this mutant G protein was able to link receptors, which are normally exclusively coupled to $G_{i/o}$, to the PI pathway, indicating that the receptors are able to recognize the mutant G protein via the C-terminal five amino acids (Conklin *et al.*, 1993; Liu *et al.*, 1995; Gomeza *et al.*, 1996b; Kostenis *et al.*, 1997a). Likewise, the $G_{q/11}$-linked M_3 muscarinic and V1a vasopressin receptors were shown to be able to stimulate adenylyl cyclase through interaction with a mutant $G\alpha_s$-subunit, in which the last five amino acids were

exchanged against homologous $G\alpha_q$ sequence (Conklin *et al.* 1996; Kostenis *et al.*, 1997c). This strategy was successfully applied to many other receptor/G protein systems (reviewed by Wess, 1998).

More detailed mutational studies identified specific residues among the C-terminal five amino acids of Gα that play key roles in determining receptor/G protein coupling selectivity. Gain-of-function studies showed that two single amino acids of α_i, even when individually substituted into α_q, could alter the receptor selectivity of the recipient Gα-subunit (Kostenis *et al.*, 1997a). The two crucial residues were a cysteine in position –4 and a glycine at –3 (Fig. 1.10). Consistent with this finding, mutational and biochemical analysis of $G\alpha_t$ (transducin) had demonstrated earlier that these two residues, as well as a hydrophobic amino acid at –1, are essential for productive rhodopsin/$G\alpha_t$ coupling (Garcia *et al.*, 1995; Osawa and Weiss, 1995; Martin *et al.*, 1996). Interestingly, the –4 cysteine and –3 glycine residues are conserved only among $G_{i/o}$ class G proteins, reflecting their importance in regulating receptor/$G_{i/o}$ protein coupling selectivity (Fig. 1.10).

Kostenis *et al.* (1997c) also identified residues at the C-terminus of $G\alpha_q$ that are required for activation of G_q by G_q-linked receptors. To this goal, individual $G\alpha_q$ amino acids were substituted into $G\alpha_s$ and the resulting mutant G proteins were assayed for their ability to be activated by the $G_{q/11}$-coupled M_3 muscarinic and V1a vasopressin receptors. Two single point mutants were identified that allowed G protein activation by the $G_{q/11}$-linked receptors. The two α_q residues that mediated this interaction were a glutamate at position –5 and an asparagine at –3 (Fig. 1.10). Again, these two residues are highly conserved only among members of the $G_{q/11}$ family. It is likely that the distal C-terminus of Gα adapts different spatial arrangements that contribute to the specificity with which receptors recognize G proteins (Dratz *et al.*, 1993).

Another interesting mechanism by which receptor/G protein coupling can be regulated was described by Kostenis *et al.* (1997b). The G_q and G_{11} proteins are characterized by a six amino acid extension at their N-terminus, which is attached to the plasma membrane via fatty acid modifications. Interestingly, this short stretch of amino acids appears to contribute to the selectivity of receptor/$G_{q/11}$ coupling. Removal of the N-

terminal six amino acids yielded a mutant G_q protein, which could be activated by many $G_{i/o}$- or G_s-linked receptors that normally do not recognize wt G_q (Kostenis *et al.*, 1997b). Possibly, the N-terminal extension represents a sterical hindrance preventing $G_{i/o}$- and G_s-coupled receptors from accessing functionally important αq residues. Alternatively, the N-terminus of $G\alpha_q$ may modulate the conformation of other $G\alpha_q$ domains that are involved in receptor coupling.

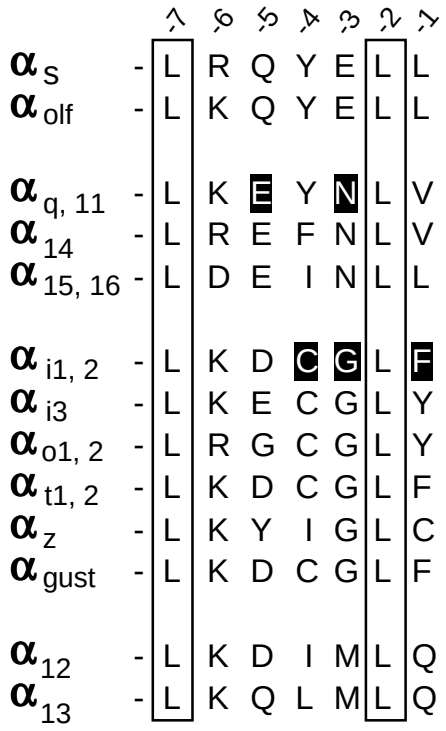


Fig. 1.10 Comparison of the C-terminal 7 amino acids of different classes of G protein α-subunits. The $\alpha_{q/11}$ and $\alpha_{i1/2}$ residues highlighted in black are known to be intimately involved in determining the selectivity of receptor/G protein interactions, as demonstrated in gain-of-function mutagenesis studies (Conklin *et al.*, 1993, 1996; Liu *et al.*, 1995; Kostenis *et al.*, 1997b, c). The boxed leucine residues at position –2 and –7 are conserved among all mammalian α-subunits and have been shown to be essential for receptor/G protein coupling (Garcia *et al.*, 1995; Osawa and Weiss, 1995; Martin *et al.*, 1996). Adapted from Wess, 1998.

1.5 Aim of the thesis

GPCRs form one of the largest protein family in nature regulating many fundamental physiological processes (Balwin, 1994; Strader *et al.*, 1994; Gudermann *et al.*, 1995). Despite the diversity of their activating ligands, GPCRs recognize and interact only with a limited set of the many different G proteins that are expressed within a cell (Hedin *et al.*, 1993; Conklin and Bourne, 1993). The spectrum of cellular responses triggered by activation of a specific GPCR is largely determined by the type of G proteins recognized by the activated receptor (Dohlman *et al.*, 1991; Strader *et al.*, 1994; Gudermann *et al.*, 1996; Wess, 1998). Therefore, elucidating the molecular basis governing the selectivity of receptor/G protein interactions is of fundamental importance for understanding cellular signal transduction.

As discussed in chapter 1.4, multiple intracellular domains are involved in receptor/G protein coupling selectivity (Strosberg, 1991; Savarese and Fraser, 1992; Hedin *et al.*, 1993). The G protein coupling domains of biogenic amine receptors such as the muscarinic acetylcholine (Wess, 1993) and adrenergic receptors (Dohlman *et al.*, 1991; Strader *et al.*, 1994) have been analyzed in great detail. In contrast, only little is known about the molecular mechanisms underlying the coupling selectivity of GPCRs that are activated by peptide ligands. However, peptide receptors constitute one of the largest GPCR subfamily comprising more than 60 different receptors (Watson and Arkinstall, 1994). Peptide GPCRs play key roles in regulating many fundamental physiological processes. Studies aimed at elucidating the structural basis of the G protein coupling selectivity displayed by these receptors have been impeded by the fact that most members of a given peptide receptor subfamily couple to similar G proteins. All CCK, endothelin, neurokinin, and bombesin receptors, for example, are preferentially coupled to G proteins of the $G_{q/11}$ class, whereas various opioid and somatostatin receptors are all selectively linked to G proteins of the $G_{i/o}$ class (Watson and Arkinstall, 1994). This pattern has impaired investigations into receptor/G protein coupling selectivity by the use of hybrid receptor approaches.

The vasopressin receptor family, however, differs from virtually all other peptide receptor subfamilies in that its members clearly differ in their G protein coupling profile. Therefore, the vasopressin receptor subtypes constitute an attractive model system to study peptide receptor domains that are critical for G protein recognition. The V1a and V1b receptors are selectively linked to G protein of the $G_{q/11}$ class (Laszlo *et al.*, 1991), which mediate the activation of distinct isoforms of phospholipase Cβ, resulting in the breakdown of PI lipids. In contrast, the V2 receptor preferentially couples to the G protein G_s (Laszlo *et al.*, 1991), which results in the activation of adenylyl cyclase and subsequent elevation of intracellular cAMP levels.

In a former study, Liu and Wess (1996) used a hybrid receptor approach to broadly map the receptor domains that regulate the coupling selectivity of the V1a and V2 receptors. They found that the coupling profile of the two receptor subtypes is determined by different single receptor domains. The presence of V1a receptor sequence in the i2 loop was critical for efficient coupling of the wt V1a or chimeric V1a/V2 receptors to $G_{q/11}$, whereas efficient interaction of the wt V2 and hybrid V1a/V2 receptors to G_s required the presence of V2 receptor sequence in the i3 loop (Liu and Wess, 1996).

The goal of this present thesis was to analyze vasopressin receptor/G protein coupling selectivity in greater structural detail, specifically to identify individual amino acids that play key roles in the coupling process. To this goal, classical mutagenesis strategies were employed in the first part and yeast expression technology in the second part of this thesis.

Structural basis of V2 vasopressin receptor/G_s coupling selectivity

The experiments described in the first part of this thesis were designed to elucidate which receptor subdomains and particularly which specific residues determine the ability of the V2 vasopressin receptor to selectively activate G_s.

This work involved the expression of hybrid V1a/V2 receptor constructs in mammalian cells and their subsequent pharmacological characterization in radioligand binding studies and functional assays. The initial goal was to map short V2 receptor

domains that confer on the V1a receptor the ability to couple to G_s (gain-of-function). Subsequently, more detailed structural information was obtained by replacing one or a combination of individual amino acids in the V1a receptor with the corresponding V2 receptor residues. To assess the importance of specific residues, deletion and alanine scanning mutagenesis was also employed.

Screening for mutant V2 vasopressin receptors with altered G protein coupling properties employing yeast expression technology

The studies described in the second part of this thesis were designed to identify mutant V2 receptors that would gain the ability to productively couple to $G_{q/11}$ proteins. To facilitate such studies, an experimental system was established that allowed the quick and efficient functional analysis of a large number of mutant receptors. Specifically, yeast expression technology (Pausch, 1997; Sommers and Dumont, 1999; Reiländer *et al.*, 2000) was employed to co-express the V2 receptor and various chimeric yeast/mammalian Gα proteins in yeast. The yeast strains employed in this study were genetically engineered, such that they required ligand-activated receptor/G protein coupling for cell growth (Pausch *et al.*, 1998). Additionally, the three $G_{q/11}$-coupled muscarinic receptors (M_1, M_3, and M_5) were also expressed in yeast to further characterize the yeast expression system.

Specifically, the i2 loop of the V2 vasopressin receptor was subjected to random mutagenesis, followed by a yeast genetic screen that allowed the identification of mutant receptors with altered coupling properties. The i2 loop was chosen as the primary target for this analysis, because this domain plays a key role in determining the $G_{q/11}$ coupling selectivity of the V1a receptor (Liu and Wess, 1996).

2. Materials and Methods

2.1 Materials

2.1.1 Commercially available compounds and materials

Compounds

Agar (Sigma, St. Louis, MO, USA)

Amino acids (various) (Bio 101, now Qbiogene, Carlsbad, CA, USA)

3-Amino-1,2,4-triazole [AT] (Sigma, St. Louis, MO, USA)

Ammonium sulfate (Sigma, St. Louis, MO, USA)

^{8}Arginine-vasopressin [AVP] (Calbiochem, San Diego, CA, USA)

Atropine sulfate (Sigma, St. Louis, MO, USA)

Bacitracin (activity: 70U/mg) (Amersham Lifescience, Piscataway, NJ, USA)

Bovine serum albumine [BSA] (Sigma, St. Louis, MO, USA)

Carbamoylcholine chloride [carbachol] (Sigma, St. Louis, MO, USA)

Chloroquine (Sigma, St. Louis, MO, USA)

Dithiothreitol [DTT] (Sigma, St. Louis, MO, USA)

Dropout powder: complete supplement mixture [CSM] minus amino acids used for selection (Bio 101, now Qbiogene, Carlsbad, CA, USA)

Gelatin (porcine skin) (Sigma, St. Louis, MO, USA)

3-Isobutyl-1-methylxanthin [IBMX] (Sigma, St. Louis, MO, USA)

Phenylmethylsulfonylfluoride [PMSF] (Sigma, St. Louis, MO, USA)

V2 receptor antagonist (non-peptide): SR 121463 (1-[4-(N-tert-butylcarbamoyl)-2-methoxybenzene sulfonyl]-5-ethoxy-3-spiro-[4-(2-morpholinoethoxy)-cyclohexane]-indol-2-one, monophosphate salt) was synthesized at Sanofi-Synthelabo, Toulouse, France

Yeast nitrogen base [YNB] (Bio 101, now Qbiogene, Carlsbad, CA, USA)

All other reagents used were purchased from Sigma (St. Louis, MO, USA), unless otherwise noted.

Solutions

Dulbecco's modified Eagle's medium [DMEM] (with glucose: 4.5g/l) (Biofluids, Rockville, MD, USA)

Ethylenediaminetetraacetate [EDTA] (0.5 M) (Quality Biological Inc., Gaithersburg, MD, USA)

Fetal calf serum [FCS](Gemini Bio Products, Woodland, CA, USA)

Hank's balanced salt solution [HBSS] (Biofluids, Rockville, MD, USA)

$MgCl_2$ (2 M) (Quality Biological Inc., Gaithersburg, MD, USA)

Phosphate-buffered saline [PBS] (with or without Ca^{2+}/Mg^{2+}) (Biofluids, Rockville, MD, USA)

Tris-HCl (1 M, pH 7.4) (Quality Biological Inc., Gaithersburg, MD, USA)

Penicillin G/Streptomycin/Glutamine (100X) (Gibco BRL, Grand Island, NY, USA)

Trypsin (0.05%)/Versene (0.02%) (Biofluids, Rockville, MD, USA)

Radioactive compounds

[^{3}H]-Adenine (18 Ci/mmol) (American Radiolabeled Chemicals Inc., St. Louis, MO, USA)

[^{3}H]-Arg-vasopressin ([^{3}H]AVP: 81 Ci/mmol) (NEN Life Science Products, Boston, MA, USA)

[^{3}H]-*Myo*-inositol (20-23 Ci/mmol) (American Radiolabeled Chemicals Inc., St. Louis, MO, USA)

[^{3}H]-N-methylscopolamine ([^{3}H]NMS; 82 Ci/mmol) (NEN Life Science Products, Boston, MA, USA)

[^{3}H]-SR121463 (47.5 Ci/mmol) (NEN Life Science Products, Les Ulis, France).

Plasmids

p416GPD (yeast expression plasmid) (American Type Culture Collection [ATCC], Rockville, MD, USA)
p426GPD (yeast expression plasmid) (American Type Culture Collection [ATCC], Rockville, MD, USA)

Antibodies for Immunoblotting

Anti-HA monoclonal antibody (12CA5) (Boehringer Mannheim, now Roche, Indianapolis, IN, USA)
Anti-mouse IgG antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ, USA)

Other materials

Alumina [Al_2O_3] (dry mesh size: 60-325) (Fisher Chemicals, Fair Lawn, NJ, USA)
Dowex AG-1X8 (dry mesh size: 100-200) (Bio-Rad Laboratories, Herkules, CA, USA)
Dowex AG 50W-X8 (dry mesh size: 200-400) (Bio-Rad Laboratories, Herkules, CA, USA)
Glass beads (425-600 microns, acid-washed) (Sigma, St. Louis, MO, USA)
Nitrocellulose membranes (0.45 μm) [Hybond-C Extra] (Amersham Pharmacia Biotech, Piscataway, NJ, USA)
Enhanced chemiluminescence [ECL] detection kit (NEN Life Sciences, Boston, MA, USA)

All other chemicals and materials used for SDS-PAGE and western blotting were from Bio-Rad Laboratories, Herkules, CA, USA.

All enzymes used for molecular cloning were from New England Biolabs, Beverly, MA, USA.

2.1.2 Media and buffer solutions

2.1.2.1 Mammalian cell culture related media and buffer solutions

DMEM

Dulbecco's modified Eagle's medium supplemented with

Glucose	4.5 g/l
Fetal calf serum (FCS)	10%
Penicillin G	100 I.U./ml
Streptomycin	100 µg/ml
Glutamine	4 mM

Transfection mixture

For 100 mm dish	
PBS (+Ca^{2+}/Mg^{2+})	850 µl
DEAE-dextran (10mg/ml)	55 µl
Plasmid-DNA	4 µg
For 150 mm dish	
PBS (+Ca^{2+}/Mg^{2+})	2125 µl
DEAE-dextran (10mg/ml)	238 µl
Plasmid-DNA	4 µg

Peptide binding buffer[$]

Tris-HCl (pH 7.4)	50 mM
$MgCl_2$	3 mM
EDTA	1 mM
BSA	0.1%
Bacitracin	0.1 mg/ml

cAMP buffers

Trichloroacetic acid (TCA) solution[$]

TCA	5 %
cAMP	1 mM
ATP	1 mM

Regeneration buffer (Dowex columns)[$]

HCl	1 M

Regeneration buffer (Alumina columns)[$]

Tris-HCl (pH 7.5)	100 mM

IP buffers

Glycerol phophate elution buffer[$]

Sodium borate	5 mM
Sodium formate	60 mM

Elution buffer[$]

Formic acid	0.1 M
Ammonium formate	0.2 M

[$] non-sterile solutions

Regeneration buffer (Dowex columns)[$]

Formic acid	0.1 M
Ammonium formate	3 M

2.1.2.2 Yeast related media and buffer solutions

YPD (YEPD) media

Bacto-yeast extract	1 %
Bacto-peptone	2 %
Dextrose	2 %

YPD (YEPD) plates

Bacto-yeast extract	1 %
Bacto-peptone	2 %
Dextrose	2 %
Bacto-agar	2 %

Synthetic complete (SC) and drop-out media

Yeast nitrogen base (YNB) w/o amino acids	0.67 %
Ammonium sulfate	0.5 %
Dextrose	2 %
Drop-out mix*	0.2 %

SC and drop-out plates

Yeast nitrogen base (YNB) w/o amino acids	0.67 %
Ammonium sulfate	0.5 %
Dextrose	2 %
Drop-out mix*	0.2 %
Bacto-agar	2 %

**Drop-out mix:*
Drop-out mix is a combination of the following ingredients minus the appropriate supplement. It should be mixed very thoroughly by turning end-over-end for at least 15 minutes; adding a couple of clean marbles helps.

Adenine	0.5 g	Leucine	10 g
Alanine	2 g	Lysine	2 g
Arginine	2 g	Methionine	2 g
Asparagine	2 g	para-Aminobenzoic acid	0.2 g
Aspartic acid	2 g	Phenylalanine	2 g
Cysteine	2 g	Proline	2 g
Glutamine	2 g	Serine	2 g
Glutamic acid	2 g	Threonine	2 g
Glycine	2 g	Tryptophane	2 g
Histidine	2 g	Tyrosine	2 g
Inositol	2 g	Uracil	2 g
Isoleucine	2 g	Valine	2 g

SE solution

Sorbitol	1 M
EDTA	100 mM
	pH 7.5

Lysis buffer

Tris-HCl	50 mM
EDTA	1 mM
PMSF	0.1 mM
for storage of yeast membranes also add	
Glycerol	10 %

PEG mixture for transformation

PEG 3550	40%
Lithium acetate	0.1 M

Lämmli sample buffer

Tris-HCl, pH 6.8	125 mM
Glycerol	20 %
Dithiotreitol (DTT)	100 mM
SDS	4 %
Bromphenol blue	0.01 %

Yeast peptide binding buffer[$]

Tris-HCl, pH 8.1	50 mM
$MgCl_2$	2 mM
EDTA	1 mM
BSA	0.1 %
Bacitracin	0.1 %

Yeast phosphate binding buffer[$]

Sodium phosphate	25 mM
$MgCl_2$	5 mM
	pH 7.4

All media, buffers, and solutions were made from commercially available chemicals of analytical grade purity and purified water (Millipore Milli-Q plus) or double distilled water. Reaction buffer for modification and elution/storage buffers for samples of DNA were prepared from ultrapure water. Unless otherwise noted, media and buffer solutions were either autoclaved or sterile filtered.

[$] non-sterile solutions

2.1.3 Gifts

The mammalian expression rat V1a-pcD-SP6/T7 was kindly provided by Michael Brownstein**,** Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, Maryland 20892, U.S.A.

The yeast strains used in this study (see chapter 2.2.2.5) were kindly provided by Mark Pausch, Molecular Genetic Screen Design, Wyeth-Ayerst Research, Princeton, NJ 08543-8000, U.S.A.

2.2 Methods

2.2.1 Mammalian expression technologies

2.2.1.1 Genetic engineering

2.2.1.1.1 Mammalian expression plasmid pcD

The coding sequences of the rat V1a and human V2 vasopressin receptors were inserted into a pcD-based expression vector (Okayama and Berg, 1983), resulting in V1a-pcD-SP6/T7 (Morel *et al.*, 1992) and V2pcD/PS (Schöneberg *et al.*, 1996), respectively. The pcD vectors are mammalian expression vectors that contain a Simian virus 40 (SV40) origin of replication, an SV40 promoter and an SV40 polyadenylation sequence. The binding of the SV40 large T-antigen to the SV40 origin of replication drives replication of the plasmid. Therefore, the pcD vector can replicate to high copy number in mammalian cell lines that constitutively express the SV40 large T-antigen. Thus, the pcD vector produces high levels of transient expression of receptor genes in COS-7 cells, a mammalian cell line that has been SV40 transformed.

The pcD vector also contains a pBR322 origin of replication and an ampicillin-resistance gene. These two features allow the propagation of the plasmid in *E.coli* and selection of the plasmid by the presence of ampicillin.

2.2.1.1.2 Construction of mammalian expression plasmids

The construction of the various chimeric V1a/V2 and mutant vasopressin receptors expressed from the mammalian pcD vector is described in chapter 3.1.2 (the precise amino acid composition of the individual V1a/V2 hybrid receptors is given in Table 3.1 and Fig. 3.1, chapter 3.1). Standard molecular biology techniques (Sambrook *et al.*, 1989) were used for generating cDNA constructs in mammalian expression vectors. Enzymes that were used for genetic engineering of plasmid DNA were purchased from New England Biolabs (Beverly, MA, USA) or BRL (Gaithersburg, MD, USA). The oligonucleotides were obtained from Gibco-BRL (Grand Island, NY, USA).

Oligonucleotides longer than 50 bases were purified on a 6% polyacrylamide gel as described by Sambrook *et al.* (1989). DNA fragments generated by polymerase-chain-reaction (PCR) or digestion with restriction enzymes were gel-purified (0.7-1.0% agarose gel) for subcloning purposes and extracted using the Geneclean^R II gel purification kit from Bio101 (Vista, CA, USA). For cDNA cloning, DNA fragments were ligated and the resulting ligation mixture was transformed into competent DH5α cells prepared by the $CaCl_2$ method (according to Inoue *et al.*, 1990). Plasmid DNA was purified from bacterial cultures by using the Qiagen Miniprep or Maxiprep kit (Chatsworth, CA, USA). The identity of all mutant constructs and the correctness of all PCR-derived coding sequences were verified by dideoxy sequencing of the mutant plasmids (Sanger *et al.*, 1977) and restriction enzyme analysis.

2.2.1.2 Cell culture

Expression plasmids coding for the wt V1a, wt V2 and mutant V2 receptors were individually expressed in COS-7 cells, which lack endogenous vasopressin receptors, as determined in radioligand binding studies conducted with COS-7 cells transfected with the empty pcD vector (also see chapter 2.2.1.5)

COS-7 cells were obtained from the American Type Culture collection (Rockville, MD, USA). COS-7 is a fibroblast-like cell line derived from CV-1 simian cells (African green monkey kidney; ATCC CCL 70) transformed by an origin-defective mutant of SV40 which codes for the wt T antigen. Cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 I.U./ml penicillin G, 100 µg/ml streptomycin, and 4 mM glutamine at 37°C in a humidified 5% CO_2 incubator.

2.2.1.3 Transient expression of cloned receptors in COS-7 cells

For transfections, 1 x 10^6 cells were seeded into 100 mm dishes. About 24 h later, cells were transfected with the various vasopressin receptor constructs (4 µg of plasmid

DNA per dish) by a diethylaminoethyl-(DEAE)-dextran method (Cullen, 1987). Plasmid DNA was mixed with 850 µl PBS(+Ca^{2+}/Mg^{2+}) and 55 µl DEAE-dextran (10mg/ml). Prior to transfection, culture dishes were rinsed twice with PBS (+Ca^{2+}/Mg^{2+}). After aspirating the washing buffer, the transfection mixture was added dropwise to the culture dishes. During a subsequent 3-hr incubation, the dishes were gently shaken in 10-min intervals to prevent the cells from drying out. Then, 7 ml of medium supplemented with 80 µl chloroquine (Sigma, St. Louis, MO, USA) were added and the cells were incubated for another 4 hrs at 37°C (5% CO_2). Eventually, the incubation mix was aspirated and fresh medium was added. Cells were harvested about 48 or 72 hrs after transfection to be used for functional experiments or radioligand binding studies, respectively (see below).

2.2.1.4 Preparation of membrane homogenates

Transfected cells were washed once with PBS (w/o Ca^{2+}/Mg^{2+}) and once with ice-cold peptide binding buffer (50 mM Tris (pH 7.4), 3 mM $MgCl_2$, 1 mM EDTA, 0.1% bovine serum albumin, and 0.1 mg/ml bacitracin). Then, cells were scraped into 5 ml of ice-cold binding buffer and homogenized for 30 sec (setting: 5) using a Brinkmann Homogenizer (Brinkmann Instruments, Westbury, NY, USA). Protein concentrations were determined according to Bradford (1976) using a Bio-Rad (Richmond, CA, USA) protein assay kit. Membranes were used fresh for radioligand binding assays.

2.2.1.5 Radioligand binding assays

For radioligand binding studies, COS-7 cells were harvested approximately 70-72 hrs after transfections, and membrane homogenates were prepared as described above. Binding buffer consisted of 50 mM Tris (pH 7.4), 3 mM $MgCl_2$, 1 mM EDTA, 0.1% bovine serum albumin, and 0.1 mg/ml bacitracin. Assays were conducted in duplicate in the presence of the radioligand, [^{3}H]-8-arginine vasopressin ([^{3}H]AVP, 59 Ci/mmol; Dupont NEN), in a 0.5 ml volume. For saturation binding studies, six different concentrations of [^{3}H]AVP (0.125-4 nM) were used. In some cases, membrane

homogenates were incubated with only two saturating concentrations of [^{3}H]AVP, 4 and 8 nM. Since the two concentrations detected similar numbers of [^{3}H]AVP binding sites for each individual mutant receptor, binding activities obtained with 8 nM [^{3}H]AVP could be considered approximations of B_{max} values. Specific binding was defined as the difference in [^{3}H]AVP binding determined in the absence (= total binding) and presence of 5 µM AVP (nonspecific binding). No specific [^{3}H]AVP binding activity could be detected with membranes prepared from vector-transfected COS-7 cells.

Incubations were carried out for 1 hr at room temperature (22°C). Binding reactions were terminated by rapid filtration through a Brandel cell harvester onto Whatman GF/C filters (Brandel, Gaithersburg, MD, USA). Membranes were washed three times with 5 ml of ice-cold binding buffer, dried, transferred to 7 ml of scintillation fluid (Biosafe II^R, RPI, Mount Prospect, IL, USA), and counted in an LKB liquid scintillation counter (Pharmacia, Gaithersburg, MD, USA) at a counting efficiency of 59%.

Data from saturation binding studies were analyzed according to the one-site model of binding:

$$L + R \underset{K_2}{\overset{K_1}{\Leftrightarrow}} LR$$

R = receptor; L = ligand; K_1 = association rate constant; K_2 = dissociation rate constant

According to the law of mass action, at equilibrium: $K_2\,[RL] = K_1\,[L] \cdot [R]$

and with $K_2/K_1 = K_D$ = equilibrium dissociation constant (molar units)

$K_1/K_2 = K_A$ = equilibrium association constant (units of molar^{-1})

$[RL] = K_A\,[R] \cdot [L]$

Definitions: [R] = concentration of unoccupied receptors

[L] = free ligand concentration, later denoted as *F*

[RL] = concentration of ligand-receptor complex. Since this, by definition, is equal to the concentration of ligand **bound** to R, [RL] is later denoted as *B*

$[R_{TOT}]$ = total receptor concentration = B_{max}

The concentration of occupied receptor [RL] can be calculated from the equation:

$$[R_{TOT}] = [R] + [RL]$$

Rearrangement gives:

$$[RL] = \frac{[R_{TOT}] \cdot K_A \cdot [L]}{1 + K_A [L]}$$

or with $K_A = 1/K_D$

$$[RL] = \frac{[R_{TOT}] \cdot [L]}{K_D + [L]}$$

K_D is the concentration of ligand that half-maximally occupies the receptor $[RL] = [R_{TOT}]/2$.

Scatchard plot

Rearrangement and transformation of the equation above to a linear expression y = mx + *b* gives:

$$B/F = -1/K_D \cdot B + B_{max}/K_D$$

Consequently, when a single ligand is interacting with a single population of receptors possessing a single affinity for the ligand, a plot of *B*/*F* versus *B* is a straight line and possesses a slope of $-1/K_D$. The x intercept (y = 0) is an estimate of B_{max}.

The computer program LIGAND (Munson and Rodbard) was used to calculate the dissociation constant K_D and the binding capacity B_{max}, fitting the specific and non-specific binding as determined from saturation experiments.

2.2.1.6 cAMP Assays

Approximately 20-24 hrs after transfections, COS-7 cells were transferred into six-well plates (about 0.3-0.4 x 10^6 cells/well), and 2 µCi/ml of [^{3}H]adenine (18 Ci/mmol; American Radiolabeled Chemicals Inc.) were added to the growth medium.

Determination of adenylyl cyclase activity was then performed according to Salomon *et al.* (1974). After a 24-hr labeling period, cells were preincubated in Hanks' balanced salt solution containing 20 mM Hepes and 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 20 min (37°C). IBMX serves as competitive inhibitor of nucleotide phophodiesterases and prevents degradation of cAMP. After the 20-min incubation, the cells were stimulated with 1 mM AVP for 30 min at 37°C (total volume per well: 1 ml). The incubation time was shown to be within the linear range of cAMP accumulation (Liu and Wess, unpublished results). To generate complete concentration-response curves, seven different concentrations of AVP (ranging from 10^{-13} to 10^{-6} M) were used. Incubations were terminated by aspiration of medium and addition of 1 ml ice-cold 5% trichloroacetic acid denaturing all proteins containing 1 mM ATP and 1 mM cAMP. Increases in intracellular [^{3}H]cAMP levels were then determined by anion exchange chromatography. The aqueous phase was added to columns containing 1 ml AG 50W-X8 resin (dry mesh size: 200-400). The columns were washed with 3 ml dH_2O, placed over a set of columns containing 1 ml alumina (Al_2O_3, dry mesh size: 60-325) and eluted with 10 ml dH_2O. Finally, [^{3}H]cAMP was eluted from the alumina columns with twice 3 ml Tris-HCl (100 mM, pH 7.5) into scintillation vials, mixed with 13 ml Hydrofluor (National Diagnostics, Atlanta, GA, USA) and counted in an LKB liquid scintillation counter (Pharmacia, Gaithersburg, MD, USA) at a counting efficiency of 59%.

2.2.1.7 PI Assays

About 20-24 hrs after transfections, cells were split into six-well dishes (approximately 0.3-0.4 x 10^6 cells/well) and labeled with 3 μCi/ml [^{3}H]myo-inositol (20 Ci/mmol; American Radiolabeled Chemicals Inc.). After a 20-24-hr labeling period, cells were preincubated for 20 min at room temperature with 2 ml of Hanks' balanced salt solution containing 20 mM Hepes and 10 mM LiCl, which inhibits the action of inositol monophophatases (Berridge *et al.*, 1982), thus preventing the recycling of inositol. Cells were then stimulated with 1 mM AVP for 1 hr at 37°C (total volume per well: 1 ml). The incubation time was shown to be within the linear range of the assay (Liu and Wess,

unpublished results). Incubations were terminated by aspiration of medium. Cells were lysed by addition of 750 µl ice-cold formic acid (20 mM), followed by a 35-min incubation at 4°C. The incubation mixture was neutralized by addition of 250 µl of amonium hydroxide (60 mM) and increases in intracellular inositol monophosphate (IP_1) levels were determined by anion exchange chromatography (Berridge *et al.*, 1982) using columns containing 1 ml of Dowex AG-1X8 resin (dry mesh size: 100-200). After washing the columns with glycerole phophate elution buffer, [^{3}H]IP_1 was eluted with twice 2 ml of IP_1-elution buffer (0.1 M formic acid, 0.2 M ammonium formate), mixed with 15 ml Hydrofluor (National Diagnostics, Atlanta, GA, USA) and counted in an LKB liquid scintillation counter (Pharmacia, Gaithersburg, MD, USA) at a counting efficiency of 59%.

2.2.2 Yeast expression technologies

2.2.2.1 Genetic engineering

2.2.2.1.1 Yeast expression plasmid p416GPD

The yeast expression plasmid p416GPD is based on the pRS4XX vector series of standard yeast expression plasmids (Sikorski and Hieter, 1989). Basically, the pRS4XX vectors contain either no replication element (= integrating plasmid, pRS4**0**X) or a replication element for single copy (CEN: pRS4**1**X) or multicopy (2µ: pRS4**2**X) propagation in yeast. Furthermore, they contain selectable markers that allow maintenance of the yeast expression plasmids in yeast strains that are auxotrophic for these markers: *HIS3* (pRS4X**3**), *TRP1* (pRS4X**4**), *LEU2* (pRS4X**5**), or *URA3* (pRS4X**6**). The yeast expression plasmid p416GPD contains the powerful yeast *GPD* promoter (*GPD*: gene encoding glyceraldehyde-3-phosphate dehydrogenase) and the *CYC1* terminator (*CYC*: gene encoding cytochrome-c oxidase), which are inserted into the polylinker region between *KpnI* and *XhoI* (*GPD* promoter) and *XbaI* and *SacI* (*CYC1* terminator) (Mumberg *et al.*, 1995) (Fig. 2.1).

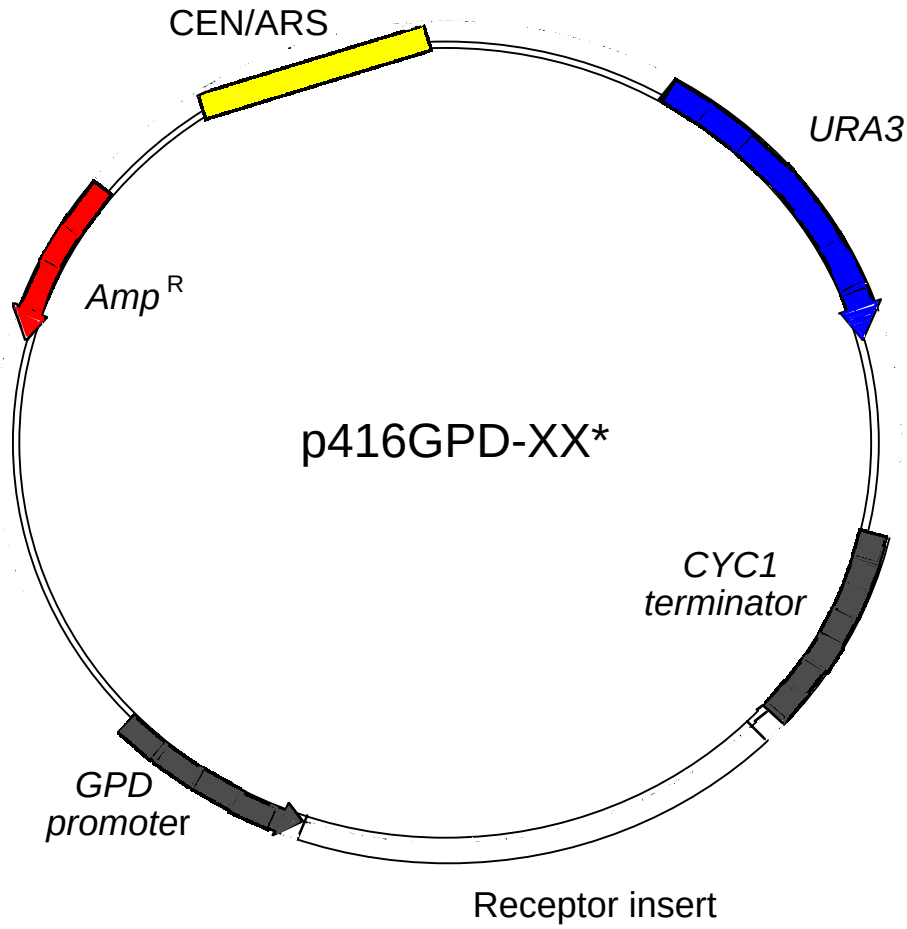


Fig. 2.1 Features of the expression plasmid, p416GPD, used for the heterologous expression of muscarinic receptors in yeast (*S. cerevisiae*). The p416GPD was developed by Mumberg et al. (1995) based on pRS416 (Christianson et al., 1992). It is a single copy plasmid containing the CEN/ARS element, the *URA3* gene as selectable marker for maintenance in yeast, and the *Amp* gene for propagation in *E. coli*. The *GPD* promoter and the *CYC1* terminator flank the receptor insert. Different muscarinic receptor sequences were cloned into the polylinker of p416GPD as indicated under "Materials and Methods". *XX: receptor insert.

To allow propagation in *E. coli*, the pRS-based yeast expression plasmids contain the *Amp* resistance gene. Since miniprep preparations made directly from yeast yield only minute amounts of DNA and transformation into *E. coli* is an indispensable step to recover yeast vectors for further manipulations such as subcloning, sequencing, and

transformation of yeast strains. Since these expression plasmids can be propagated both in yeast and *E. coli*, they are also called "shuttle vectors".

2.2.2.1.2 Construction of wt V2 and mutant V1a/V2 vasopressin receptor plasmids

The p416GPD-based yeast expression plasmid coding for the human V2 vasopressin receptor was constructed using standard molecular biological techniques. A 1.26 kb *EcoR*I-*Xho*I fragment containing the human V2 receptor coding sequence was cut out from the mammalian expression plasmid, hV2-pcD (Schöneberg *et al.*, 1996), and cloned into the corresponding sites in the polylinker of the yeast expression plasmid, p416GPD (Fig. 2.1), resulting in hV2-p416GPD. The final V2 receptor expression construct, hV2-p416GPD, contained 6 basepairs (bp) of 5' untranslated sequence (CCCACC) following the *EcoR*I cloning site. The translation start codon was followed by a 27 bp sequence coding for the hemagglutinin (HA) epitope tag (YPYDVPDYA) (Schöneberg *et al.*, 1996). Previous studies showed that the presence of the HA tag did not affect the ligand-binding and G protein-coupling properties of the V2 vasopressin receptor (Schöneberg *et al.*, 1996**,** Liu and Wess, 1996). The hV2-p416GPD construct also contained 125 bp of 3' untranslated sequence followed by the *Xho*I cloning site.

Mutant V2 receptor genes were constructed employing the "gap-repair" method by Raymond *et al.* (1998) as described below (chapter 2.2.2.2). The precise amino acid composition of the individual V2 receptor mutants is given in Table 3.6**,** chapter 3.2. The identity of all receptor constructs was confirmed by restriction enzyme analysis, and the correctness of all PCR-derived sequences was verified by dideoxy sequencing.

2.2.2.1.3 Construction of wt and mutant muscarinic receptor plasmids

All muscarinic receptor coding sequences were cloned into the polylinker of the yeast expression plasmid, p416GPD (Fig. 2.1). Specifically, the human M_1, rat M_3, and human M_5 muscarinic receptor coding sequences were cut out from Hm1pcD (Bonner *et al.*, 1988; Dörje *et al.*, 1991), Rm3pcD-N-HA (Bonner *et al.*, 1997; Schöneberg *et al.*,

1995), and Hm5pcDp2 (Bonner *et al*., 1988) and inserted into the polylinker of p416GPD, yielding pJW36 (p416GPD-M_1), pMP289 (p416GPD-M_3), and pJW38 (p416GPD-M_5), respectively. The following cloning sites present in the p416GPD polylinker were used for cloning purposes: M_1 and M_5, *Spe*I and *Sma*I; M_3, *Spe*I and *BamH*I.

To improve the efficiency of translation initiation in yeast (Price *et al*., 1995, 1996), the 5' flanking sequences of all receptor inserts were removed and replaced with a AAA triplet by using standard PCR mutagenesis techniques (Higuchi, 1989). The receptor inserts still contained 3' flanking sequences of differing lengths (M_1, 32 bp; M_3, 437 bp; M_5, 187 bp). The M_3 receptor construct contained a nine-amino acid HA epitope tag (YPYDVPDYA) inserted after the initiating methionine codon (Schöneberg *et al*., 1995). Previous studies have shown that the presence of the HA tag has no effect on the ligand-binding and G protein-coupling properties of the M_3 muscarinic receptor (Schöneberg *et al*., 1995).

An additional set of yeast expression constructs were generated that encoded M_1, M_3, and M_5 muscarinic receptors in which major portions of their i3 loops had been deleted by standard PCR mutagenesis techniques (Higuchi, 1989). The resulting expression plasmids are referred to as pJW37 (p416GPD-$M_1\Delta$i3), pMP290 (p416GPD-$M_3\Delta$i3), and pJW39 (p416GPD-$M_5\Delta$i3). The encoded receptor proteins are designated $M_1\Delta$i3, $M_3\Delta$i3, and $M_5\Delta$i3, respectively. Specifically, the following receptor segments were removed in the different deletion constructs (see also Fig. 3.18): $M_1\Delta$i3, Pro231-Gly345; $M_3\Delta$i3, Ala274-Lys469; $M_5\Delta$i3, Thr237-Pro413. Except for the i3 loop deletions, the sequences of the $M_1\Delta$i3, $M_3\Delta$i3, and $M_5\Delta$i3 yeast expression plasmids were identical to those coding for their full-length receptor counterparts. The identity of all receptor constructs was confirmed by restriction enzyme analysis, and the correctness of all PCR-derived sequences was verified by dideoxy sequencing.

2.2.2.2 Manipulation of yeast expression plasmids employing the "gap-repair" method

Mutations were introduced into yeast expression plasmids by using the "gap-repair" method (Raymond *et al.*, 1999), taking advantage of the high rate of homologous recombination occurring in yeast. When a yeast expression is linearized ("gapped") within the region target for mutagenesis and co-transformed into yeast with a mutagenized PCR fragment that contains at either side at least 30 bp unmutagenized sequence homologous to vector sequence, the PCR fragment replaces the corresponding or missing region of the plasmid via homologous recombination (Fig. 2.2). This recombination is highly efficient with at least 80-90% of the recovered circular plasmids containing the desired PCR fragment (Sommers and Dumont, 1999; Raymond *et al.*, 1999). The use of linearized vectors requires only one restriction site as long as the cut lies well within the sequence of the PCR product (Sommers and Dumont, 1999; Raymond *et al.*, 1999). Cloning of yeast expression plasmids using homologous recombination can also be done using uncut vector and PCR fragment (Raymond *et al.*, 1999). In this case, homologous recombination is somewhat less efficient than with the use of cut plasmids, yet this method allows cloning independent of restriction sites.

Linearized ("gapped") plasmid DNA was dephosphorylated and gel purified to diminish re-circularization of the vector backbone. Also, prior to transformation the mutagenized PCR fragment was purified using the Qiagen PCR purification kit (Chatsworth, CA, USA). For cloning purposes 0.1 µg of both plasmid DNA and PCR fragment were used for co-transformation experiments. For details about the application of this method for generating mutant V2 receptor libraries employing random mutagenesis techniques, see chapter 2.2.2.6.

After co-transformation and subsequent homologous recombination of linearized plasmid and PCR fragment in yeast, the circular yeast expression plasmids were recovered (for details, see chapter 2.2.2.3) and transformed into competent DH5α cells. Plasmid DNA was purified from bacterial cultures by using the Qiagen Miniprep kit

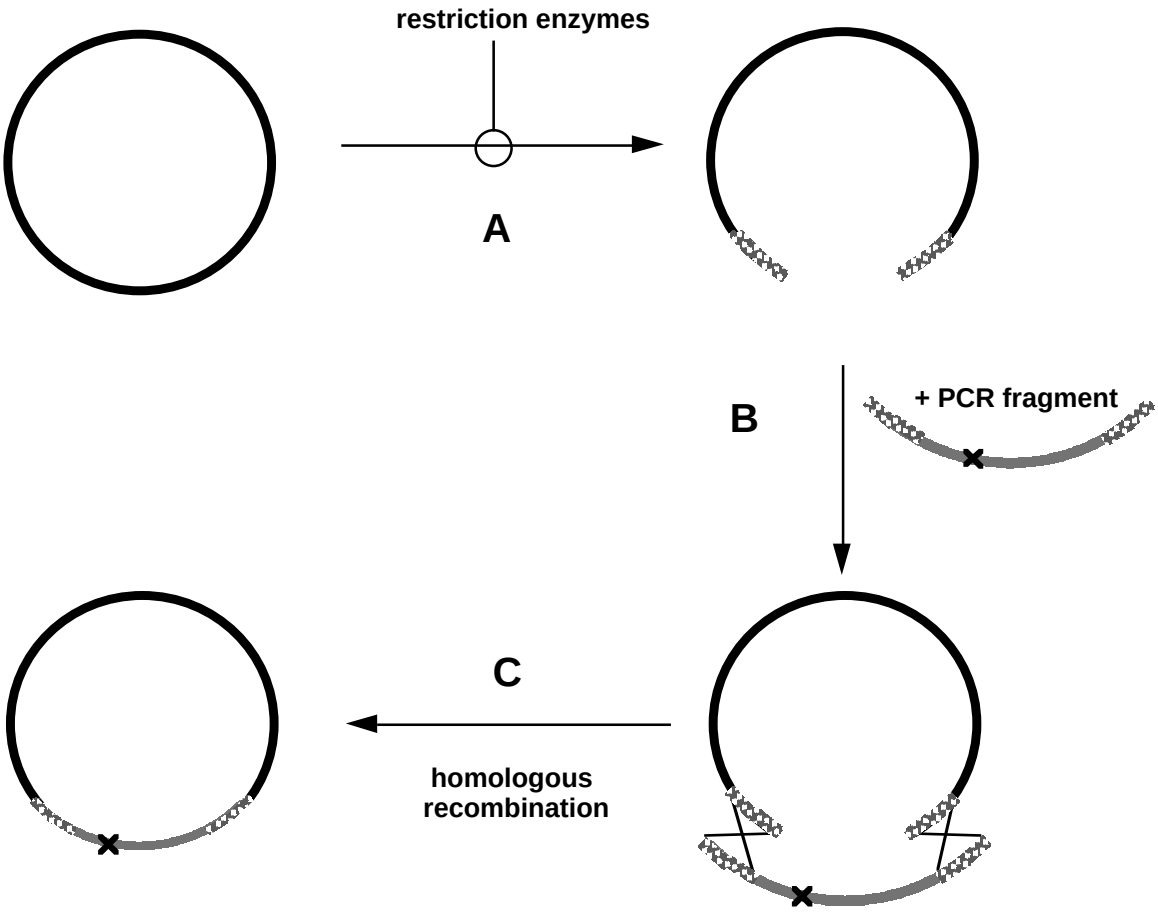


Fig. 2.2 Manipulation of yeast expression plasmids employing the "gap-repair" method (Raymond *et al.*, 1999). *A,* A yeast expression plasmid is linearized (cut with one restriction enzyme) or "gapped" (cut with two restriction enzymes). The procedure works with single-cut plasmid as long as the cut lies well within the sequence of the PCR product. When uncut vector is used, the recombination efficiency tends to be lower. *B,* Linearized vector is co-transformed with a PCR fragment that contains the desired mutational alterations (**X**) by employing the lithium method described by Gietz and Wood (1994). The PCR fragment to be incorporated into the vector should contain at least 30 bp homologous vector sequence at either side. *C,* In yeast, the PCR fragment replaces the corresponding or missing region of the plasmid via homologous recombination. This method can be applied for subcloning purposes as well as for creating libraries of mutant proteins generated by random PCR mutagenesis.

(Chatsworth, CA, USA). The identity of all mutant constructs and the correctness of all PCR-derived coding sequences were verified by dideoxy sequencing of the mutant plasmids (Sanger *et al.*, 1977).

2.2.2.3 Transformation of yeast strains

DNA-mediated yeast transformation was carried out using a lithium acetate (LiAc) method (Gietz and Woods, 1994). For transformation, yeast cells were grown in rich medium (YPD medium containing **y**east extract, **p**eptone, and **d**extrose) and harvested during the logarithmic growth phase (OD: 0.5-1; 1-2x10^7 cells/ml). Cells were spun down, washed once with dH_20 and resuspended in 0.1 M LiAc to a concentration of 2 x 10^9 cells per ml. Incubation of this cell suspension in a 30°C water bath for 15 min yielded competent yeast cells. 10 µl of the cell suspension was mixed with p416GPD-based receptor expression constructs and/or PCR fragments to be transformed and a freshly prepared PEG mixture (1 ml PEG mixture contains: 800 µl 50% PEG 3550, 100 µl 1 M LiAc, 100 µl dH_20) was added at a ratio of 1:6 (µg cDNA : µl PEG mixture). This suspension was incubated at 30°C for 30 min and then heatshocked at 42°C for 10 min. For better recovery, cells were spun down after the incubation, the transformation solution was aspirated, and cells were resuspended in synthetic complete medium (SC) lacking uracil. Finally, the cell suspension was plated onto SC plates lacking uracil. Following a 72-hr incubation at 30°C individual transformants were isolated. Three individual transformants from each transformation were chosen for further analysis.

2.2.2.4 Yeast miniprep

Yeast strains were grown in 10 ml of the appropriate SC medium that allowed maintenance of the yeast expression plasmid to be extracted, to a density of 10^7 cells/ml (OD: 0.5). Cells were harvested by centrifugation at 4000 g for 5 min, washed twice with dH_20 and resuspended in 1 ml of SE (1 M sorbitol, 100 mM EDTA, pH 7.5). In order to generate spheroblasts, 10 U of lyticase, an enzyme that removes the cell wall, was added. The cell suspension was incubated at 37°C for 1 to 2 hrs until at least 70% of the yeast cells were transformed into spheroblasts as confirmed by microscopy. Spheroblasts were then carefully spun down (3,000 x g for 1 min), the aqueous layer was aspirated, and the cell pellet was processed using a Qiagen Miniprep kit (Chatsworth, CA, USA) according

to the manufacturer's instructions. 0.5 µl of the final yeast miniprep were transformed into 150 µl of competent DH5α cells. Plasmids were recovered from bacterial cultures using the Qiagen Miniprep kit (Chatsworth, CA, USA).

2.2.2.5 Yeast strains used in this study

The genotypes of the yeast (*S. cerevisiae*) strains used in this study (MPy578fc, MPy578q5, and MPy578s5) are summarized in Table 2.1. These strains are derived from a typical laboratory yeast strain, which was genetically engineered in order to establish a sensitive bioassay to study mammalian GPCRs in yeast. A key feature of this strain is that yeast growth requires activation of the yeast pheromone pathway. Specifically, the following genetic modifications were introduced into a typical laboratory yeast strain (Table 2.1, also see Fig. 3.13, chapter 3.2):

3 Cell cycle arrest in response to pheromone is mediated by the product of the *FAR1* gene. Deletion of this gene therefore allows for continued growth and transcriptional induction of pheromone-responsive genes in the presence of an activated mating pathway.

4 The Sst2p protein is involved in mediating desensitization of the activated yeast pheromone pathway. Therefore, deletion of the *SST2* gene results in hypersensitivity to the presence of pheromone and inability to recover from pheromone-induced cell cycle arrest.

5 The *FUS1* gene, which encodes a pheromone-inducible membrane protein, was replaced with a reporter gene construct made by fusing the *FUS1* promoter to *HIS3* coding sequence, thereby placing expression of His3p under the control of the pheromone pathway. Thus, receptor stimulation by an agonist leads to activation of the pheromone response cascade, induction of the *FUS1* promoter, and increased His3p expression, permitting growth of auxotrophic (*his3*) yeast strains on medium lacking histidine (Price *et al.*, 1995, 1996).

6 The endogenous yeast GPCR gene (*STE2*) was deleted, making it possible to study the function of heterologously expressed mammalian GPCRs in a 'clean' background.

The construction of MPy578fc has been described previously (Pausch *et al.*, 1998). MPy578q5 and MPy578s5 were derived from MPy578fc by using standard yeast mutagenesis techniques (see below). All three strains are isogenic except for the *GPA1* gene coding for the yeast G protein α subunit. MPy578fc contains the wt *GPA1* gene, whereas MPy578q5 and MPy578s5 harbor genomically integrated mutant versions coding for hybrid Gpa1 proteins in which the last five amino acids of Gpa1p (KIGII) were replaced with the homologous mammalian $G\alpha_q$ (EYNLV) and $G\alpha_s$ (QYELL) sequences, respectively (Hadcock and Pausch, manuscript in preparation).

Table 2.1 ***Genotypes of yeast strains used in this study***

Strain	Genotype
MPy578fc	*MAT**a** GPA1 far1::LYS2 fus1::FUS1-HIS3 sst2::SST2-G418^r ste2::LEU2 fus2::FUS2-CAN1 ura3 lys2 ade2 his3 leu2 trp1 can1*
MPy578s5	same as MPy578fc, with *gpa1::Gs5*[a]
MPy578q5	same as MPy578fc, with *gpa1::Gq5*[a]

[a]The structure of the encoded mutant Gpa1 proteins are shown in Fig. 3.14.

A two-step integrative replacement strategy (Rothstein, 1991) was employed to generate strains MPy578q5 and MPy578s5. A 2.6 kb *Eco*RI-*Sal*I fragment of the *GPA1* gene was cloned into the corresponding sites in the *URA3*-containing integrating plasmid, YIp5 (Rothstein, 1991). Sequences coding for the five C-terminal amino acids of Gpa1p were replaced with the corresponding sequences from $G\alpha_s$ and $G\alpha_q$ by oligonucleotide-mediated mutagenesis. The resulting plasmids were used to transform Mpy578fc to ura prototrophy under conditions in which the plasmids should integrate into the *GPA1* locus. Chromosomal DNA isolated from resulting ura+ transformants was examined for integration of the plasmids at the *GPA1* locus by PCR. Positive strains were patched onto 5-fluoroorotic acid plates to select for loss of the *URA3* gene (Rothstein, 1991). Chromosomal DNA was isolated from the resulting ura-revertants and checked from replacement of the C-terminal coding sequences by PCR.

The use of chimeric Gα-subunits proved advantageous since yeast strains transformed with wt mammalian Gα subunits were characterized by excessive background growth (growth even in the absence of ligand). A possible explanation for this observation is that mammalian Gα-subunits show reduced affinity to the yeast βγ-complexes (Pausch *et al.*, 1998), leading to constitutive activation of the yeast pheromone pathway due to the presence of free βγ-complexes (for details, see chapter 3.2.2).

2.2.2.6 Random mutagenesis strategy and creation of mutant receptor library

2.2.2.6.1 Library construction

To construct a mutant V2 receptor library, the "gap-repair" method by Raymond *et al.* (1998) was applied (Fig. 2.2). hV2-p416GPP was digested with *BstX*I, which removed a 52 bp fragment fragment corresponding in sequence to most of the i2 loop from the hV2-p416GPD construct. The PCR fragment was generated by an oligonucleotide-directed random mutagenesis approach was employed which introduced mutations into the V2 receptor region coding for the i2 loop. The following oligonucleotide coding for V2 receptor residues 138-160 was used (Genemed, S.San Francisco, CA; underlines denote bases doped with 10% non-wt nucleotides): ACG CTG

GAC CGC CAC CGT GCC ATC TGC CGT CCC ATG CTG GCG TAC CGC CAT GGA AGT GGG GCT CAC TGG AAC CGG CCG GTA CTA GTG GCT TGG. The underlined mutagenized sequence codes for amino acids His138 to Val160. This oligonucleotide was used as a primer for PCR amplification together with oligonucleotide ON2 (5'-GGC CCA GCA GTC AGT GAC CCC GCT GCC ACC TTC CAC GTT GCG CTG-3'; corresponding in sequence to amino acid codons 180-194) as anti-sense primer and hV2-p416GPD as a template. The resulting 183 bp PCR fragment was then used as a template for a second round of PCR using a sense primer that overlapped with the first 12 nucleotides of the mutagenic oligonucleotide (ON30; 5'-CAG ATG GTG GGC ATG TAT GCC TCC TCC TAC ATG ATC CTG GCC ATG ACG CTG GAC CGC-3'; corresponding in sequence to amino acid codons 119-137) and ON2 as the antisense primer. The resulting 228 bp PCR fragment contained 57 bp of wt sequence 5' and 102 bp of wt sequence 3' of the mutagenized sequence, respectively.

For construction of the mutant receptor library 0.2 µg of linearized hV2-p416GPD was co-transformed with 0.2 µg of PCR product into MPy578q5 following the transformation protocol described in chapter 2.2.2.3. Following transformation into yeast, the ends of the mutagenized PCR fragment recombined with the homologous ends of the gapped receptor plasmid to regenerate circular receptor expression plasmids (Muhlrad *et al*, 1992; Staples and Diekman, 1992)(Fig. 2.2).

To assess the quality of the mutant V2 receptor library, the plasmids of 15 randomly picked colonies were recovered. Sequencing of the mutagenized region resulted in a mutagenesis rate of 9%, which was in good agreement with the predicted rate (10%). The mutations were evenly distributed, suggesting that no bias occurred within the targeted receptor region.

2.2.2.6.2 Receptor selection

In order to screen for mutant V2 receptors with altered G protein coupling preferences, conditions were established that allowed V2 receptor/Gs5 co-expressing yeast strains to grow in an AVP-dependent fashion on SD-ura-his plates, but did not

allow a hormone-dependent growth response of V2 receptor/Gq5 co-expressing strains. First, to suppress background growth, various concentrations of 3-amino-1,2,4-triazol (AT) were tested. AT is a competitive inhibitor of the *HIS3* gene product, imidazole glycerolphosphate dehydratase, which is being produced at low level due to low basal constitutive activity of the *FUS1* promoter. The presence of 10 mM AT proved to efficiently suppress background growth. Yeast strain carrying the empty vector (p416GPD) were unable to grow in the presence of 10 mM AT (Fig. 2.3, first row). Moreover, various concentrations of AVP were tested to assess a concentration of hormone that allowed growth of V2 receptor/Gs5 co-expressing yeast strains but not of the V2 receptor and the Gq5 chimeric G protein. A concentration of $4x10^{-7}$ M AVP was sufficient to stimulate growth of V2 receptor/Gs5 co-expressing yeast cells. No background growth could be observed in the absence of ligand. Also, under identical conditions, V2 receptor/Gq5 co-expressing yeast cells were unable to grow (Fig. 2.3, second row). Thus, replica plating of Gq5-expressing yeast colonies transformed with a randomly mutagenized V2 receptor library under the conditions described above allowed screening for mutant V2 receptors that would be able to activate Gq5.

Specifically, the MPy578q5-based yeast expression library coding for mutant V2 vasopressin receptors was plated onto uracil-deficient SC medium to select for ura+ (plasmid-containing) transformants. To facilitate replica plating and subsequent isolation of positive colonies, the transformation mix was plated at a density of 200-300 colonies per 100 mm plate. After incubation of plates for three days at 30 ºC, ura+ colonies were transferred, via replica plating, onto plates containing SC medium lacking both uracil and histidine, but containing 10 mM AT and 0.4 µM of the agonist, AVP (an AVP stock solution was spread over the plates after the agar had cooled and hardened). For control purposes, the primary ura+ transformants were also replica-plated onto plates containing the identical selection medium but lacking AVP.

A total of approximately 30,000 yeast clones expressing mutant V2 receptors were screened. Plasmids were isolated from colonies that were able to grow, in an AVP-dependent fashion, on histidine-deficient medium, amplified in *E. coli*, and retransformed

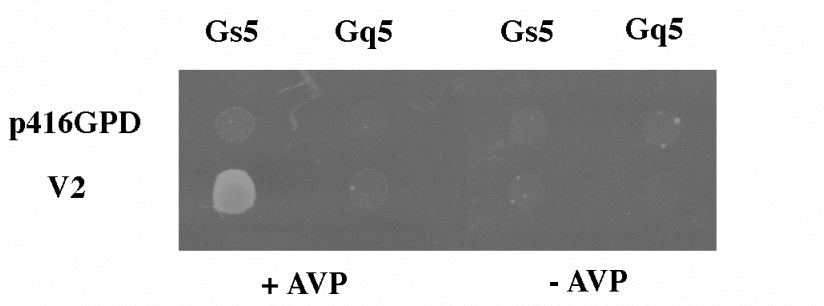


Fig. 2.3 Conditions used for screening a random mutant V2 receptor library. Yeast strains expressing either Gs5 or Gq5 were transformed with empty vector p416GPD (*row 1*) or with hV2-p416GPD (*row 2*). 5 µl of yeast cell suspensions (OD: 0.1) were dotted onto SD-ura-his plates. To suppress background growth the plates were supplemented with 10 mM AT, a competitive inhibitor of the *HIS3* gene product (see text for details). The SD-ura-his plates contained either no hormone (-AVP) or $4x10^{-7}$ M AVP (+AVP). Plates were incubated at 30°C for 3 days.

into MPy578q5 to confirm that the observed growth phenotype was indeed due to plasmid-borne V2 receptor mutations. Mutant receptor plasmids were then sequenced and, at least in some cases, re-created through site-directed mutagenesis to be used for further studies.

2.2.2.7 Liquid bioassays

Yeast strains were cultured overnight in SC medium lacking uracil. Cells were then washed in PBS and diluted to a concentration of 10^5 cells/ml in 3 ml SC medium lacking uracil and histidine (pH 6.9). To suppress basal yeast growth, different concentrations of AT, a competitive inhibitor of the *HIS3* gene product (imidazole glycerolphosphate dehydratase), was added to the growth medium (8 mM AT in the case of the V2 vasopressin receptor-expressing strains, 9 mM in the case of muscarinic receptor-expressing MPy578fc and MPy578q5 strains, and 18 mM in the case of muscarinic receptor-expressing MPy578s5 strains). Subsequently, aliquots of yeast cell

suspensions (180 µl) were dispensed into wells of sterile 96-well microtiter dishes containing 20 µl of serially diluted samples of AVP or carbachol, respectively (final concentrations: 10^{-4} – 10^{-13} M). The plates were incubated at 25°C for 3 days under gentle agitation. Growth was monitored by recording increases in absorbance at 630 nm using a microplate reader. Assays were conducted in triplicate, using three independent transformants. Growth rate measurements were obtained during the logarithmic phase of yeast cell growth. AVP and carbachol concentration-response curves were analyzed using the computer program Kaleidagraph (Synergy Software).

2.2.2.8 Isolation of yeast membranes

Yeast cells from a one liter overnight culture (OD: 0.5–1; 1–2 x10^7 cells/ml) were collected by centrifugation (4,000 x g at 4°C for 10 min). All subsequent manipulations were conducted on ice. Cells were washed in water and recentrifuged. The cell pellet was then weighed and resuspended in ice-cold lysis buffer (1.6 times the volume of the pellet weight) containing 50 mM Tris-HCl, 1 mM EDTA, and 0.1 mM PMSF. Glass beads (Mesh 400–600) were added to the suspension to a final volume of 70%, and the cells were broken by vigorous vortexing. To minimize protein denaturation, the sample was put on ice for 1 min after each 1 min of vortexing. This procedure was repeated four times. The homogenate was then diluted with lysis buffer (1.6 times the volume of the pellet, see above) and extracted. This procedure was repeated five times. The extracted samples were combined and centrifuged at 5,000 x g (4°C) for 10 min. Subsequently, membranes were pelleted by centrifugation at 40,000 x g (4°C) for 40 min. The resulting membrane pellet was resuspended in the same volume of lysis buffer (containing 10% glycerol) that was used to respuspend the cell pellet (see above) and homogenized using a Dounce tissue grinder. Protein concentrations were determined using the Pierce BCA kit with BSA as a standard.

2.2.2.9 Radioligand binding assays with yeast membranes expressing wt and mutant vasopressin receptors

Radioligand binding studies were carried out with membrane homogenates (100-200 µg protein per sample) prepared as described above from yeast strains expressing different vasopressin receptors. Incubations were carried out in a 50 mM Tris-HCl buffer, pH 8.1, containing 2 mM $MgCl_2$, 1 mM EDTA, 0.1 % BSA, 0.1 % bacitracin and increasing concentrations (0.1 - 15 nM) of the radioligand, [^{3}H]-SR121463, a selective V2 receptor antagonist (Serradeil-Le Gal *et al.*, 1996, 2000). Binding reactions were started by the addition of yeast membranes (100-200 µg protein/assay tube) and allowed to proceed for 45 min at 25°C. In [^{3}H]-SR121643 saturation binding experiments, eight different radioligand concentrations (0.1 – 15 nM) were used. Bound and free ligand were separated by rapid filtration over Whatman GF/B filters (presoaked in 0.3% polyethyleneimine) using a Brandel cell harvester. After several washes with ice-cold binding buffer, radioactivity bound to the filters was determined by liquid scintillation counting. Non-specific binding was determined in the presence of 1 µM SR121463. Data for equilibrium binding (apparent equilibrium dissociation constant (K_d), maximum binding density (B_{max})) were analyzed by nonlinear least-squares curve-fitting procedures using the program LIGAND (Munson and Rodbard, 1980).

2.2.2.10 Radioligand binding assays with yeast membranes expressing wt and mutant muscarinic receptors

Radioligand binding studies were carried out with membrane homogenates (250-500 µg protein per sample) prepared as described above from yeast strains expressing different muscarinic receptors. Incubations were carried out for 3 h at 22°C in a 25 mM sodium phosphate buffer (pH 7.4) containing 5 mM $MgCl_2$. In [^{3}H]NMS saturation binding experiments, seven different radioligand concentrations (0.03 – 3.2 nM) were used. Bound and free ligand were separated by rapid filtration over Whatman GF/B filters (presoaked in 0.3% polyethyleneimine) using a Brandel cell harvester. After several

washes with ice-cold binding buffer, radioactivity bound to the filters was determined by liquid scintillation counting. Nonspecific binding was assessed in the presence of 10 µM atropine. Binding data were analyzed by nonlinear least-squares curve-fitting procedures using GraphPAD Prism (GraphPAD Software).

2.2.2.11 Western blotting analysis

For immunoblotting studies, yeast membranes were isolated as described above. Membrane preparations (10 µg protein per sample) were mixed with an equal volume of 2-fold concentrated Laemmli sample buffer (125 mM Tris-HCl (pH 6.8), 20% glycerol, 100 mM DTT, 4% SDS and 0.01% bromphenol blue) and incubated at room temperature for 30 min. Subsequently, proteins were separated on a 4-20% gradient SDS-polyacrylamide gel. Proteins were then transferred onto nitrocellulose membranes via electroblotting. Membranes were blocked with 0.1% gelatin in PBS containing 0.1% of Tween 20 (PBS-T/gelatin; 1 hr at room temperature) and then incubated with the mouse 12CA5 (anti-HA) monoclonal antibody (dilution 1:16,000 in PBS–T/gelatin; 1 hr at room temperature). Bound antibody was detected by incubation with a secondary anti-mouse antibody conjugated to horseradish peroxidase (1:5,000 dilution in PBS-T/gelatin; 1 hr at room temperature). After extensive washing of blots with PBS-T/gelatin, proteins were visualized using an ECL detection kit.

3. Results and Discussion

3.1 Structural basis of V2 vasopressin receptor/G_s coupling selectivity

3.1.1 Aim of the study

During the past decade, biogenic amine GPCRs, including the muscarinic acetylcholine (Wess, 1996) and adrenergic receptors (Dohlman *et al.*, 1991; Savarese and Fraser, 1992; Strader *et al.*, 1994), have been subjected to systematic structure-function studies. This analysis has led to a detailed insight into the structural elements that control the G protein coupling selectivity of this class of receptors. In contrast, the molecular mechanisms determining the coupling selectivity of GPCRs activated by peptide ligands are not well understood. To shed light on this issue, the $G_{q/11}$-linked V1a and the G_s-coupled V2 vasopressin peptide receptors were used as model systems. In a former study (Liu and Wess, 1996), a hybrid receptor strategy was employed to broadly map V1a and V2 receptor domains determining the coupling properties of these two vasopressin receptor subtypes. Biochemical and functional analysis revealed that efficient coupling to G_s by the wt V2 or chimeric V1a/V2 receptors was largely dependent on the presence of V2 receptor sequence in the i3 loop. To explore the structural basis underlying the ability of the V2 receptor to selectively recognize G_s in greater molecular detail, a gain-of-function study was employed. Distinct V2 receptor segments or single amino acids were systematically substituted into the V1a receptor. The resulting mutant vasopressin receptors were then studied for their ability to mediate hormone-dependent cAMP production. This strategy appeared particularly attractive since the wt V1a receptor essentially offered a “null background”, in that stimulation of this receptor subtype has virtually no effect on intracellular cAMP levels (Liu and Wess, 1996).

Table 3.1 ***Amino acid composition of V1a/V2 hybrid vasopressin receptors used in this study***

Chimeric Receptor	V1a receptor residues replaced with V2 receptor sequence[a]
CR 1	V1a (Tyr238-Phe300) -> V2 (Glu225-Leu274)
CR 2	V1a (Val153-Ala172) -> V2 (Ile141-Leu161) and V1a (Tyr238-Phe300) -> V2 (Glu225-Leu274)
CR 3	V1a (Tyr238-Phe300) -> V2 (Glu225-Leu274) and V1a (Cys349-Thr424) -> V2 (Thr320-Ser371)
CR 4	V1a (Cys349-Thr424) -> V2 (Thr320-Ser371)
CR 5	V1a (Cys349-Pro370) -> V2 (Thr320-Leu340)
CR 6	V1a (Phe369-Thr424) -> V2 (Leu339-Ser371)
CR 7	V1a (Tyr238-Phe300) -> V2 (Glu225-Leu274) and V1a (Cys349-Pro370) -> V2 (Thr320-Leu340)
CR 8	V1a (Tyr238-Val278) -> V2 (Glu225-Gly254)
CR 9	V1a (Lys252-Phe300) -> V2 (Gly239-Leu274)
CR 10	V1a (Ser284-Phe300) -> V2 (Pro256-Leu274)
CR 11	V1a (Tyr238-Ser255) -> V2 (Glu225-Gln242)
CR 12	V1a (Tyr238-Ile249) -> V2 (Glu225-Leu236)
CR 13	V1a (Tyr238-Val278) -> V2 (Glu225-Gly254) and V1a (Cys349-Thr424) -> V2 (Thr320-Ser371)
CR 14	V1a (Ser284-Thr424) -> V2 (Pro256-Ser371)
CR 15	V1a (Tyr238-Gly239) -> V2 (Glu225-Val226)
CR 16	V1a (Cys242-His244) -> V2 (Phe229-Gln231)
CR 17	V1a (Trp246-Asn248) -> V2 (His233-Ser235)
CR 18	V1a (Tyr238-Gly239) -> V2 (Glu225-Val226) and V1a (Lys252-Phe300) -> V2 (Gly239-Leu274)
CR 19	V1a (Cys242-His244) -> V2 (Phe229-Gln231) and V1a (Lys252-Phe300) -> V2 (Gly239-Leu274)
CR 20	V1a (Trp246-Asn248) -> V2 (His233-Ser235) and V1a (Lys252-Phe300) -> V2 (Gly239-Leu274)
CR 22	V1a (Gly239-Val278) -> V2 (Val226-Gly254)
CR 23	V1a (Tyr238) -> V2 (Glu225) and V1a (Phe240-Val278) -> V2 (Leu227-Gly254)
CR 24	V1a (Tyr238-Phe240) -> V2 (Glu225-Leu227) and V1a (Tyr243-Val278) -> V2 (Arg230-Gly254)
CR 25	V1a (Tyr238-Cys242) -> V2 (Glu225-Phe229) and V1a (His244-Val278) -> V2 (Gln231-Gly254)
CR 26	V1a (Tyr238-Tyr243) -> V2 (Glu225-Arg230) and V1a (Trp246-Val278) -> V2 (His233-Gly254)
CR 27	V1a (Tyr238-Ile249) -> V2 (Glu225-Leu236) and V1a (His258-Val278) -> V2 (Gly245-Gly254)
CR 28	V1a (Tyr238-Ile249) -> V2 (Glu225-Leu236) and V1a (His258-Val278) -> GGAAAGRRTG
CR 29	V1a (Tyr238-Ile249) -> V2 (Glu225-Leu236) and V1a (His258-Val278) -> GGRRRGAATG
CR 30	V1a (Tyr238-Ile249) -> V2 (Glu225-Leu236) and V1a (His258-Val278) -> AARRRARRAA
CR 31	V1a (Tyr238-Ile249) -> V2 (Glu225-Leu236) and V1a (His258-Val278) -> AAAAAAAAAA
CR 32	V1a (Tyr258-Val278) -> V2 (Gly245-Gly254)
CR 33	V1a (Tyr238-Ser255) -> V2 (Glu225-Gln242) and V1a (Thr279-Phe300) -> V2 (Ser255-Leu274)

[a]Numbers refer to amino acid positions in the rat V1a (Morel *et al.*, 1992; Innamorati *et al.*, 1996) and human V2 (Birnbaumer *et al.*, 1992) vasopressin receptors

3.1.2 Construction of mutant V1a/V2 vasopressin receptors

To generate hybrid V1a/V2 vasopressin receptor constructs, expression plasmids coding for the rat V1a receptor, V1apcD-SP6/T7 (Morel *et al.*, 1992), and the human V2 receptor, V2pcD-PS (Schöneberg *et al.*, 1996), were used. The wt V2 receptor construct contained a nine-amino acid HA tag (YPYDVPDYA) inserted after the initiating methionine codon. To facilitate the construction of V1a/V2 mutant receptors, the following silent restriction sites were introduced into the V1a receptor expression plasmid: *Spe*I (codons 173/174), *BspE*I (codons 224/225), *Kpn*I (codons 235/236), and *Eco47*III (codons 304/305). Chimeric V1a/V2 receptor genes were constructed by using standard PCR mutagenesis techniques (Higuchi *et al.*, 1989). The precise amino acid composition of the individual V1a/V2 hybrid receptors is given in Table 3.1 (see also Fig. 3.1). The correctness of all polymerase chain reaction-derived sequences was confirmed by dideoxy sequencing of the mutant plasmids (Sanger *et al.*, 1977).

3.1.3 Results

When transiently expressed in COS-7 cells, the wt V2 vasopressin receptor, upon stimulation with the hormone AVP, produced a pronounced increase in intracellular cAMP levels (11±1-fold above basal; EC_{50} = 0.56±0.14 nM). In contrast, the V1a vasopressin receptor was unable to mediate an appreciable increase in adenylyl cyclase activity (Figs. 3.2 and 3.3, Tables 3.2 and 3.3). To elucidate the structural basis underlying this selectivity, a gain-of-function mutagenesis approach was employed. Distinct segments/amino acids of V2 receptor sequence were substituted into the V1a receptor subtype, and the resulting hybrid receptors were then examined for their ability to mediate increases in intracellular cAMP levels. All studies were carried out with COS-7 cells transiently expressing the different wt and mutant vasopressin receptors.

Table 3.2 ***Expression levels (B_{max}) of wt and mutant V1a/V2 vasopressin receptors***

B_{max} values (fmol/mg membrane protein) were determined in [^{3}H]AVP binding assays using membrane homogenates prepared from transfected COS-7 cells as described under "Materials and Methods". Unless indicated otherwise, membrane homogenates were incubated with two saturating concentrations of [^{3}H]AVP, 4 and 8 nM. Since these two concentrations detected similar numbers of [^{3}H]AVP binding sites for each individual mutant receptor, binding activities obtained with 8 nM [^{3}H]AVP can be considered approximations of B_{max} values. For several key receptors a (see text for details), complete [^{3}H]AVP saturation binding curves were generated. Mean values ± S.E. are given (n=2-4).

Receptor	B_{max}	Receptor	B_{max}
	fmol/mg		*fmol/mg*
V1	368 ± 45[a]	CR 15	303 ± 30
V2	493 ± 34[a]	CR 16	423 ± 5
		CR 17	258 ± 3
CR 1	166 ± 8[a]	CR 18	77 ± 8
CR 2	40 ± 4[a]	CR 19	140 ± 16[a]
CR 3	231 ± 11[a]	CR 20	75 ± 12
CR 4	343 ± 21		
CR 5	243 ± 5	CR 22	318 ± 14
CR 6	353 ± 16	CR 23	227 ± 9
CR 7	197 ± 13[a]	CR 24	202 ± 18
		CR 25	217 ± 8
CR 8	316 ± 41[a]	CR 26	297 ± 40
CR 9	120 ± 8[a]		
CR 10	91 ± 2	CR 27	374 ± 68[a]
CR11	280 ± 3	CR 28	537 ± 70
CR 12	424 ± 39[a]	CR 29	451 ± 31
CR 13	305 ± 40	CR 30	377 ± 64
CR 14	222 ± 27	CR 31	492 ± 14[a]
		CR 32	465 ± 100
		CR 33	173 ± 13[a]

a For these receptors, complete [^{3}H]AVP saturation binding curves were generated (for [^{3}H]AVP K_D values, see Table 3.3).

Table 3.3 ***Pharmacological properties of hybrid V1a/V2 vasopressin receptors***

[^{3}H]AVP saturation binding and cAMP assays were carried out using transfected COS-7 cells as described under "Materials and Methods". K_D values were determined using the program LIGAND (Munson and Rodbard, 1980). EC_{50} and E_{max} values were obtained from AVP concentration-response curves, using the computer program Kaleidagraph (Synergy Software). Data are given as means ± S.E. of 2-4 (binding assays) or 3-9 (cAMP assays) independent experiments, each carried out in duplicate (binding assays) or triplicate (cAMP assays), respectively.

Receptor	[^{3}H]AVP binding[a]	cAMP production	
	K_D	Maximum increase in cAMP above basal	AVP EC_{50}
	nM	%	*nM*
V1a	0.38 ± 0.02	3 ± 1	nd[b]
V2	0.85 ± 0.06	100	0.56 ± 0.14
CR 1	0.19 ± 0.01	76 ± 2	1.1 ± 0.2
CR 2	0.22 ± 0.07	56 ± 7	1.2 ± 0.4
CR 3	0.50 ± 0.01	99 ± 1	1.2 ± 0.2
CR 7	0.41 ± 0.04	98 ± 2	1.1 ± 0.2
CR 8	0.55 ± 0.07	71 ± 2	1.2 ± 0.1
CR 9	0.18 ± 0.01	11 ± 1	nd[b]
CR 12	0.70 ± 0.05	29 ± 7	4.1 ± 0.2
CR 19	0.39 ± 0.07	44 ± 3	4.1 ± 0.4
CR 27	0.79 ± 0.07	73 ± 3	1.7 ± 0.2
CR 31	0.92 ± 0.14	78 ± 7	1.4 ± 0.3
CR 33	0.21 ± 0.04	33 ± 6	3.7 ± 0.2

[a] For B_{max} values, see Table 3.2.

[b] nd, not determinable with sufficient accuracy.

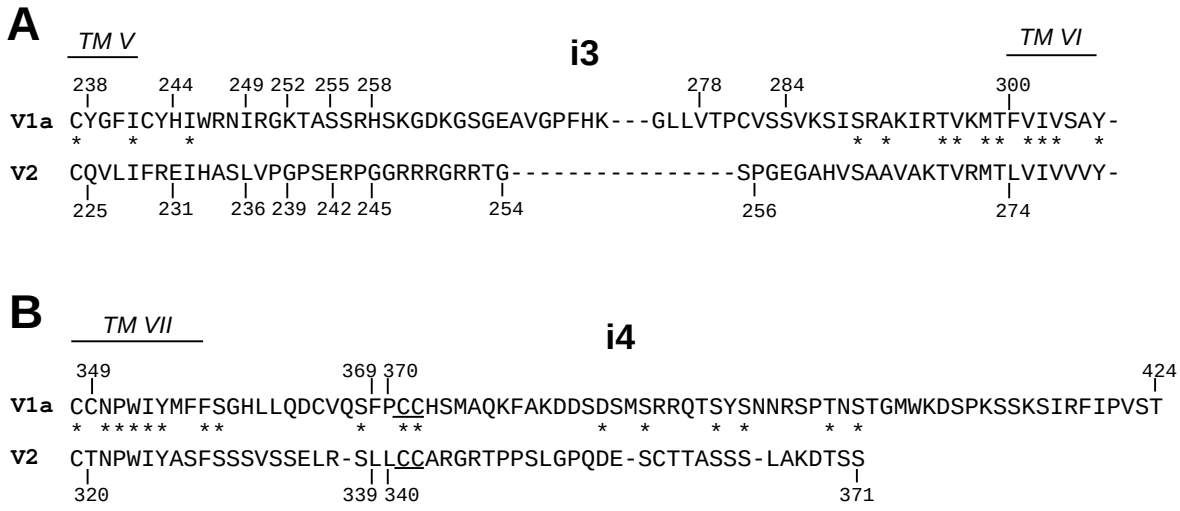


Fig 3.1 Comparison of amino acid sequences of i3 and i4 domains of the rat V1a and human V2 vasopressin receptors. *A,* i3 loop sequences. *B,* i4 domain sequences. The cytoplasmic ends of TM V, VI, and VII are also shown. Positions at which both receptor subtypes have identical residues are marked with *asterisks*. The two adjacent conserved cysteine residues in the i4 domain are *underlined*. Numbers next to individual residues refer to amino acid positions within the rat V1a (Morel *et al.*, 1992; Innamorati *et al.*, 1996b) and human V2 (Birnbaumer *et al.*, 1992) receptor sequences. Gaps were introduced to allow for maximal sequence identity**.**

[^{3}H]AVP binding studies showed that most of the mutant receptors were expressed at levels comparable to those found with the two wt receptors (Table 3.2). In only a few rare cases (e.g. CR2), B_{max} values were found to be strongly reduced (see below for details).

3.1.3.1 Importance of the i4 domain in coupling selectivity of the V2 receptor

In a previous study, Liu and Wess (1996) demonstrated that a mutant V1a receptor (CR1) in which the i3 loop (Tyr238-Phe300; Fig. 3.1) was replaced with the corresponding V2 receptor sequence (Glu225-Leu274; Fig. 3.1) gained the ability to

stimulate adenylyl cyclase with high efficacy (Figs. 3.2 and 3.3, Table 3.2). This observation suggested that the i3 loop of the V2 receptor contains major structural elements determining the coupling selectivity of this receptor subtype. However, it was also noted that the maximum cAMP response mediated by CR1 was about 25% smaller than that observed with the wt V2 receptor (Figs. 3.2 and 3.3; Table 3.3), suggesting that other V2 receptor domains, besides the i3 loop, are also critical for optimum G_s coupling efficiency.

Molecular genetic and biochemical studies with biogenic amine receptors and other classes of GPCRs have shown that residues within the i2 loop and portions of the cytoplasmic tail (i4) also contribute to G protein coupling (Dohlman *et al.*, 1991; Savarese and Fraser, 1992; Strader *et al.*, 1994; Kobilka, 1992; Wess, 1997). Therefore, two additional mutant receptors, CR2 and CR3 (Fig. 3.2), were created by substituting V2 receptor sequences into the i2 loop (CR2) or the i4 domain (CR3) of CR1. Fig. 3.2 shows that maximum cAMP responses mediated by CR2 were smaller than those observed with CR1 (E_{max} = 56±7% compared to wt V2). However, [^{3}H]AVP radioligand binding studies showed that CR2 was poorly expressed (B_{max} = 40 fmol/mg; Table 3.2), making the loss-of-function data difficult to interpret. In contrast to CR2, CR3 (which is expressed at similar levels as CR1; Table 3.2) gained the ability to stimulate adenylyl cyclase with the same efficacy as the wt V2 receptor, indicating that the last 52 amino acids of the V2 receptor (Thr320-Ser371; Fig. 3.1) make a critical contribution to V2 receptor/G_s coupling selectivity. Consistent with this finding, substitution of this C-terminal V2 receptor sequence directly into the wt V1a receptor resulted in a hybrid receptor, CR4, that gained the ability to stimulate cAMP production to a significant extent (E_{max} = 28±4%; Fig. 3.2).

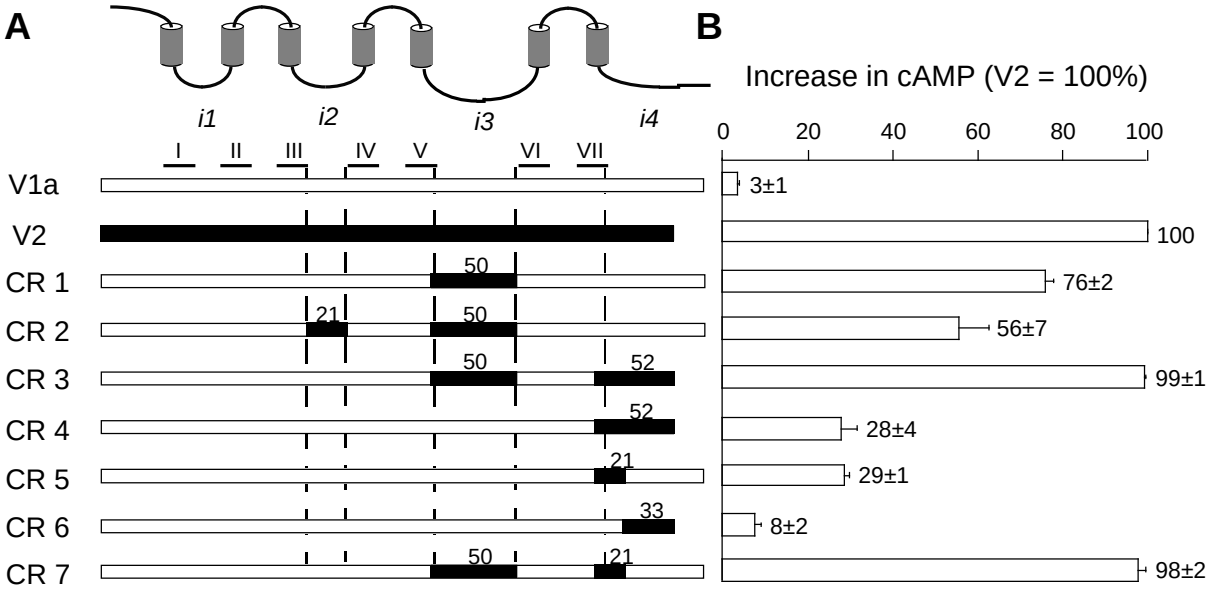


Fig. 3.2 Stimulation of cAMP production by hybrid V1a/V2 vasopressin receptors, CR1-CR7. *A,* structure of the CR1-CR7 hybrid receptors (for exact amino acid composition, see Table 3.1 and Fig. 3.1). The positions of the seven TM domains (*I-VII*) and the four intracellular receptor domains (*i1-i4*) are marked. The number of V2 receptor residues present in each construct is indicated above the *filled bars*. The fact that the two receptors differ in the size of their i3 loops is ignored in this scheme. *B,* COS-7 cells transiently expressing the various wt and mutant receptors were stimulated with AVP (1μM), and the resulting increases in intracellular cAMP levels (fold stimulation above basal) were determined as descried under "Materials and Methods." Basal cAMP levels (no ligand added) were similar for the different receptor constructs (data not shown). In the case of the wt V2 receptor, basal levels amounted to 780 ± 60 cpm/well. In each individual experiment, the wt V2 receptor response was set equal to 100%. Data are expressed as means ± S.E. of three to nine independent experiments, each carried out in triplicate.

Based on these results, two additional mutant V1a receptors, CR5 and CR6, were created next, in which the N-terminal portion of the i4 domain (including several residues predicted to be located at the cytoplasmic end of TM VII) and the distal portion of the i4 region (the region C-terminal of the conserved Cys-Cys pair) were replaced with the corresponding V2 receptor sequences (Thr320-Leu340 and Leu339-Ser371, respectively; Fig. 3.1). Functional studies showed that CR5 was capable of mediating ligand-dependent cAMP accumulation in a fashion similar to CR4 (E_{max} = 29±1%; Fig. 3.2). In contrast, CR6 displayed only residual functional activity, similar to the wt V1a receptor (Fig. 3.2).

When the V2 receptor sequence present in CR5 (Thr320-Leu340) was substituted into CR1 (which contains V2 receptor sequence in the i3 loop), the resulting chimeric receptor, CR7, showed a functional profile very similar to that of the wt V2 receptor (Figs. 3.2 and 3.3). As shown in Fig. 3.3, AVP concentration-response curves generated for CR7 and the wt V2 receptor were almost superimposable (for E_{max} and EC_{50} values, see Table 3.3), further highlighting the functional importance of the Thr320-Leu340 V2 receptor segment.

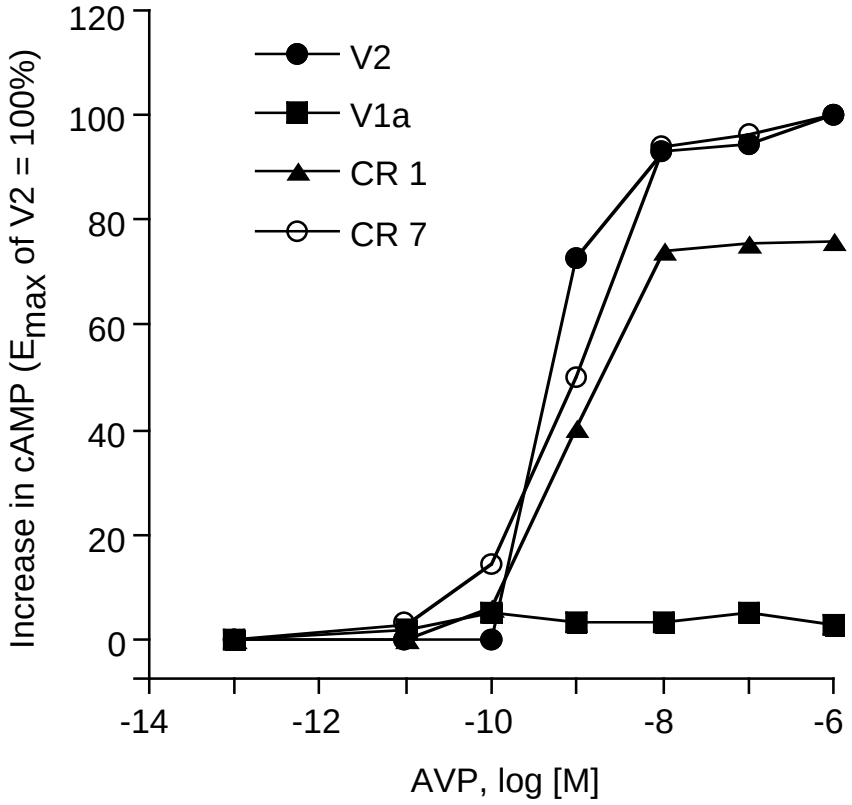


Fig. 3.3 Concentration-response curves for CR1- and CR7-mediated stimulation of adenylyl cyclase. Experiments were carried out with transfected COS-7 cells as detailed in the legend to Fig 3.2. and under "Materials and Methods." AVP EC_{50} and E_{max} values (as well as K_D and B_{max} values determined in [^{3}H]AVP saturation binding studies) are given in Tables 3.2 and 3.3. Results (means) from a representative experiment carried out in triplicate are shown; two additional experiments gave similar results.

3.1.3.2 Role of the N-terminal portion of the i3 loop in determining V2 receptor/G_s coupling selectivity

The next goal was to examine which specific regions/amino acids within the i3 loop of the V2 vasopressin receptor are of primary importance for proper recognition of G_s. To address this question, a series of mutant V1a receptors (CR8-CR12; Fig. 3.4) were created in which distinct segments of the i3 loop were replaced with the corresponding V2 receptor sequences. As shown in Fig. 3.4, CR9 and CR10, in which C-terminal portions of the i3 loop contained V2 receptor sequence (residues Gly239-Leu274 and Pro256-Leu274, respectively), were unable to mediate stimulation of adenylyl cyclase to a significant extent.

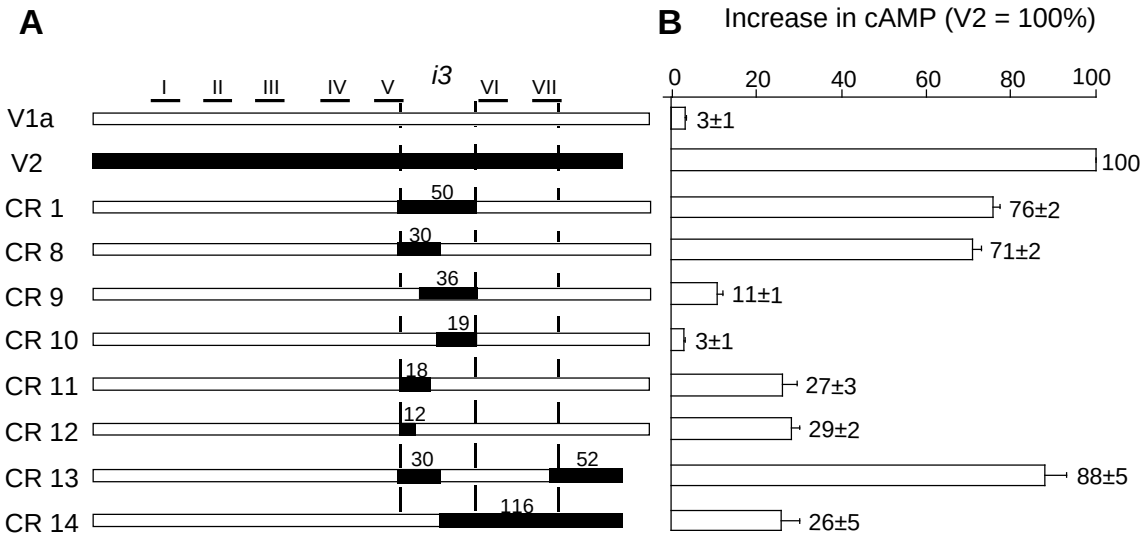


Fig. 3.4 Stimulation of cAMP accumulation by hybrid V1a/V2 vasopressin receptors, CR8-CR14. *A,* structure of the CR8-CR14 hybrid receptors (for exact amino acid composition, see Table 3.1 and Fig. 3.1). The locations of the TMI-VII and the i3 loop are indicated. The fact that the two receptors differ in the size of their i3 loops is ignored. The number of V2 receptor residues present in each construct is indicated above the *filled bars*. For comparison, CR1 was also included in this set of experiments. *B,* COS-7 cells transiently expressing the various wt and mutant receptors were stimulated with AVP (1 μM), and the resulting increases in intracellular cAMP levels (fold stimulation above basal) were determined as described under "Materials and Methods." Basal cAMP levels (no ligand added) were similar for the different receptor constructs (data not shown). In each experiment, the wt V2 receptor response was set equal to 100%. Data are given as means ± S.E. of four to eight independent experiments, each carried out in triplicate.

In agreement with this observation, CR14 in which the entire C-terminal third of the receptor protein (excluding the N-terminal portion of the i3 loop) was derived from the V2 receptor (residues Pro256-Ser371) did not display an increase in functional efficacy (E_{max} = 26±5%; Fig. 3.4), as compared to CR4 or CR5 which contain smaller substitutions within their i4 domains (Fig. 3.2).

On the other hand, CR8 which contains 30 amino acids of V2 receptor sequence (Glu225-Gly254) at the beginning of the i3 loop (including several residues predicted to be located at the cytoplasmic end of TM V) gained the ability to efficiently stimulate ligand-dependent cAMP accumulation (E_{max} = 71±2%; Fig. 3.4).

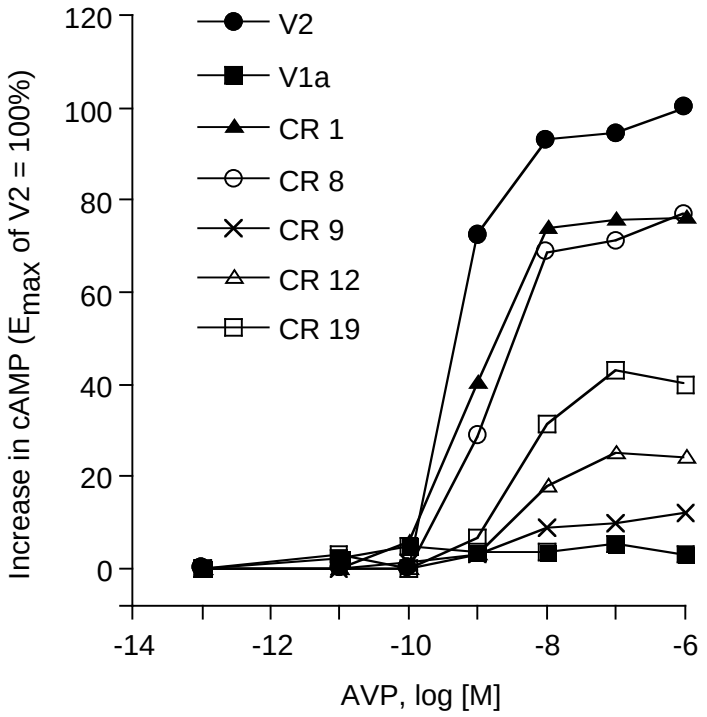


Fig. 3.5 Concentration-response curves for CR1-, CR8-, CR9-, CR12-, and CR19-mediated stimulation of adenylyl cyclase. Experiments were carried out with transfected COS-7 cells as detailed in the legend to Fig. 3.4 and under "Materials and Methods." AVP EC_{50} and E_{max} values (as well as K_D and B_{max} values determined in [^{3}H]AVP saturation binding studies) are given in Tables 3.2 and 3.3. Results (means) from a representative experiment carried out in triplicate are shown; two additional experiments gave similar results. Note that the curves shown in this figure and in Fig. 3.3 were generated in the same set of experiments, explaining that the curves for the two wt receptors and for CR1 are identical in these two figures. Two additional experiments gave similar results.

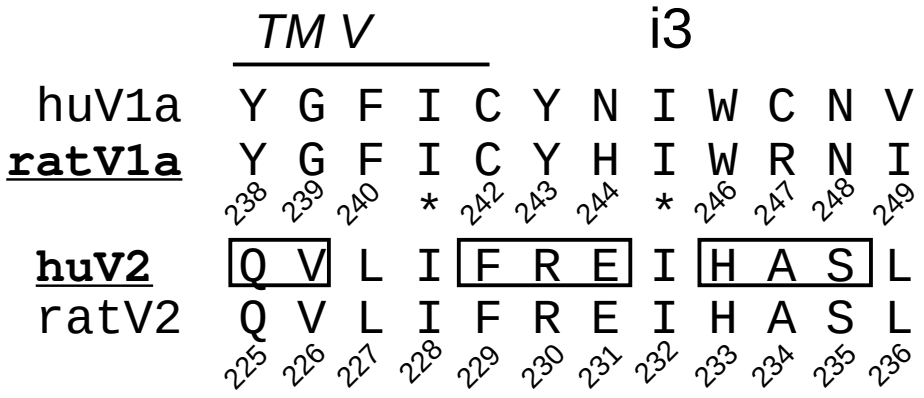


Fig. 3.6 Comparison of V1a and V2 vasopressin receptor sequences present at the TM V/i3 loop junction. Rat and human (*hu*) sequences are shown. The *boxed* V2 receptor residues clearly differ in their physicochemical properties from the corresponding amino acids present in the V1a receptor. Positions at which the receptors have identical residues are marked with *asterisks*. Numbers indicate amino acid positions within the rat V1a (Morel *et al.*, 1992) and human V2 (Birnbaumer *et al.*, 1992) receptor sequences. The other sequences were taken from Thibonnier *et al.* (1994) (human V1a) and Lolait *et al.* (1992) (rat V2).

Comparison of complete AVP concentration-response curves showed that CR8 behaved functionally very similar to CR1 in which the entire i3 loop consists of V2 receptor sequence (Fig. 3.5, Table 3.3). Consistent with results described in the previous section, replacement of the i4 domain of CR8 with the corresponding V2 receptor sequence (Thr320-Ser371; yielding CR13) led to a further improvement in G_s coupling efficacy (E_{max} = 88±5%; Fig. 3.4).

Progressive shortening of the V2 receptor sequence present in CR8 resulted in CR11 and CR12 (Fig. 3.4) which contained only 18 (Glu225-Gln242) and 12 (Glu225-Leu236) V2 receptor residues, respectively. Fig. 3.4 indicates that both hybrid receptors retained the ability to activate the G_s/adenylyl cyclase system (E_{max} = 27-29%), though maximum cAMP responses were reduced as compared to CR8 (for a complete functional characterization of CR12, see Table 3.3).

3.1.3.3 Identification of single residues at the TMV/i3 loop junction critical for V2 receptor/G_s coupling

As outlined in the previous section, CR12 which contains only 12 amino acids of V2 receptor sequence (Glu225-Leu236) gained significant coupling to G_s (Fig. 3.4). This short V2 receptor sequence is shown enlarged in Fig. 3.6, aligned with the corresponding V1a receptor segment (both rat and human sequences are shown). Fig. 3.6 indicates that there are three groups of V2 receptor receptor residues (Glu225/Val226, Phe229-Gln231, and His233-Ser235; boxed in Fig. 3.6) which clearly differ in their physicochemical properties from the corresponding amino acids present in the V1a receptor.

To test the potential importance of these V2 receptor residues for efficient G_s coupling, these three groups of amino acids were substituted directly into the wt V1a receptor, creating hybrid receptors CR15-17 (Fig. 3.7A). However, none of these mutant receptors gained significant coupling to the G_s/adenylyl cyclase system (Fig. 3.7B). On the other hand, when the same substitutions were introduced into CR9, which also contained V2 receptor sequence in the central and C-terminal portions of the i3 loop (yielding CR18-CR20), two mutant receptors were identified, CR18 and CR19, which showed clearly improved cAMP responses (Fig. 3.7). As shown in Fig. 3.7B, CR18 (containing V2-Glu225 and V2-Val226) and CR19 (containing the V2 receptor residues Phe229/Arg230/Gln231) displayed 2- and 4-fold increases in E_{max} values, respectively, as compared to CR9. No such increase in maximum cAMP responses was observed for CR20 which contains the V2 receptor triplet, His233/Ala234/Ser235 (Fig. 3.7B).

Next, a loss-of-function mutagenesis approach was employed to evaluate the potential functional importance of Glu225, Val226, Phe229, Arg230, and Gln231 in more detail. Starting from the CR8 mutant receptor which is able to activate G_s with high efficacy (Fig. 3.4), five mutant receptors (CR22-CR26) were created in which these five amino acids were individually replaced with the corresponding V1a receptor residues (Fig. 3.8A). Three of these mutant receptors (CR23, CR24, and CR25; containing Val226->Gly, Phe229->Cys, and Arg230->Tyr point mutations, respectively) behaved functionally very similar to CR8 (Fig. 3.8B). In contrast, two of the introduced point

mutations, Glu225->Tyr and Gln231->His, yielded hybrid receptors (CR22 and CR26, respectively) that showed strong reductions (by about 60-70% as compared to CR8) in receptor-mediated cAMP responses (Fig. 3.8B).

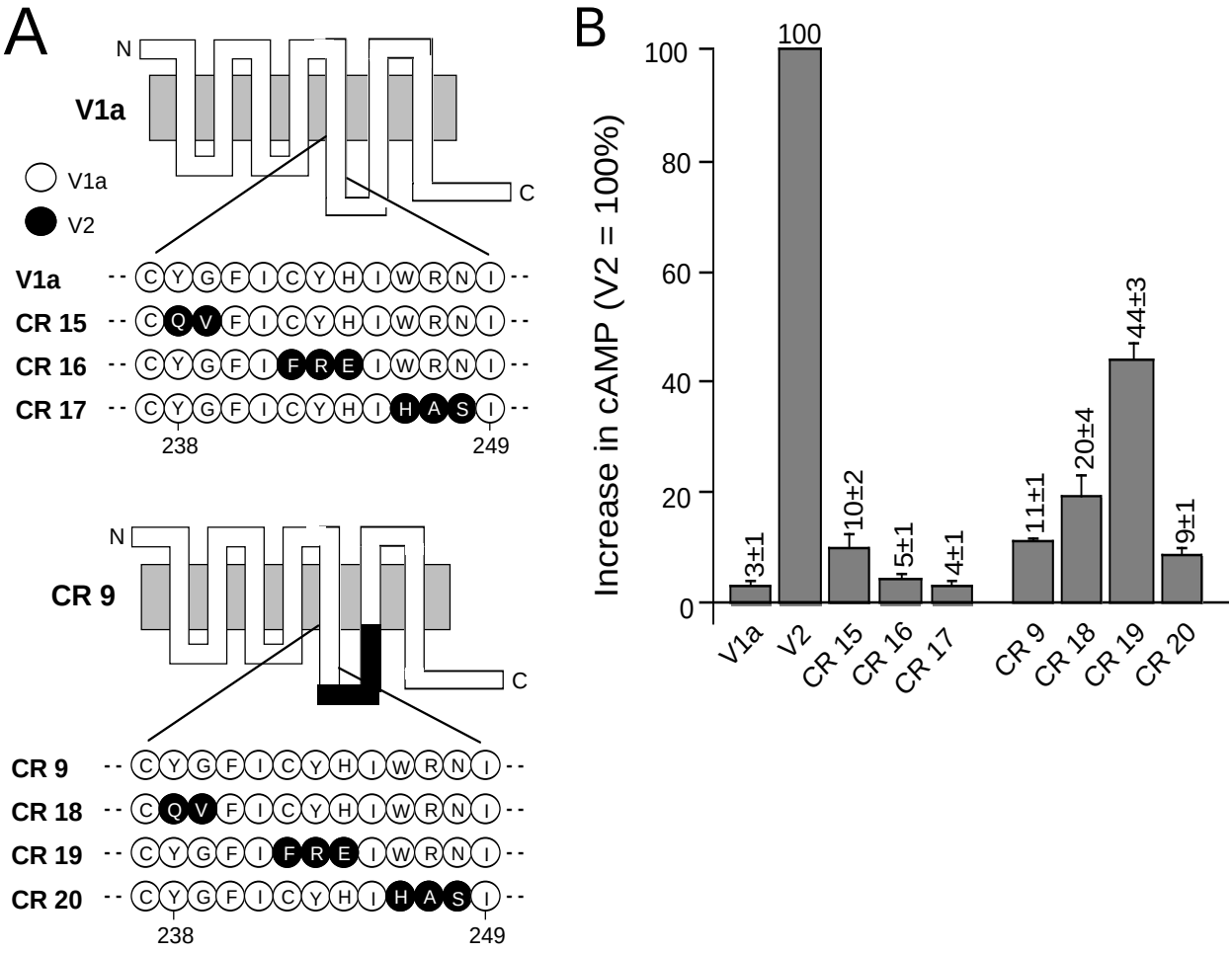


Fig. 3.7 Stimulation of cAMP formation by mutant V1a/V2 vasopressin receptors, CR15-CR20. *A,* structure of the CR15-CR20 mutant receptors (for exact amino acids composition, see Table 3.1 and Fig. 3.1). The fact that the two receptors differ in the size of their i3 loops is ignored. CR15-CR17 and CR18-CR20 are derived from the wt V1a receptor and CR 9, respectively. The N-terminal portion of the i3 loop is shown enlarged. Numbers next to individual residues refer to amino acid positions within the rat V1a receptor sequence. *B,* COS-7 cells transiently expressing the various wt and mutant receptors were stimulated with AVP (1 μM), and the resulting increases in intracellular cAMP levels (fold stimulation above basal) were determined as described under "Materials and Methods." Basal cAMP levels (no ligand added) were similar for the different receptor constructs (data not shown). In each individual experiment, the wt V2 receptor response was set equal to 100%. Data are given as means ± S.E. of three to six independent experiments, each carried out in triplicate.

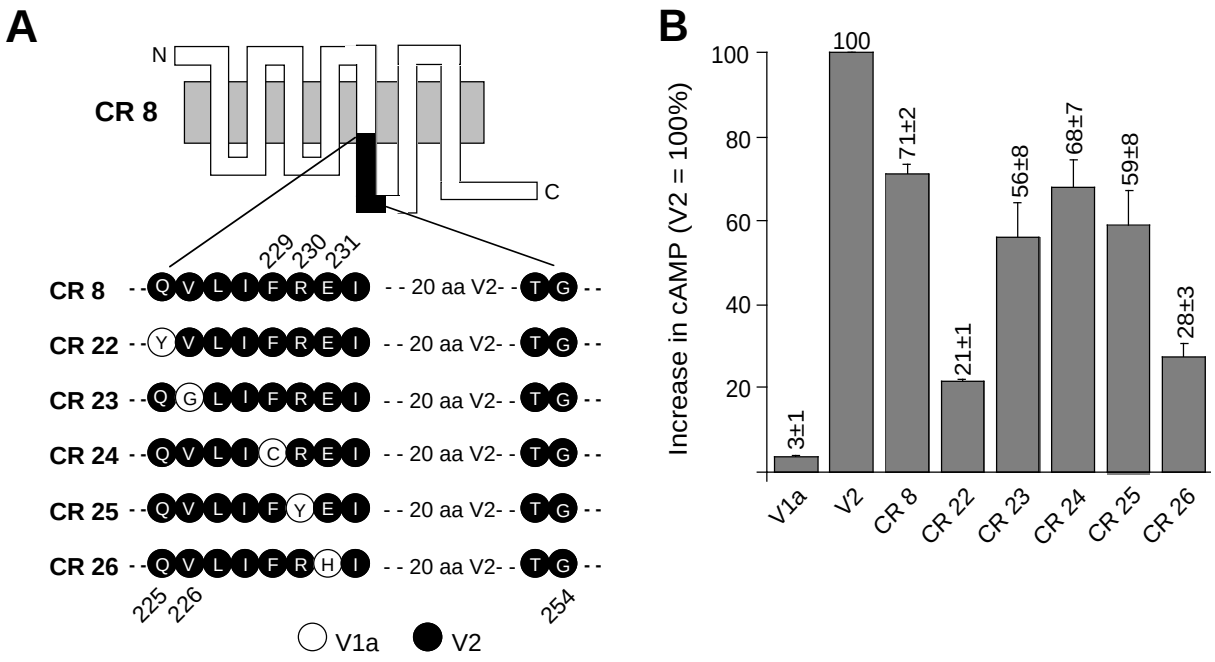


Fig. 3.8 Stimulation of cAMP production by hybrid V1a/V2 vasopressin receptors, CR22-CR26. *A,* structure of the CR22-CR26 mutant receptors (for exact amino acid composition, see Table 3.1 and Fig. 3.1). CR22-CR26 are derived from CR8, which contains 30 amino acids of V2 receptor sequence (Gln225-Gly254) at the beginning of the i3 loop. Numbers next to individual residues refer to amino acid positions within the human V2 receptor sequence. V2 receptor residues were systematically substituted with the corresponding V1a receptor residues. As a control, CR8 was also included in this set of experiments. *B,* COS-7 cells transiently expressing the various wt and mutant receptors were stimulated with AVP (1 μM), and the resulting increases in intracellular cAMP levels (fold stimulation above basal) were determined as described under "Materials and Methods." Basal cAMP levels (no ligand added) were similar for the different receptor constructs (data not shown). In each individual experiment, the wt V2 receptor response was set equal to 100%. Data are given as means ± S.E. of three independent experiments, each carried out in triplicate.

3.1.3.4 Role of charged amino acids in the central portion of the i3 loop in regulating receptor/G_s coupling efficiency

As shown in Fig. 3.4, CR8 (which contains 30 amino acids of V2 receptor i3 loop sequence) displayed an E_{max} that was approximately 2.5-fold greater than that observed with CR12 (which contains only 12 amino acids of V2 receptor i3 loop sequence). This observation suggested that the central portion of the i3 loop of the V2 receptor, in addition to residues at the TM V/i3 loop junction, might also be important for efficient coupling to G_s. To further test this hypothesis, a hybrid receptor, CR27 (Fig. 3.9A), was

generated, in which the central portion of the i3 loop of CR12 (V1a receptor residues His258-Val278) was replaced with V2 receptor residues Gly245-Gly254 (note that the introduced V2 receptor segment is 11 amino acids shorter than the replaced V1a receptor segment, reflecting differences in i3 loop sizes between the two receptor subtypes; Fig. 3.1). Interestingly, CR27 gained the ability to stimulate cAMP production with markedly increased efficacy (E_{max} = 73±3%), in a fashion similar to CR8 (Fig. 3.9B).

The introduced V2 receptor segment, Gly245-Gly254, contains two groups of arginine residues (Fig. 3.9A), suggesting that these positively charged residues might be important for G_s coupling. However, groupwise replacement in CR27 of these charged amino acids with alanine residues (resulting in CR28 (Arg247/Arg248/Arg249->AlaAlaAla) and CR29 (Arg251/Arg252->AlaAla) (Fig. 3.9A) did not lead to a reduction in cAMP responses (Fig. 3.9B).

Similarly, simultaneous replacement of all five non-charged residues located within Gly245-Gly254 (CR30) or even replacement of this entire sequence element with a string of 10 alanine residues (CR31) still yielded mutant receptors that allowed very efficient coupling to G_s (E_{max}= 78-95%; Fig. 3.9B). Fig. 3.10 shows that AVP concentration-response curves generated for CR27 and CR31 were essentially superimposable (for E_{max} and EC_{50} values, see also Table 3.3). As shown in Fig. 3.1, the central portion of the i3 loop of the V1a receptor is 13 amino acids longer than the corresponding V2 receptor region. These data therefore suggest that the ability of CR27-CR31 to couple to G_s with clearly improved efficacy (as compared to CR12) is primarily due to the shortening of the central portion of the i3 loop rather than the presence of specific V2 receptor residues within this domain.

To further test this hypothesis, two additional hybrid receptors, CR32 and CR33, were constructed (Fig. 3.9A). CR32 differs from CR27 in that the His258-Val278 (V1a sequence) -> Gly245-Gly254 (V2 sequence) substitution was introduced directly into the wt V1a receptor (rather than into CR12). CR33 is derived from CR1 by replacing Arg243-Gly254 (V2 sequence) with the longer Ser256-Val278 (V1a sequence) segment. Fig. 3.9B shows that CR32, in contrast to CR27, did not gain efficient coupling to G_s and showed only residual functional activity. Moreover, CR33 proved to be considerably

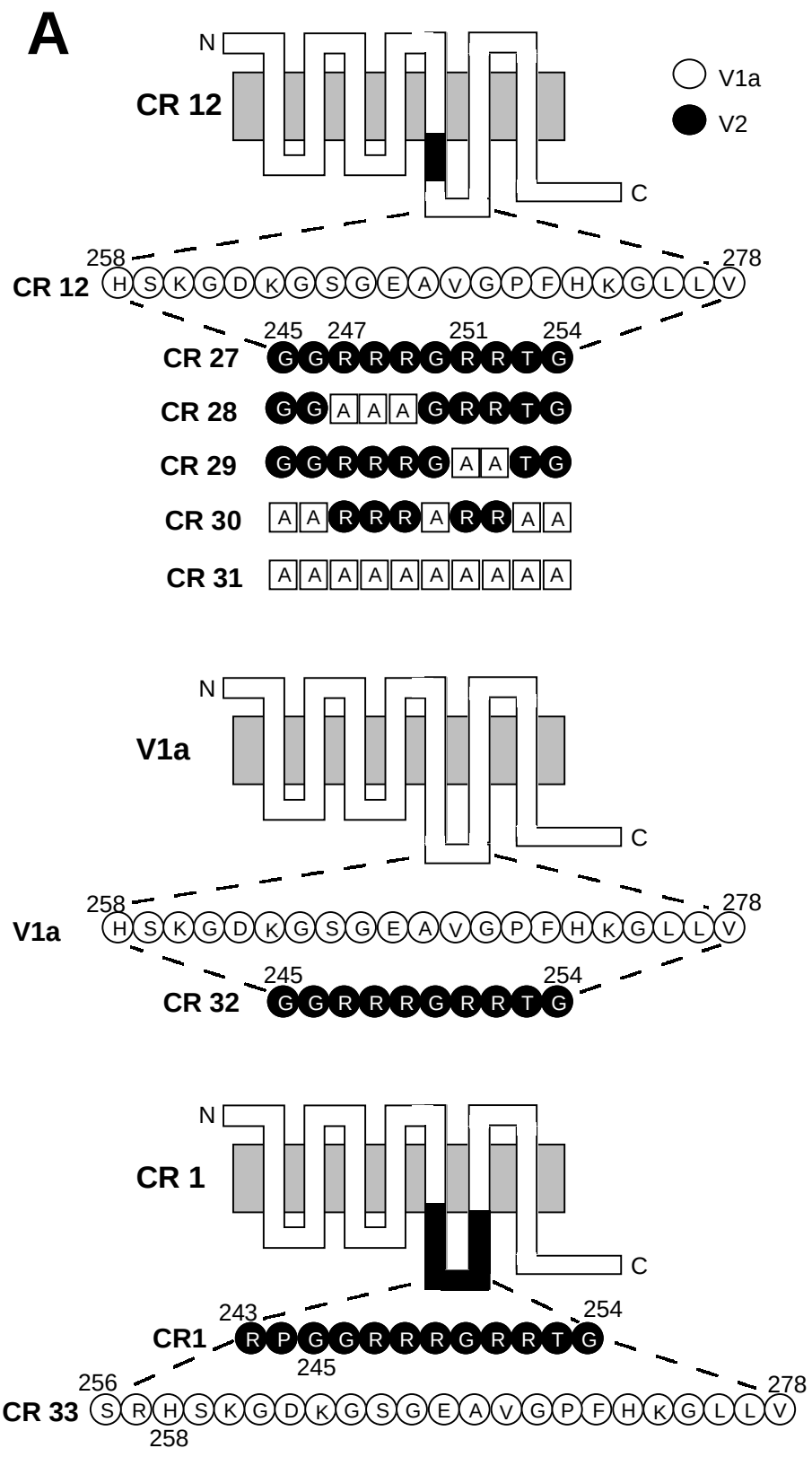

A
N
C
V1a
V2
CR 12
258
278
CR 12 H S K G D K G S G E A V G P F H K G L L V
245 247 251 254
CR 27 G G R R R G R R T G
CR 28 G G A A A G R R T G
CR 29 G G R R R G A A T G
CR 30 A A R R R A R R A A
CR 31 A A A A A A A A A A
V1a
258
278
V1a H S K G D K G S G E A V G P F H K G L L V
245 254
CR 32 G G R R R G R R T G
CR 1
243 254
CR1 R P G G R R R G R R T G
245
256 278
CR 33 S R H S K G D K G S G E A V G P F H K G L L V
258

Fig. 3.9

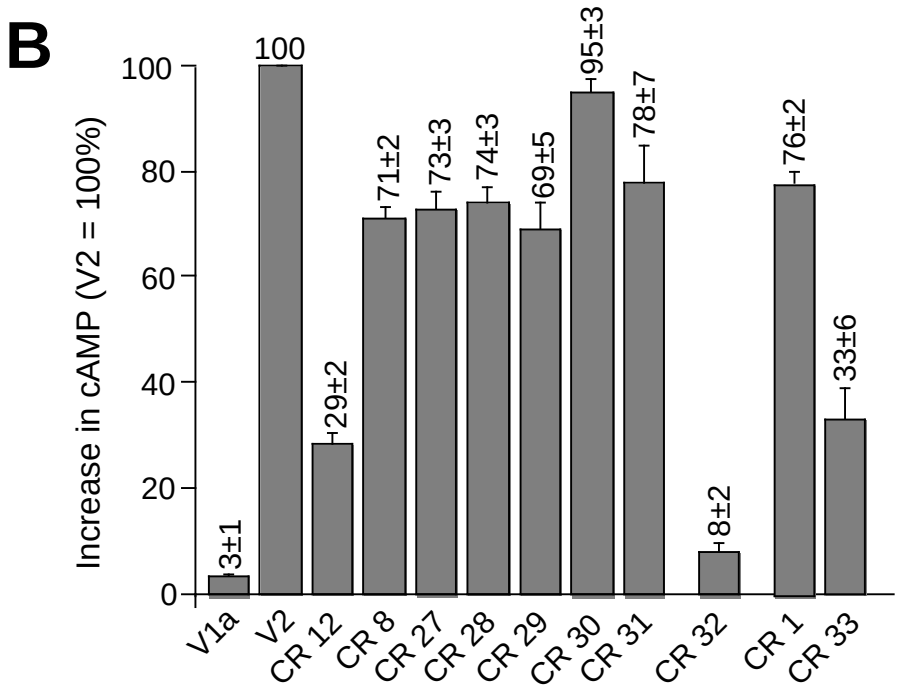


Fig. 3.9 Stimulation of cAMP formation by mutant V1a/V2 vasopressin receptors, CR27-CR33. *A,* structure of the CR27-CR33 chimeric receptors (for exact amino acid composition, see Table 3.1 and Fig. 3.1). Numbers next to individual residues refer to amino acid positions within the rat V1a and the human V2 receptor sequences. The central portion of the i3 loop is shown enlarged. CR27-CR31 are derived from CR12, which contains 12 amino acids of V2 receptor sequences at the beginning of the i3 loop. In CR27, the V1a receptor sequence His258-Val278 (21 amino acids) was replaced with the V2 receptor segment, Gly245-Gly254 (10 amino acids) (see also Fig. 3.1). In CR32, the same substitution was introduced into the wt V1a receptor background. CR33 was generated by introducing a reciprocal substitution into the CR1 hybrid receptor. Please note that the sequences that were exchanged in CR32 and CR33 slightly differ in length, due to technical reasons (presence of useful restriction sites) relating to the construction of the hybrid receptors. For comparison, CR1, CR8, and CR12 were also included in this set of experiments. *B,* COS-7 cells transiently expressing the various wt and mutant receptors were stimulated with AVP (1µM), and the resulting increases in intracellular cAMP levels (fold stimulation above basal) were determined as described under "Materials and Methods." Basal cAMP levels (no ligand added) were similar for the different receptor constructs (data not shown). In each individual experiment, the wt V2 receptor response was set equal to 100%. Data are given as means ± S.E. of three to seven independent experiments, each carried out in triplicate.

less active (reduction in E_{max} by approximately 2.5-fold) than its "parent" receptor, CR1 (Fig. 3.9B; for a complete AVP concentration-response curve of CR33, see Fig. 3.10).

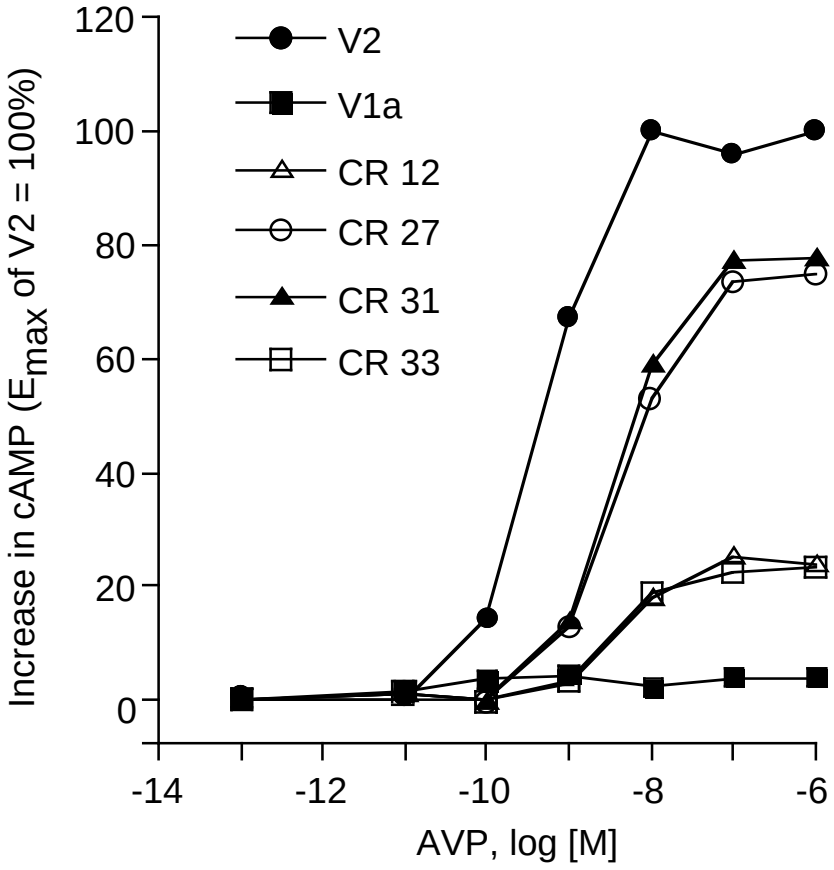


Fig. 3.10 Concentration-response curves for CR12-, CR27-, CR31-, and CR33-mediated stimulation of adenylyl cyclase. Experiments were carried out with transfected COS-7 cells as detailed in the legend to Fig. 3.8 and under "Materials and Methods." AVP EC_{50} and E_{max} values (as well as K_D and B_{max} values determined in [^{3}H]AVP saturation binding studies) are given in Tables 3.2 and 3.3. Results (means) from a representative experiment carried out in triplicate are shown; two additional experiments gave similar results.

3.1.3.5 PI assays with hybrid receptors unable to stimulate cAMP production

Several of the hybrid V1a/V2 vasopressin receptors analyzed in this study (e.g. CR6, CR9, CR10, CR15-18, CR20, and CR32) exhibited only residual activity in the cAMP assays. To exclude the possibility that this lack of functional activity was due to improper folding of the intracellular receptor surface, these receptors were also tested for stimulate PI hydrolysis (mediated by G proteins of the G_q/G_{11} family) as long as their i2 loops contain V1a receptor sequence. As shown in Fig. 3.11, all examined mutant their

ability to mediate AVP-dependent stimulation of PI hydrolysis. A previous study (Liu and Wess, 1996) demonstrated that chimeric V1a/V2 receptors can efficiently receptors that coupled to G_s inefficiently were still able to stimulate the accumulation of inositol phosphates with high efficacy (in the presence of 1 μM AVP). In all cases, maximum PI responses were similar to those observed with the wt V1a receptor (increase in IP_1 production: 22±2-fold above basal).

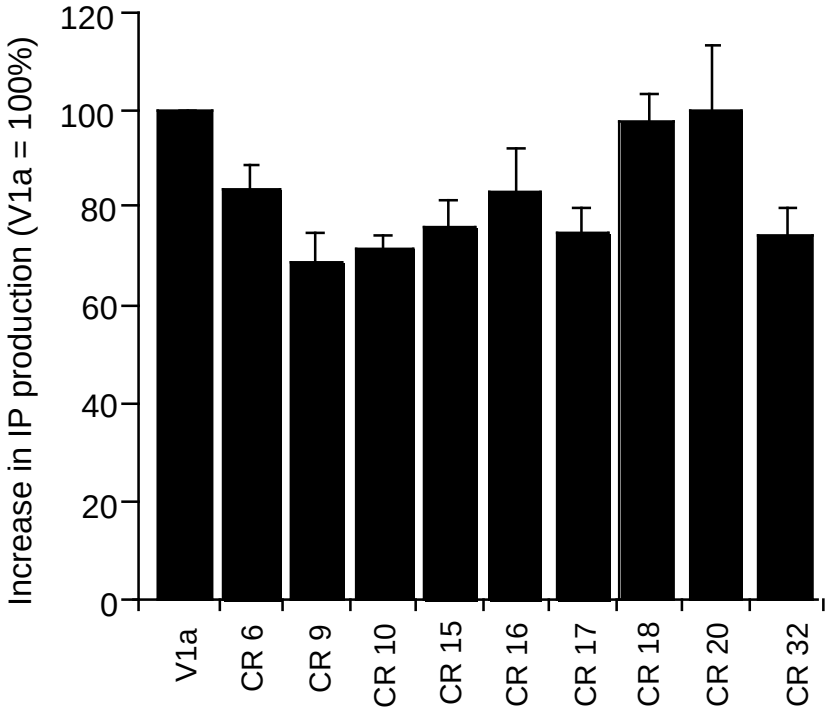
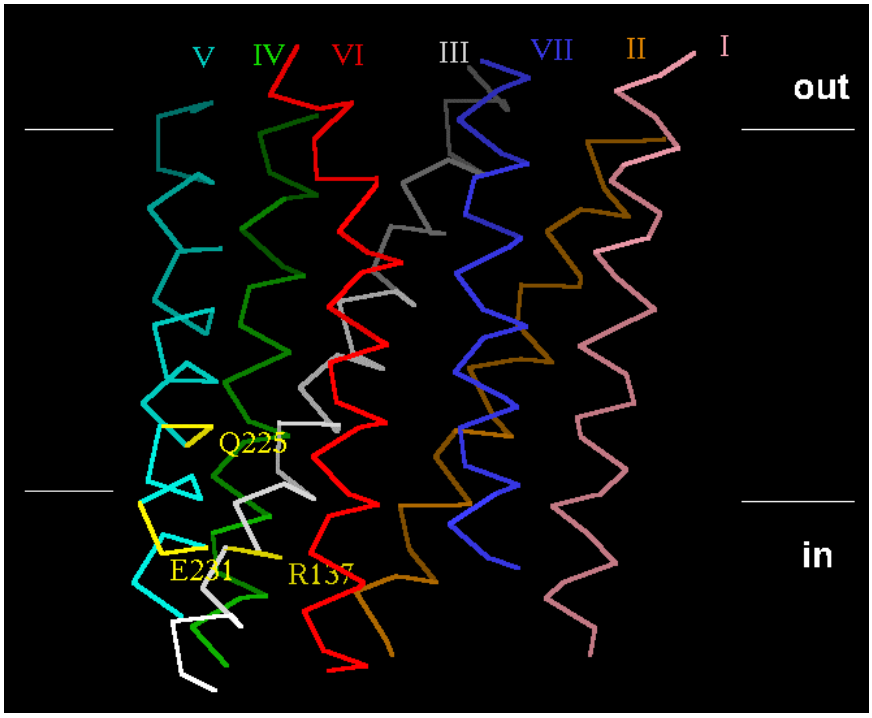
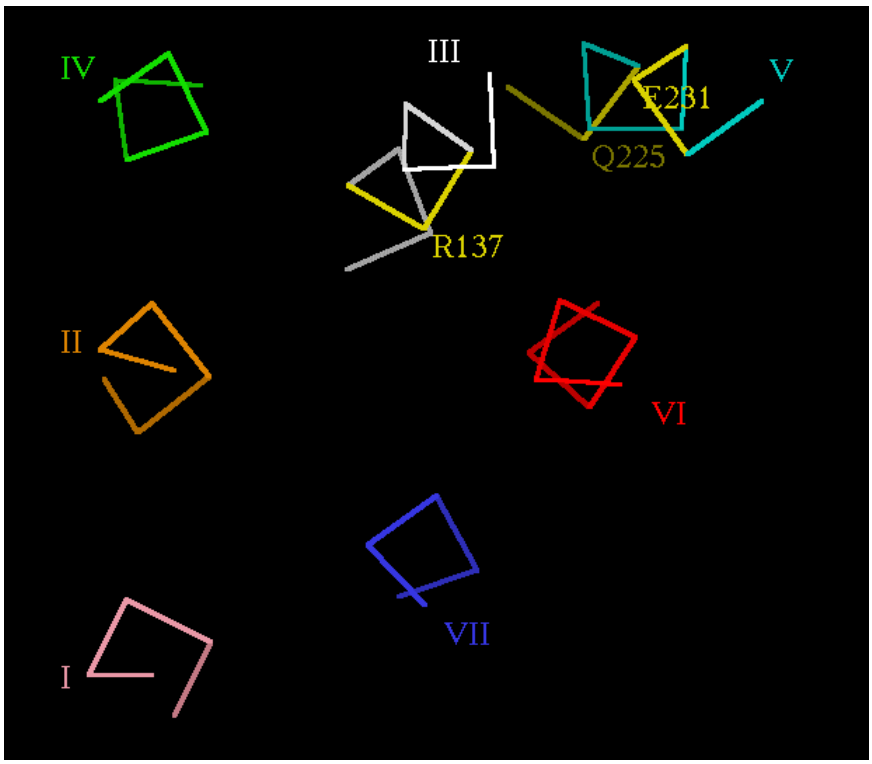


Fig. 3.11 Stimulation of PI hydrolysis by hybrid V1a/V2 receptors poorly coupled to cAMP production. The structures of the various hybrid receptors are given in Table 3.1 and Fig. 3.2, 3.4, 3.7, and 3.9. COS-7 cells transiently expressing the wt V1a and the indicated mutant receptors were stimulated with AVP (1 μM), and the resulting increases in intracellular IP_1 levels (fold stimulation above basal) were determined as described under "Materials and Methods." Basal IP_1 levels (no ligand added) were similar for the different receptor constructs (data not shown). In the case of the wt V1a receptor, basal IP_1 levels amounted to 301 ± 39 cpm/well. In each individual experiment, the IP_1 response mediated by the wt V1a receptor was set equal to 100%. Data are given as means ± S.E. and are representative of three independent experiments, each carried out in triplicate.

A


V
IV
VI
III
VII
II
I
out
in
Q225
E231
R137

B


IV
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3.1.4 Discussion

A gain-of-function mutagenesis strategy was employed to elucidate the molecular basis underlying the ability of the V2 vasopressin receptor to selectively stimulate the G_s/adenylyl cyclase system. In contrast to the V2 receptor which mediates a robust increase in intracellular cAMP levels, hormone stimulation of the structurally closely related V1a receptor subtype leaves intracellular cAMP levels virtually unaffected (Figs. 3.2 and 3.3). To identify V2 receptor residues determining this coupling selectivity, we systematically replaced distinct V1a receptor sequences (or single amino acids) with the corresponding V2 receptor sequences and studied whether the resulting hybrid receptors would gain the ability to mediate ligand-dependent cAMP accumulation.

Consistent with a previous study (Liu and Wess, 1996), we found that substitution of the i3 loop of the V2 receptor into the V1a receptor subtype yielded a mutant receptor (CR1) that gained the ability to efficiently stimulate cAMP production (E_{max} about 75%, as compared with the wt V2 receptor; Fig. 3.2). Interestingly, a mutant receptor (CR3) that contained V2 receptor sequence in both the i3 and i4 domains was able to activate the G_s/adenylyl cyclase system with the same high efficacy as the wt V2 receptor (E_{max} = 99±1%; Fig. 3.2), suggesting that the i4 domain of the V2 receptor (Thr320-Ser371) also contains residues that are critical for efficient G_s activation. Functional analysis of the CR7 hybrid receptor showed that a stretch of 21 amino acids of V2 receptor sequence (Thr320-Leu340) located at the TM VII/i4 domain junction can fully mimic the effects of the Thr320-Ser371 substitution (Figs. 3.2, 3.3).

Fig. 3.12 Putative structure of the transmembrane core of GPCRs of the rhodopsin family. The arrangement depicted here is based on the α-carbon model proposed by Baldwin *et al.* (1997). *A,* Side view of TM I-VII. Functionally critical V2 vasopressin receptor residues are highlighted in yellow (backbone α-carbons). Glu225 and Gln231 play critical roles in determining the ability of the V2 receptor to selectively couple to G_s (see text for details). Arg137 at the cytoplasmic end of TM III is found in virtually all GPCRs of the rhodopsin family and is known to be important for G protein activation (Rosenthal *et al.*, 1993). *B,* Model of the intracellular surface of rhodopsin-like GPCRs, as viewed from the cell interior. A cross-section through the transmembrane core of the human V2 vasopressin receptor (at a level approximately corresponding to the membrane/cytoplasm boundary) is shown. Please note that due to the inclinations of TM III and other TM helices, the relative positions of TM III and IV are predicted to be reversed on the cytoplasmic side of the membrane (Palczewski *et al.*, 2000).

Substitution of Thr320-Leu340 or Thr320-Ser371 directly into the wt V1a receptor led to mutant receptors (CR4 and CR5, respectively) that also gained the ability to activate the cAMP cascade (E_{max} about 30%; Fig. 3.2). The ability of CR4 (previously referred to as V1i4; ref. Liu and Wess, 1996) to mediate a small but significant cAMP response remained undetected in our initial analysis of V1a/V2 hybrid receptors (Liu and Wess, 1996). One possible explanation for this discrepancy is that a modified transfection procedure was used in the present study (lower cell plating density: 1 x 10^6 versus 2 x 10^6 cells/plate; prolonged transfection time: 3 hrs versus 1 hr).

The Thr320-Leu340 V2 receptor sequence is followed by a pair of cysteine residues which are conserved among all vasopressin receptor subtypes (Fig. 3.1). Similarly, most GPCRs contain one or more highly conserved cysteine residues within their C-terminal i4 domains. Studies with a great number of different GPCRs, including the V2 vasopressin receptor (Sadeghi *et al.*, 1997), have shown that these cysteines are modified by covalent attachment of palmitic acid (for reviews, see Bouvier *et al.*, 1995; Ross, 1995), which may provide a lipophilic membrane anchor to create a fourth intracellular loop (i4 loop). Our data therefore suggest that this putative i4 loop (corresponding to helix VIII in the X-ray structure of rhodopsin (Palczewski *et al.*, 2000)) makes an important contribution to V2 receptor/G_s coupling selectivity.

Studies with hybrid α_{2A}/β_2-adrenergic (Liggett *et al.*, 1991) and hybrid mGluR1/mGluR3 glutamate receptors (Pin *et al.*, 1994; Gomeza *et al.*, 1996) also suggest that the membrane-proximal portion of the i4 domain plays a role in regulating the selectivity of G protein recognition in other GPCR families. In both cases, however, it was noted that substitution of this region alone of the donor receptor into the functionally different recipient receptor was not sufficient to confer a novel coupling profile, but required additional substitutions involving i2 or i3 loop sequences. To the best of our knowledge, the present study therefore provides the first example that exchange of the i4 loop alone between two functionally distinct receptor subtypes can lead to mutant receptors with qualitatively different G protein coupling profiles.

Another goal of this study was to examine which subdomains/specific amino acids within the i3 loop of the V2 receptor (Glu225-Leu274) are of primary importance

for efficient G_s recognition and activation. To address this issue, we created and analyzed a series of mutant V1a receptors in which distinct segments of the i3 loop were systematically replaced with the corresponding V2 receptor sequences. These studies showed that the C-terminal segments of the i3 loop of the V2 receptor do not make a significant contribution to G_s coupling selectivity and efficiency (see, for example, CR9 and CR10; Fig. 3.4). The relative lack of G_s coupling seen with CR9 and CR10 is unlikely to be due to improper folding of the intracellular receptor surface, since both receptors retained the ability to mediate the stimulation of PI hydrolysis with high efficacy (Fig. 3.11).

In contrast, substitution into the V1a receptor of V2 receptor sequences located at the N-terminus of the i3 loop (Ni3) allowed the resulting hybrid receptors to activate the G_s/adenylyl cyclase system. A mutant V1a receptor containing 30 amino acids of V2 receptor Ni3 sequence (Glu225-Gly254; CR8) quantitatively mimicked the cAMP responses mediated by CR1 (E_{max} = 71-76%; AVP EC_{50} = 1.1-1.2 nM; Figs. 3.4 and 3.5, Table 3.3), suggesting that this short receptor segment contains the key structural elements of the V2 receptor i3 loop critical for G_s coupling.

CR12 which contains only 12 amino acids of V2 receptor Ni3 sequence (Glu225-Leu236) still retained the ability to activate the cAMP pathway, though with reduced efficacy (E_{max} about 30%; Fig. 3.4, Table 3.3). A sequence comparison showed (Fig. 3.6) that this sequence element contains three groups of amino acids (Glu225/Val226, Phe229-Gln231, and His233-Ser235) which clearly differ in their physicochemical properties from the corresponding residues present in the V1a receptor. Groupwise substitution of these amino acid pairs/triplets into CR9 (which contains V2 receptor sequences in the central and C-terminal portions of the i3 loop) suggested that residues contained within the Glu225/Val226 pair and the Phe229/Arg230/Gln231 triplet make an important contribution to G_s coupling efficiency (Fig. 3.7). Using the CR8 mutant receptor (which contains 30 amino acids of V2 receptor Ni3 sequence) as a "background" for single amino acid substitutions, loss-of-function mutagenesis studies showed that only two of these five residues, Glu225 and Gln231, are required for efficient activation of G_s (Fig. 3.8).

Based on biochemical, molecular genetic, and biophysical studies with other classes of GPCRs, Glu225 and Gln231 are thought to be located in a receptor region (TM V/i3 loop junction) predicted to be α-helically arranged (Strader *et al.*, 1994; Wess, 1997; Altenbach *et al.*, 1996). Guided by an improved low-resolution electron density map of frog rhodopsin (Unger *et al.*, 1997) and structural information gathered from the analysis of approximately 500 different GPCRs, Baldwin and coworkers (Baldwin *et al.*, 1997) proposed an updated model for the α-carbon positions in the seven TM helices of CPCRs of the rhodopsin family (which includes the vasopressin receptors). In this model, Glu225 and Gln231 are predicted to project into a cavity formed by TM III, V, and VI (Figs. 3.12A and 3.12B), consistent with the recently published high-resolution structure of bovine rhodopsin (Palczewski *et al.*, 2000). Another functionally critical residue projecting into this cavity is Arg137 (located at the cytoplasmic end of TM III) which is conserved in most GPCRs of the rhodopsin family and plays a key role in triggering G protein activation (Dohlman *et al.*, 1991; Savarese and Fraser, 1992; Strader *et al.*, 1994; Kobilka, 1992; Wess, 1997). Rosenthal *et al.* (1993) and Barak *et al.*, (2001) have shown, for example, that mutational modification of this residue in the V2 vasopressin receptor severely impairs V2 receptor/G_s coupling. Arg137, Glu225, and Gln231 are therefore likely to define a distinct site on the intracellular surface of the V2 receptor that is critical for G_s recognition and activation.

In agreement with the data presented here, studies with other classes of GPCRs including the muscarinic (reviewed in Wess, 1997) and adrenergic receptors (reviewed in Dohlman *et al.*, 1991; Savarese and Fraser, 1992; Strader *et al.*, 1994; Kobilka, 1992) have also demonstrated that residues at the TM V/i3 loop junction are critically involved in regulating receptor/G protein coupling selectivity. These studies have shown that the residues that are of primary importance for proper G protein recognition are hydrophobic or non-charged. Interestingly, the functionally critical V2 receptor residues, Glu225 and Gln231, are highly polar and charged, respectively, indicating that the contribution of the Ni3 region to receptor/G protein coupling selectivity is not limited to hydrophobic contacts.

Comparison of the functional properties of CR8 (containing 30 amino acids of V2 receptor sequence; E_{max} = 71%) and CR12 (containing 12 amino acids of V2 receptor sequence; E_{max} = 29%) suggested that the central portion of the i3 loop also makes a contribution to V2 receptor/G_s coupling efficiency. Consistent with this notion, substitution of Gly245-Gly254 (V2 receptor sequence) into CR12 yielded a hybrid receptor (CR27) that gained the ability to stimulate intracellular cAMP levels with the same high efficacy as CR8 (Fig. 3.9, Table 3.3). This sequence element (Gly245-Gly254) is particularly rich in arginine and glycine residues (Fig. 3.9A). However, systematic alanine substitution mutagenesis showed that these residues are not critical for the ability of CR27 to efficiently mediate G_s activation. Most strikingly, a CR27-derived mutant receptor in which the Gly245-Gly254 sequence was replaced with a string of 10 alanine residues (CR31) was able to mediate AVP-dependent cAMP production with the same high efficacy and AVP potency as CR27 (Figs. 3.9 and 3.10, Table 3.3).

Fig. 3.1 indicates that the central portion of the i3 loop of the V1a receptor is 13 amino acids longer than the corresponding V2 receptor region. Thus, during the construction of CR27 (as well as of CR28-CR31; Fig. 3.9A), 21 amino acids of V1a receptor sequence (His258-Val278) were replaced with only 10 V2 receptor residues (Gly245-Gly254). This observation, in addition to the observed functional profiles of CR27-CR31, suggested that the relative length of the central portion of the i3 loop (rather than the specific amino acid sequence of this region) may play a role in regulating receptor/G_s coupling selectivity. To further test this hypothesis, two additional mutant V1a/V2 receptors, CR32 and CR33, were constructed and functionally analyzed (Fig. 3.9). CR32, which contains the His258-Val278 -> Gly245-Gly254 substitution in the wt V1a receptor background, did not gain the ability to couple to the G_s/adenylyl cyclase system to a significant extent. Since CR27 (which was highly active in the cAMP assays) differs from CR32 only in the presence of 12 amino acids of V2 receptor sequence at the beginning of the i3 loop, this observation suggests that shortening of the central portion of the i3 loop by itself is not sufficient to allow efficient G_s coupling but requires the simultaneous presence of V2 receptor residues at the TM V/i3 loop junction (see above). However, when the central portion of the i3 loop of CR1 (E_{max} = 76%) was extended in

length by replacing Arg243-Gly254 (V2 receptor sequence) with Ser256-Val278 (V1a receptor sequence), the resulting hybrid receptor (CR33) displayed a pronounced loss of activity in the cAMP assays (E_{max} = 33%) (Fig. 3.9). Taken together, these results are consistent with the novel concept that the central portion of the i3 loop, though predicted not to be directly involved in receptor/G protein interactions, can modulate receptor/G protein coupling selectivity by regulating G protein access (e.g. via steric hindrance) to functionally important recognition sites on the receptor protein (such as the TM V/i3 loop junction).

In conclusion, the structural elements determining the ability of the V2 vasopressin peptide receptor to selectively activate G_s were studied in molecular detail. These findings indicate that V2 receptor coupling selectivity depends on several different structural features, some of which have not been observed previously in studies using other classes of GPCRs. This work highlights the diversity of mechanisms by which receptor/G protein-coupling selectivity can be achieved.

3.2 Single amino acid substitutions that alter the G protein coupling properties of the V2 vasopressin receptor identified in yeast by receptor random mutagenesis

3.2.1 Aim of the study

The first part of this thesis was designed to investigate the structural basis underlying the ability of the V2 receptor to selectively recognize G_s. To complement this analysis, it was of interest to examine why the V2 receptor lacked efficient coupling to $G_{q/11}$. Thus, the present mutagenesis study has aimed at identifying mutant V2 receptors that would gain coupling to G proteins of the $G_{q/11}$ class.

To facilitate such studies, the availability of an experimental system that would allow the quick and efficient functional analysis of a large number of mutant receptors would be highly desirable. To overcome the limitations of classical mutagenesis approaches which usually allow the analysis of only a relatively small number of mutant receptors, yeast expression technology was employed (Pausch, 1997; Sommers and Dumont, 1999; Reiländer *et al.*, 2000). The yeast expression system offers the attractive possibility that powerful genetic selection techniques can be used to assess with relative ease the functional properties of thousands of mutant receptors (which can be generated by random mutagenesis), without having to rely on specific structural hypotheses or preconceived notions of GPCR function (Konopka *et al.*, 1996; Sommers and Dumont, 1997; Dosil *et al.*, 1998; Baranski *et al.*, 1999; Sommers *et al.*, 2000; Geva *et al.*, 2000).

The first part of the present study deals with the functional co-expression of the V2 receptor and various chimeric yeast/mammalian Gα proteins in yeast. The yeast strains were genetically engineered, such that they required ligand-activated receptor/G protein coupling for cell growth (Pausch *et al.*, 1998). Additionally, the $G_{q/11}$-coupled muscarinic M_1, M_3, and M_5 and various mutant vasopressin and muscarinic receptors were also expressed in yeast to further validate the yeast expression system.

Subsequently, to take advantage of the powerful genetic selection procedures that are possible in yeast, random mutagenesis techniques were employed to isolate mutant V2 receptors with altered G protein coupling properties. A former study demonstrated

that the i2 loop of the V1a receptor is the key component in determining the ability of the V1a receptor to selectively couple to $G_{q/11}$ (Liu and Wess, 1996). For instance, a mutant V2 receptor, in which the i2 loop was replaced with the corresponding V1a receptor sequence, gained the ability to stimulate PI hydrolysis with high efficacy (Liu and Wess, 1996).

Based on these findings, the i2 loop of the V2 receptor was subjected to saturation random mutagenesis and a yeast library was generated, co-expressing individual mutant vasopressin receptors and the Gq5 protein, a mutant Gpa1p, in which the last five amino acids were derived from mammalian Gαq. Mutant V2 receptors that gained the ability to activate Gq5 were recovered in a yeast genetic screen. Additional mutagenesis were then carried out to further delineate the structural requirements that allow the G_s-coupled V2 receptor to gain function to G_q-like proteins.

3.2.2 Yeast expression system

The baker's yeast, *Saccharomyces cerevisiae* (in the following simply referred to as yeast), exists both in haploid and diploid forms. Interestingly, haploid yeast cells, which can be further distinguished into a- and α-cells, express only one class of GPCR that can be activated by yeast pheromones (a- or α-factor), thus triggering the yeast mating response (fusion of a- and α-cells). Whereas a-cells produce a-factor and express the α-factor receptor (encoded by *STE2*), α-cells secrete the α-factor and express the a-factor receptor (encoded by *STE3*). Activated pheromone receptors catalyze dissociation of a yeast heterotrimeric G protein, encoded by the *GPA1* (α), *STE4* (β), and *STE18* (γ) genes. Gpa1-dependent inhibition of βγ is thereby relieved, allowing the βγ-complex to activate a signal transduction pathway composed of elements of a mitogen-activated protein kinase (MAP kinase) cascade. The result of activation of this pathway is cell cycle arrest (mediated by the *FAR1* gene product) and transcriptional induction of pheromone-responsive genes (mediated by *FUS1*, *FUS3*, and other genes).

The striking resemblance between the components of the yeast pheromone pathway and mammalian GPCR signaling systems makes yeast an attractive host for the

in vivo reconstitution of mammalian GPCRs, G proteins, and related signal transduction proteins.

To establish a sensitive bioassay to study mammalian GPCRs in yeast, genetically engineered yeast strains (Price *et al.*, 1995, 1996; Pausch *et al.*, 1998) were used. The three yeast strains, MPy578fc, MPy578q5, and MPy578s5, were isogenic except for the *GPA1* gene coding for the yeast Gα subunit, Gpa1p (for genotype, see Table 2.1; for construction of MPy578q5 and MPy578s5, see "Materials and Methods," chapter 2.2.2.5). A key feature of these strains is that yeast growth requires activation of the pheromone pathway. Deletion of the *FAR1* gene allows yeast to grow despite G protein-mediated activation of the pheromone pathway, which normally leads to cell cycle arrest (Pausch *et al.*, 1998). The presence of the *FUS-HIS3* reporter construct makes the production of His3 protein dependent on receptor-mediated activation of the yeast pheromone pathway. As a result, only receptors able to activate co-expressed G proteins will allow growth of auxotrophic (*his3*) yeast strains in media lacking histidine. The *SST2* gene (which codes for a protein that is homologous to mammalian RGS (**R**egulator of **G** protein **S**ignaling) proteins) was disrupted to prevent attenuation of G protein signaling mediated by the GAP activity of Sst2p. The *STE2* gene coding for the yeast α-factor (pheromone) receptor was deleted to prevent competition with heterologously expressed GPCRs for co-expressed G proteins (see Fig. 3.13).

The yeast expression system offers the great advantage that powerful genetic screens can be employed in order to identify mutant receptors or G proteins with defined phenotypes (e.g. inactive versus active receptors, receptors with altered G protein coupling selectivity, constitutively active receptors, second-site suppressor mutants, etc.). Moreover, rapid and efficient techniques are available for making genetic alterations in yeast, making it possible to study GPCR function in ways that cannot be done in mammalian expression systems.

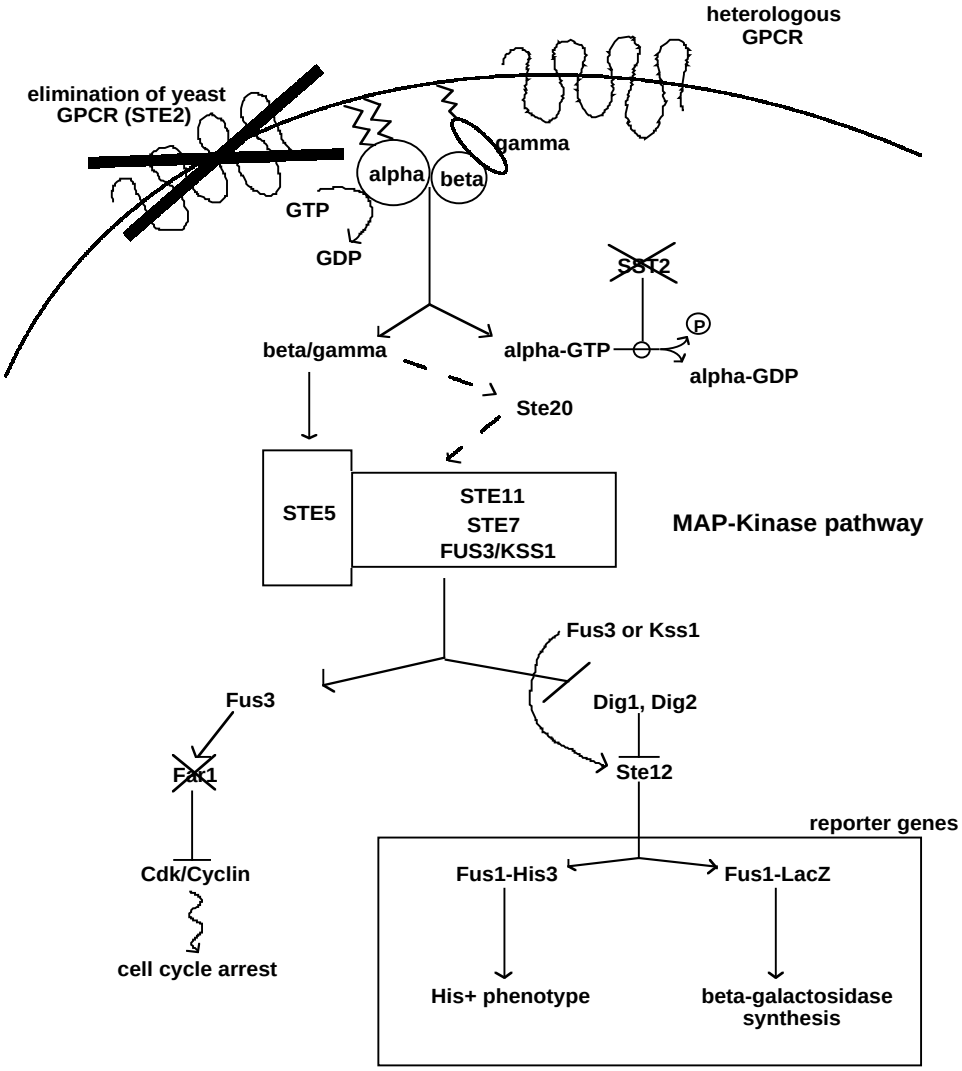


Fig. 3.13 A yeast-based expression system for studying the function of mammalian GPCRs.
The baker's yeast, S. cerevisiae, in its haploid form, expresses one GPCR and one G protein heterotrimer that are part of the yeast mating pathway. Binding of pheromone of the opposite mating type leads to activation of the yeast pheromone signal transduction pathway composed of elements of a mitogen-activated protein kinase (MAP kinase) cascade. Physiologically, activation of this pathway results in cell cycle arrest and transcriptional induction of pheromone-responsive genes.
The yeast expression system employed in the present study takes advantage of the striking resemblance of components of the pheromone pathway and mammalian GPCR signaling systems. Genetic alterations were introduced into a typical laboratory yeast strain to generate a yeast strain that required activation of the pheromone pathway for growth. Specifically, the following genetic modifications were made:

3.2.3 Results

3.2.3.1 Functional expression of the V2 vasopressin receptor in yeast

The initial goal of this study was to design a strategy that allowed the functional expression of the human V2 vasopressin receptor in yeast. First, the human V2 receptor coding sequence containing an HA epitope tag after the initiating methionine codon (Schöneberg *et al.*, 1996) was inserted into the yeast expression plasmid, p416GPD (Mumberg *et al.*, 1995), thus placing V2 receptor expression under the control of the strong yeast *GPD* promoter. The resulting expression plasmid (hV2-p416GPD) was then transformed into yeast strains that were genetically modified such that they required productive receptor/G protein coupling for growth (see "Materials and Methods" for details). Specifically, the V2 receptor expression construct was transformed into three different yeast strains (MPy578fc, MPy578q5, and MPy578s5) which were isogenic except for the *GPA1* gene coding for the endogenous yeast G protein α subunit (Pausch *et al.*, 1998, also see Table 2.1 and chapter 2.2.2.5). MPy578fc contained the wt *GPA1* gene, whereas MPy578q5 and MPy578s5 harbored mutant versions of this gene coding for mutant Gpa1 proteins in which the last five amino acids of Gpa1p were replaced with the corresponding residues present in mammalian $G\alpha_q$ and $G\alpha_s$, respectively (Fig. 3.14). In the following, the encoded Gα subunits are referred to as Gpa1p, Gq5, and Gs5, respectively.

Fig. 3.13 continued

The **Far1** protein mediates pheromone-dependent cell cycle arrest; deletion of *FAR1* therefore allows continued growth of yeast in the presence of pheromone. **Sst2p** is a prototypical RGS protein (Regulator of G protein Signaling) and increases the intrinsic GTPase activity of Gα, thus promoting desensitization of the pheromone signaling pathway. Deletion of *SST2* leads to a more robust and sustained pheromone signaling response. ***FUS1*** encodes a cell surface protein involved in cell fusion (mating); activation of the pheromone pathway strongly induces the *FUS1* promoter. The *FUS1* gene was replaced with a *FUS1-HIS3* reporter gene constuct.
In this system, agonist-induced receptor/G protein coupling triggers the activation of the yeast MAP kinase/pheromone pathway. This eventually leads to activation of the *FUS1* promoter and the expression of His3p, allowing yeast growth on histidine-deficient media (see “Materials and Methods” for details).

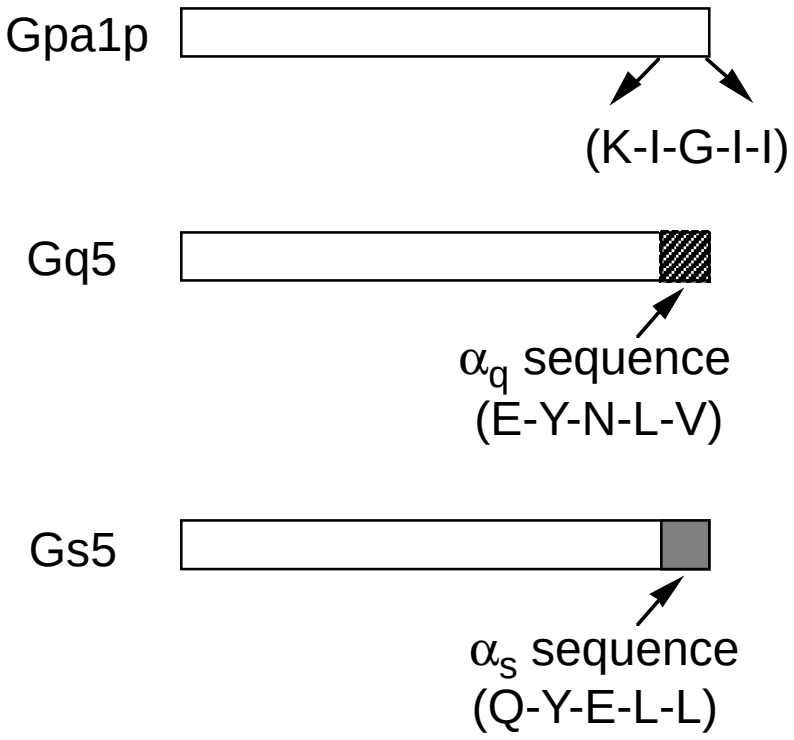


Fig. 3.14 Structure of G protein α subunits used in this study. The indicated wt and mutant G protein α subunits were co-expressed with the V2 vasopressin receptor or different muscarinic receptors in yeast. Gpa1p represents the endogenous yeast Gα subunit, whereas Gq5 and Gs5 are chimeric G proteins in which the C-terminal five amino acids of Gpa1p were replaced with the corresponding sequences derived from mammalian $G\alpha_q$ and $G\alpha_s$, respectively. The single letter amino acid code is used.

Initially, V2 receptor expression was monitored via Western blotting analysis, using the 12CA5 anti-HA mouse monoclonal antibody as the primary antibody. This analysis revealed two clusters of immunoreactive V2 receptor species, one migrating at ~ 40 kDa and the other one at ~ 80 kDA (Fig. 3.15) The ~40 kDa receptor cluster corresponds in size to monomeric forms of the V2 receptor, whereas the ~80 kDa cluster probably represents dimeric receptor species (Zhu and Wess, 1998). It is likely that the heterogeneity of the 40 and 80 kDa bands is primarilly due to heterogeneous glycosylation of the V2 receptor protein (Innamorati *et al.*, 1996; Romanos *et al.*, 1992).

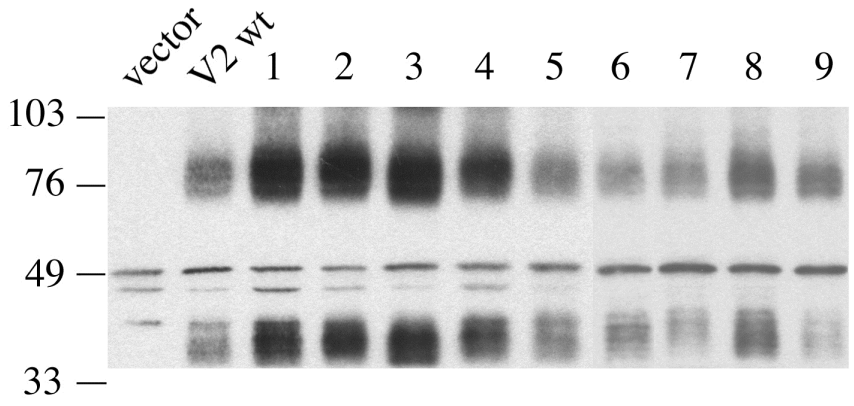


Fig. 3.15 Immunoblot analysis of wt and mutant V2 vasopressin receptors expressed in yeast. Membranes were prepared from yeast cells (strain MPy578q5) transformed with vector DNA (p416GPD) or yeast expression constructs coding for the wt V2 receptor or different mutant V2 receptors. Equal amounts of membrane proteins (10 µg) were analyzed by SDS-PAGE (4-20%) and Western blotting, using a primary antibody that was directed against the HA epitope tag present at the N-terminus of all receptor constructs (see "Materials and Methods" for details). The following mutant V2 receptor constructs were studied: V2(Ile141Δ) (*lane 1*), V2(Cys142Δ) (*lane 2*), V2(Arg143Δ) (*lane 3*), V2(Pro144Δ) (*lane 4*), V2(Met145Δ) (*lane 5*), V2(Leu146Δ) (*lane 6*), V2(Ala147Δ) (*lane 7*), V2(Tyr148Δ) (*lane 8*), and V2(Arg149Δ) (*lane 9*). Protein molecular mass standards (in kDa) are indicated.

In general, the pattern of immunoreactive V2 receptor bands that was obtained in yeast closely resembled that previously observed in transfected mammalian cells (Zhu and Wess, 1998). Also, it was found that all three yeast strains used in this study (MPy578fc, MPy578s5, and MPy578q5) expressed V2 receptor protein at similar levels (data not shown).

To determine the number of functional V2 receptors expressed in yeast, initially radioligand binding studies were carried out with the agonist ligand, [^{3}H]AVP (up to 8 nM). For reasons that are most likely related to the agonist and/or peptide nature of AVP (see below), no significant levels of [^{3}H]AVP binding sites could be detected using membranes prepared from V2 receptor-transformed yeast strains. In contrast, saturation binding experiments with the non-peptide V2 receptor antagonist, [^{3}H]-SR121463 (Serradeil-Le Gal *et al.*, 1996, 2000), consistently revealed a significant number of V2 receptor binding sites using the same yeast membrane preparations. [^{3}H]-SR121463 binding to the wt V2 receptor expressed in yeast was characterized by high affinity (K_D =

8.0 ± 2.5 nM; n=3) and a B_{max} value of 322 ± 102 fmol/mg (MPy578q5 strain; n=3) (see Table 3.7, page 145). As expected, no specific [^{3}H]-SR121463 binding activity was detected with membranes prepared from yeast strains transformed with empty vector (p416GPD).

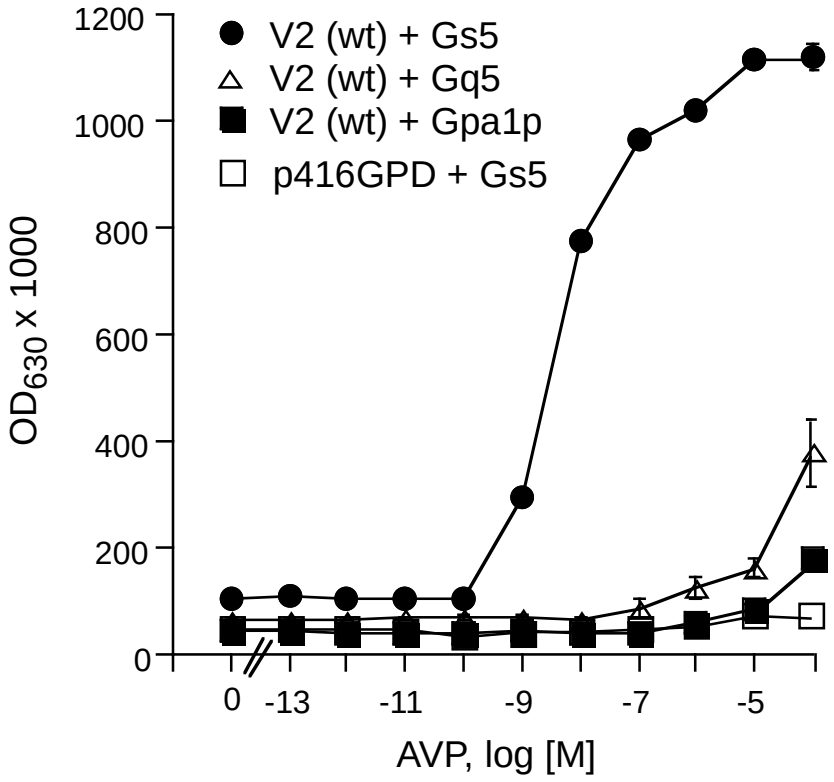


Fig. 3.16 AVP-induced yeast growth mediated by coupling of the wt V2 vasopressin receptor to a co-expressed hybrid G protein. Liquid bioassays were carried out with yeast strains co-expressing the wt V2 vasopressin receptor and the indicated G protein α subunits (Gpa1p, Gs5, or Gq5; for G protein structures, see Fig. 3.14), as described under "Materials and Methods". Yeast growth was measured by determining the absorbance at 630 nm (for AVP EC_{50} values, see Table 3.6). Results from one representative experiment, carried out with three independent transformants, are shown. Data are given as means ± S.E. Two additional experiments gave similar results.

Next, it was examined whether V2 receptors were able to productively couple to co-expressed G proteins in yeast. Specifically, liquid bioassays were carried out to examine the ability of the agonist, AVP, to stimulate growth of wt V2 receptor-expressing yeast strains in media lacking histidine (note that productive receptor/G protein coupling stimulates the production of His3 protein). As shown in Fig. 3.16, the V2 receptor was unable to efficiently interact with yeast Gpa1p and the hybrid G protein, Gq5. A residual growth response was observed only at the highest AVP concentrations used ($\geq$0.01 mM). In contrast, the wt V2 receptor was able to productively interact with co-expressed Gs5 (EC_{50} ~ 6 nM; Fig. 3.16 and Table 3.6, page 140). As shown in Fig. 3.16, this interaction was characterized by remarkably high AVP potency. Since Gpa1p, Gq5, and Gs5 differ only in their C-terminal five amino acids (Fig. 3.14), these data indicate that the V2 vasopressin receptor can selectively recognize the C-terminal α_s residues present in Gs5. Moreover, since the wt V2 receptor selectively couples to G_s in mammalian systems (Birnbaumer *et al.*, 1992; Lolait *et al.*, 1992; Burbach *et al.*, 1995; Liu and Wess, 1996; Thibonnier *et al.*, 1998), these observations confirm that the V2 receptor displays the proper G protein coupling preference in yeast.

3.2.3.2 Functional expression of muscarinic receptors in yeast

3.2.3.2.1 Wild type M_1, M_3, and M_5 muscarinic receptors are poorly expressed and do not couple efficiently to G proteins in yeast

As described above, the V2 receptor expressed in yeast displayed pronounced coupling to Gs5 but not Gq5. To rule out the possibility that the lack of interaction observed between the V2 receptor to Gq5 was due to poor expression or misfolding of Gq5, the $G_{q/11}$-coupled muscarinic M_1, M_3, and M_5 receptors were expressed in yeast. Initially, the full-length M_1, M_3, and M_5 muscarinic receptors were expressed in the yeast strain (MPy578fc) containing the endogenous yeast Gα subunit, Gpa1. To examine whether the heterologously expressed muscarinic receptors were able to couple to the endogenous yeast G protein, the ability of the muscarinic agonist, carbachol, to stimulate

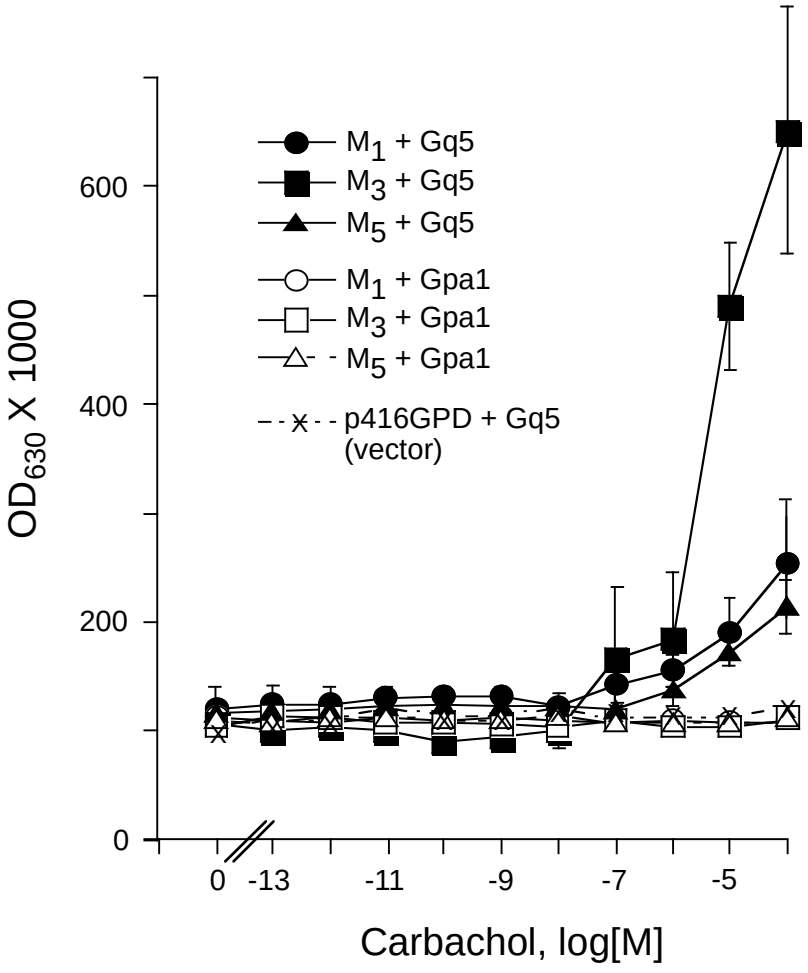


Fig. 3.17 Carbachol-induced yeast growth mediated by coupling of the wt (full-length) M_1, M_3 and M_5 muscarinic receptors to endogenous yeast G protein, Gpa1p, or the chimeric Gα subunit, Gq5. Liquid bioassays were carried out with yeast strains co-expressing the indicated muscarinic receptors and either Gpa1p or Gq5, as described under "Materials and Methods". Growth was measured by determining the absorbance at 630 nm. One representative experiment, carried out with three independent transformants, is shown. Data are given as means ± SE. Two additional experiments gave similar results.

yeast growth was determined in liquid bioassays. These studies showed that carbachol (10^{-13}-10^{-4} M) failed to promote growth of yeast strains transformed with the M_1, M_3, and M_5 receptor expression plasmids (Fig. 3.17), indicating that these receptors cannot productively interact with yeast Gpa1p.

We next expressed the M_1, M_3, and M_5 muscarinic receptors in a yeast strain (MPy578q5) expressing Gq5 (Fig. 3.14). In this system, a weak (M_1, M_5) to moderate (M_3) growth response was observed at the highest carbachol concentrations (> 1 μM), indicating that the presence of the C-terminal α_q residues in Gq5 facilitates coupling with the M_1, M_3, and M_5 muscarinic receptors (Fig. 3.17).

Table 3.4 ***Pharmacological properties of full-length and i3 loop-shortened M_1, M_3, and M_5 muscarinic receptors expressed in yeast***

	[^{3}H]NMS binding		Yeast growth assay (MPy578q5 strain)
Receptor	K_D (p*M*)	B_{max} (fmol/mg)	EC_{50} (Carbachol) *nM*
M_1	ND	<5	
$M_1\Delta i3$	188 ± 20	58± 3	120 ± 0.6
M_3	ND	<2	
$M_3\Delta i3$	574 ± 118	214 ± 15	9 ± 3
M_5	ND	<2	
$M_5\Delta i$	1,269 ± 51	53 ± 2	48 ± 1.6

ND, not determinable with sufficient accuracy.

Radioligand binding studies and liquid bioassays (yeast growth assays) were carried out as described under "Materials and Methods". In the case of the $M_1\Delta i3$, $M_3\Delta i3$, and $M_5\Delta i3$ receptors, binding parameters were calculated from complete [^{3}H]NMS saturation binding or carbachol inhibition binding curves. In the case of the full-length receptors, B_{max} values were defined as specific binding in the presence of one high saturating concentration of [^{3}H]NMS (3.8 nM). Data are given as means ± SE of three independent experiments, each carried out in duplicate (binding assays) or triplicate (liquid bioassays).

To determine receptor expression levels, [^{3}H]NMS binding studies were carried out with membranes prepared from yeast strains (based on MPy578q5) transformed with the M_1, M_3, and M_5 receptor expression plasmids. Incubation of yeast membrane homogenates with a saturating concentration of [^{3}H]NMS (3.8 nM) revealed that all three receptors were expressed at very low levels (<5 fmol/mg)(Table 3.4).

3.2.3.2.2 The $M_1\Delta i3$, $M_3\Delta i3$, and $M_5\Delta i3$ muscarinic receptors couple with high efficiency to a hybrid Gpa1p/α_q G protein

As described in the previous paragraph, the expression of the full-length M_1, M_3, and M_5 muscarinic receptors in yeast resulted in very low numbers of muscarinic binding sites and non-detectable (in the case of Gpa1p) or inefficient (in the case of Gq5) G protein coupling. The M_1-M_5 muscarinic receptors, like several other GPCRs, contain very large i3 loops ranging in size from 157 to 240 amino acids (Wess, 1996). However, studies in mammalian expression systems have shown that most of the i3 loop sequences, except for the membrane-proximal 10-15 amino acids, are not required for productive receptor/G protein coupling (reviewed in Wess, 1996).

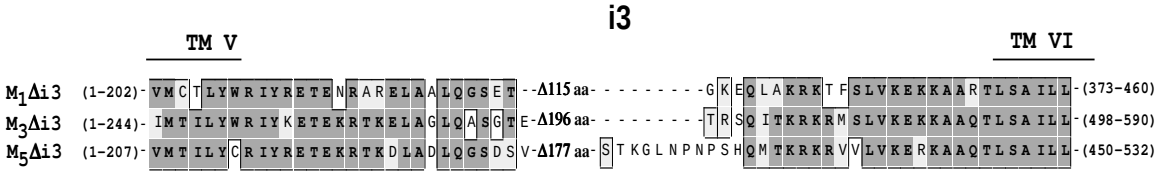
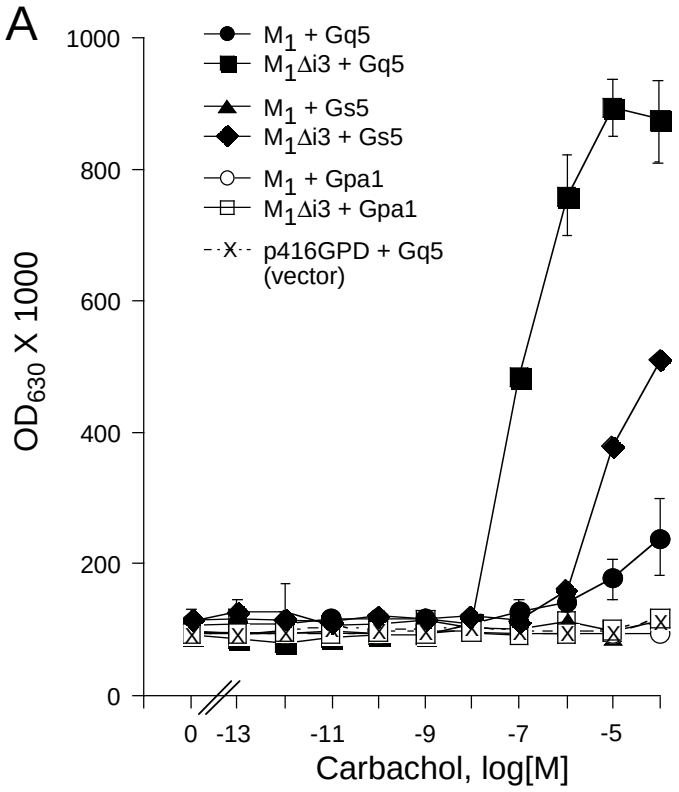


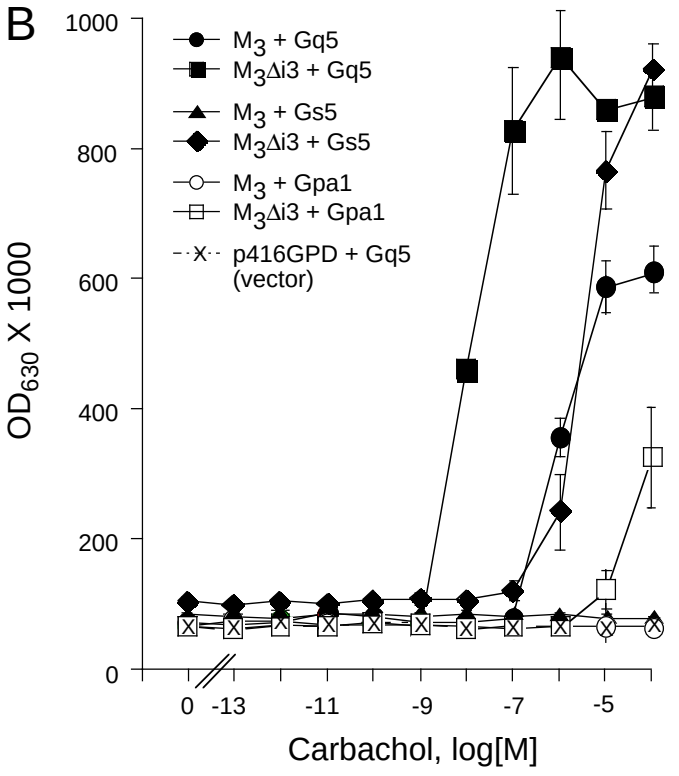
Fig. 3.18 Structure of the $M_1\Delta i3$, $M_3\Delta i3$, and $M_5\Delta i3$ mutant muscarinic receptors. The cytoplasmic ends of transmembrane domains V and VI and the membrane-proximal portions of the i3 loop are shown. To generate the $M_1\Delta i3$, $M_3\Delta i3$, and $M_5\Delta i3$ mutant muscarinic receptors, the indicated i3 loop sequences were deleted from the human M_1 (Bonner et al., 1988), rat M_3 (Bonner et al., 1987), and human M_5 (Bonner et al., 1988) muscarinic receptors. Numbers in parentheses indicate the number of amino acids that were not included in the alignment. Dark gray boxes (bold letters) indicate sites of amino acid identity; light gray boxes highlight conservative amino acid changes. The single-letter amino acid code is used.

It appeared possible that the large i3 loop domains may be detrimental to the efficient expression of muscarinic receptors in yeast, perhaps by serving as targets for yeast proteases leading to rapid receptor degradation. To test the hypothesis that removal of i3 loop sequences may lead to improved G protein coupling and increased muscarinic receptor expression levels in yeast, we generated p416GPD-based yeast expression plasmids coding for mutant M_1, M_3, and M_5 receptors ($M_1\Delta i3$, $M_3\Delta i3$, and $M_5\Delta i3$) that lacked major portions of their i3 loops (Fig. 3.18). Only the first and last 21-22 amino acids of the i3 loops still remained in the $M_1\Delta i3$, $M_3\Delta i3$, and $M_5\Delta i3$ constructs.

Liquid bioassays using yeast cells co-expressing the i3 loop-shortened versions of the M_1, M_3, and M_5 muscarinic receptors and the yeast G protein, Gpa1p, showed that $M_1\Delta i3$ and $M_5\Delta i3$ were unable to couple to yeast Gpa1p (Figs. 3.19A and 3.19C). In the case of $M_3\Delta i3$, rather weak coupling could be detected at the highest carbachol concentration used (0.1 mM) (Fig. 3.19B). Strikingly, however, the $M_1\Delta i3$, $M_3\Delta i3$, and $M_5\Delta i3$ receptors gained the ability to interact with the hybrid G protein, Gq5, with high efficiency. As shown in Figs 3.19A-C, carbachol stimulated the growth of yeast strains co-expressing $M_1\Delta i3$, $M_3\Delta i3$ or $M_5\Delta i3$ and Gq5 with high potency and efficacy (Table 3.4). These responses were abolished when yeast cells were cultured in the presence of the muscarinic antagonist, atropine (10 μM) (data not shown). As shown in Table 3.4, coupling of the $M_3\Delta i3$ mutant receptor to the Gq5 G protein was characterized by remarkably high efficiency (carbachol EC_{50}: ~ 9 nM).

To examine whether the $M_1\Delta i3$, $M_3\Delta i3$ and $M_5\Delta i3$ receptors retained proper G protein coupling selectivity in yeast, these constructs were also expressed in a yeast strain (MPy578s5) expressing a mutant version of Gpa1p (Gs5) in which the C-terminal five amino acids were replaced by the homologous $G\alpha_s$ sequence (Fig. 3.14). As shown in Fig. 3.19, the $M_1\Delta i3$, $M_3\Delta i3$, and $M_5\Delta i3$ receptors coupled to co-expressed Gs5 much less efficiently than to Gq5. In the case of $M_1\Delta i3$ and $M_3\Delta i3$, carbachol concentration response curves were shifted to the right by at least two orders of magnitude (Figs. 3.19A and 3.19B), whereas coupling of $M_5\Delta i3$ to Gs5 was virtually non-detectable (Fig. 3.19C).


A
M1 + Gq5
M1Δi3 + Gq5
M1 + Gs5
M1Δi3 + Gs5
M1 + Gpa1
M1Δi3 + Gpa1
p416GPD + Gq5 (vector)
OD630 X 1000
1000
800
600
400
200
0
0 -13 -11 -9 -7 -5
Carbachol, log[M]


B
M3 + Gq5
M3Δi3 + Gq5
M3 + Gs5
M3Δi3 + Gs5
M3 + Gpa1
M3Δi3 + Gpa1
p416GPD + Gq5 (vector)
OD630 X 1000
1000
800
600
400
200
0
0 -13 -11 -9 -7 -5
Carbachol, log[M]

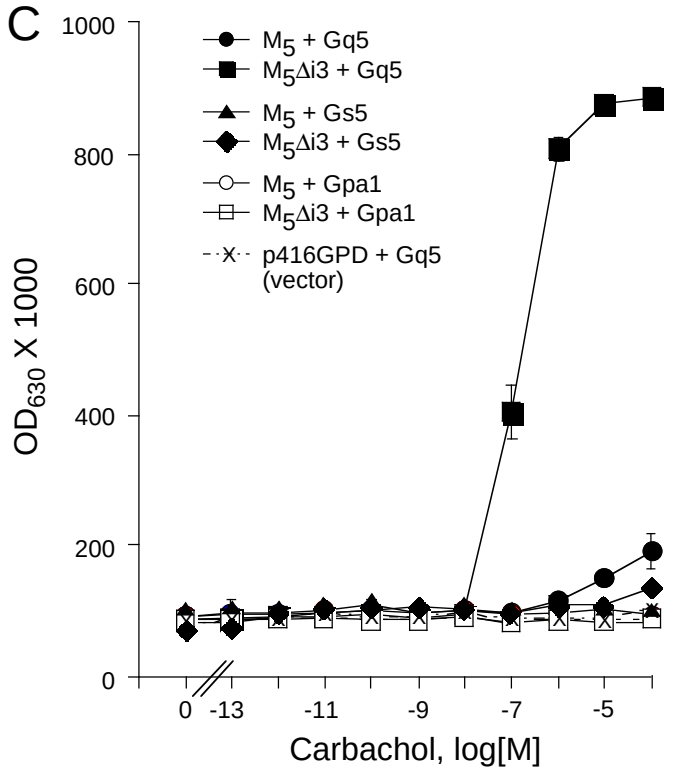


Fig. 3.19 Carbachol-induced yeast growth mediated by coupling of wt and i3-loop shortened muscarinic receptors to co-expressed wt and hybrid G proteins. *A,* Coupling properties of the full-length M_1 receptor and the $M_1\Delta i3$ deletion mutant. *B,* Coupling properties of the full-length M_3 receptor and the $M_3\Delta i3$ deletion mutant. *C,* Coupling properties of the full-length M_5 receptor and the $M_5\Delta i3$ deletion mutant. The structures of the $M_1\Delta i3$, $M_3\Delta i3$, and $M_5\Delta i3$ mutant receptors and the co-expressed Gα subunits are given in Figs. 3.15 and 3.17, respectively. Liquid bioassays were carried out with yeast strains co-expressing the indicated receptors and Gα subunits, as described under "Materials and Methods". Growth was measured by determining the absorbance at 630 nm. One representative experiment, carried out with three independent transformants, is shown. Data are given as means ± SE. Two additional experiments gave similar results.

As described in the previous paragraph, functional expression of the G_s-coupled V2 vasopressin receptor in yeast showed that this receptor interacted with Gs5 with much greater efficiency than with Gq5 or Gpa1p. The observation that the $M_1\Delta i3$, $M_3\Delta i3$, and $M_5\Delta i3$ receptors coupled to Gq5 much more efficiently than to Gs5 demonstrated that the

lack of coupling observed between the V2 receptor and Gq5 was not caused by poor expression or misfolding of the Gq5 subunit.

3.2.3.2.3 Ligand binding properties of the $M_1\Delta i3$, $M_3\Delta i3$, and $M_5\Delta i3$ muscarinic receptors expressed in yeast

To further characterize the pharmacological properties of the i3 loop-shortened muscarinic receptors, [^{3}H]NMS binding studies were carried out with membranes prepared from yeast strains (based on MPy578q5) transformed with the $M_1\Delta i3$, $M_3\Delta i3$, and $M_5\Delta i3$ receptor expression plasmids. [^{3}H]NMS saturation binding studies (Table 3.4) showed that transformation of yeast with the $M_1\Delta i3$, $M_3\Delta i3$, and $M_5\Delta i3$ constructs led to dramatic increases in the maximum number of detectable [^{3}H]NMS binding sites (range: 53-214 fmol/mg), as compared to their full-length receptor counterparts (<5 fmol/mg). [^{3}H]NMS dissociation constants (K_D values) ranged from 188 pM for the $M_1\Delta i3$ receptor to 1,269 pM for the $M_5\Delta i3$ construct (Table 3.4).

3.2.3.3 Further validation of the yeast expression system

Additional experiments were carried out to further substantiate the general usefulness of the yeast expression system described here as a model for studying mammalian receptor/G protein coupling mechanisms. Studies in mammalian expression systems have led to the identification of mutant GPCRs with altered functional properties, including receptors with modified coupling selectivity, receptors that are constitutively active, or receptors which do not couple to G proteins at all. It was of interest to examine whether these mutant receptor phenotypes could also be observed in the yeast expression system.

Zeng and Wess (unpublished results) recently identified three mutant M_3 muscarinic receptors ($M_3\Delta i3$(Gln490->Tyr), $M_3\Delta i3$(Gln490->Cys), and $M_3\Delta i3$(Gln490->Leu) (Gln490 is located at the cytoplasmic end of TM VI in the M_3 receptor)) which displayed pronounced constitutive activity when expressed in COS-7 cells. Expression of

these three mutant receptors in a yeast strain expressing the chimeric Gα-subunit, Gq5, showed that all mutant receptors retained robust constitutive activity in yeast (Fig. 3.20A).

Moreover, studies with transfected mammalian cells showed that a mutant V2 vasopressin receptor (V2i2) in which the i2 loop had been exchanged against homologous V1a receptor sequence gained the ability to interact with G proteins of the $G_{q/11}$ family with high efficiency (Liu and Wess, 1996).

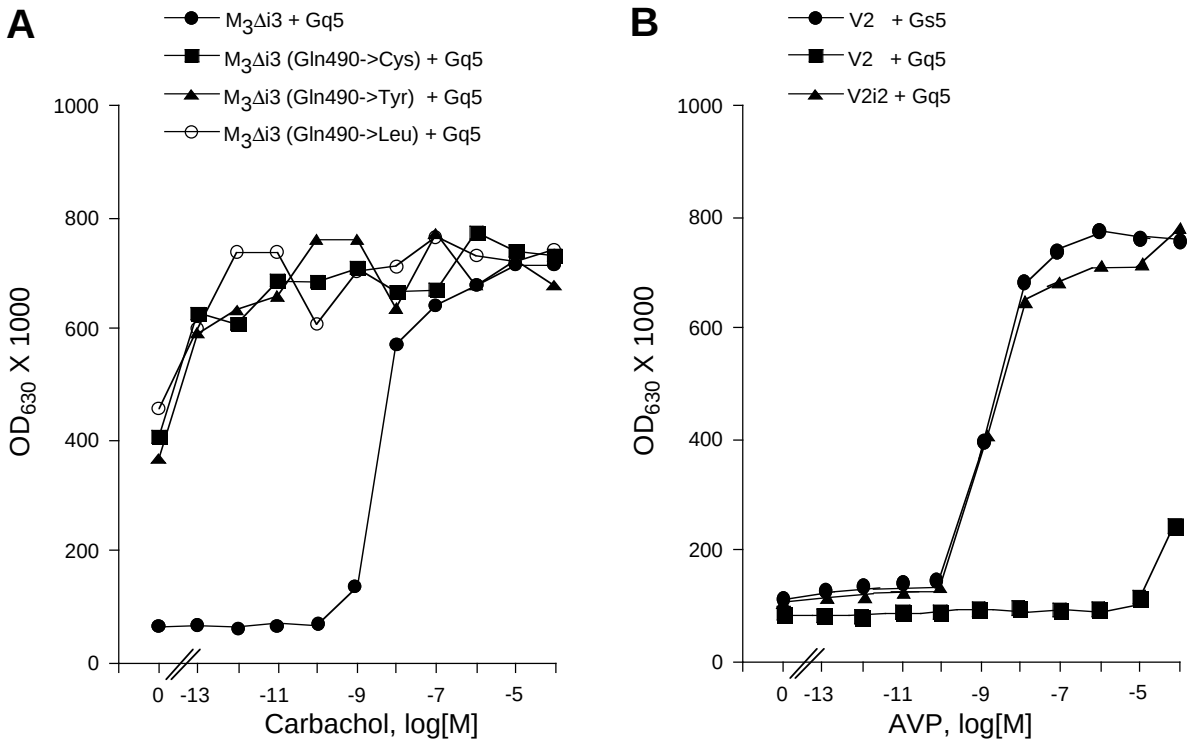


Fig. 3.20 Ligand-induced yeast growth mediated by coupling of wt and mutant muscarinic and vasopressin receptors co-expressed with hybrid G proteins. *A,* Coupling properties of the wt and mutant $M_3\Delta i3$ muscarinic receptors. The structure of the $M_3\Delta i3$ receptor is given in Fig. 3.15. *B,* Coupling properties of wt V2 and chimeric V1a/V2 vasopressin receptors. The chimeric vasopressin receptor V2i2 was created by replacing V2(138->160) with V1a(150->171) in the wt V2 receptor (also see Fig. 1.9, chapter 1.3.3). The structures of the co-expressed Gα subunits are given in Fig. 3.14. Liquid bioassays were carried out with yeast strains co-expressing the indicated receptors and Gα subunits, as described under "Materials and Methods". Growth was measured by determining the absorbance at 630 nm. One representative experiment, carried out with three independent transformants, is shown. Data are given as means ± SE. Two additional experiments gave similar results.

When expressed in the yeast expression system, this V2i2 construct gained the ability to productively couple to the Gq5 protein (Fig. 3.20B). In contrast, as indicated above, the wt V2 receptor recognized this hybrid G protein only very poorly (Fig. 3.20B).

These data strongly suggest that observations made in mammalian systems can be faithfully reproduced in the yeast expression system described here. Reciprocally, it appears reasonable to assume that results generated by using the yeast expression system will be applicable to mammalian systems.

3.2.3.4 Screening and selection of mutant receptors with altered G protein coupling properties

The powerful genetic selection procedures that can be employed in yeast should allow the isolation of mutant V2 receptors with novel G protein coupling properties. In this present study, a strategy was devised to identify mutant V2 receptors that would gain the ability to couple to the Gq5 chimeric G protein.

To test the feasibility of this approach, the i2 loop of the V2 vasopressin receptor (His138-Val160; Fig. 3.21) was subjected to oligonucleotide-based random mutagenesis. To generate a MPy578q5-based library of yeast clones expressing mutant V2 receptors, a gap-repair method was used (Muhlrad *et al.*, 1992; Staples and Dieckmann, 1993; Sommers and Dumont, 1997, 1999) that involved co-transformation of a gapped version of hV2-p416GPD with a PCR fragment coding for mutagenized i2 loop sequences into MPy578q5 (see "Materials and Methods" for details). In vivo recombination events led to the reformation of circular plasmids coding for mutant V2 vasopressin receptors. To assess the quality of the mutant V2 receptor library, plasmids were recovered from 15 randomly picked colonies grown on uracil-deficient medium to select for transformants containing receptor plasmids. Sequencing analysis of these plasmids showed that each clone contained an average of about 6.2 nucleotide changes, in good agreement with the predicted mutagenesis rate (10% at the nucleotide level). We also noted that the mutations were evenly distributed throughout the i2 loop sequence.

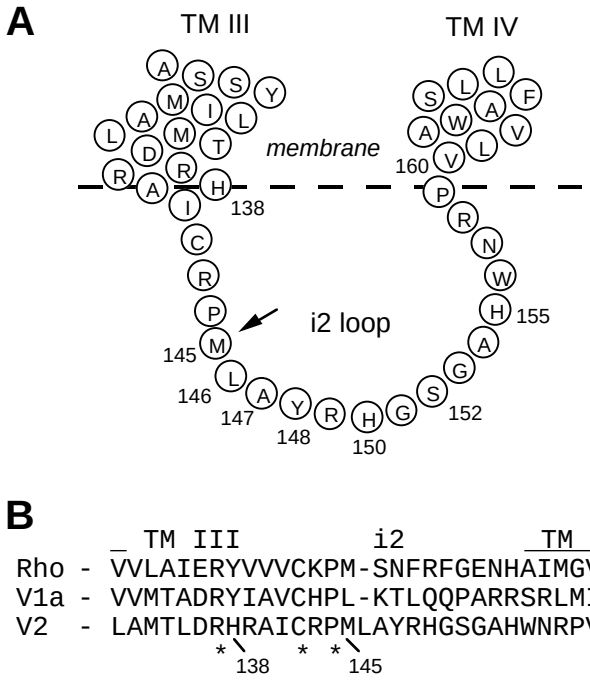


Fig. 3.21 Amino acid sequence of the i2 loop of the human V2 vasopressin receptor. *A*, Residues His138-Val160 corresponding in sequence to the i2 loop of the human V2 vasopressin receptor were subjected to random mutagenesis as described under "Materials and Methods". Met145 (highlighted by an arrow) is predicted to play a key role in regulating receptor/G protein coupling selectivity (see text for details). The putative cytoplasm/membrane boundary is indicated by a dotted line. *B*, Comparison of the i2 loop sequences of bovine rhodopsin (Rho; Nathans and Hogness, 1983), for which high resolution structural information is available (Palczewski *et al.*, 2000), the rat V1a vasopressin receptor (Morel *et al.*, 1992), and the human V2 vasopressin receptor (Birnbaumer *et al.*, 1992). *, positions of amino acid identity. Numbers refer to amino acid positions in the human V2 vasopressin receptor sequence (Birnbaumer *et al.*, 1992).

Colonies that were able to grow on uracil-deficient medium were then replica-plated onto medium that lacked both uracil and histidine but contained the agonist, AVP (0.4 μM), and 10 mM of AT (to suppress background growth). Under these conditions, yeast clones expressing the wt V2 receptor failed to grow (Fig. 3.22), obviously due to the inability of the V2 receptor to interact with Gq5 in a productive fashion. However, under identical conditions, yeast cells co-expressing the wt V2 receptor and Gs5 showed robust agonist-dependent growth (Fig. 3.22). Approximately 30,000 primary

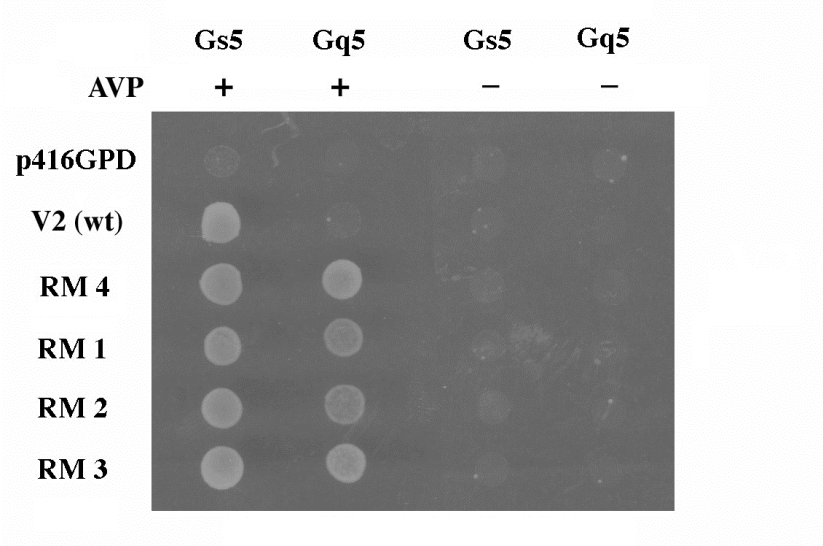


Fig. 3.22 The RM1-RM4 mutant V2 vasopressin receptors, but not the wt V2 receptor, stimulate yeast growth via interaction with Gq5. Yeast strains expressing either Gs5 or Gq5 (for G protein structures, see Fig. 3.14) were transformed with vector DNA (p416GPD) or yeast expression plasmids coding for the wt V2 receptor or the RM1-RM4 mutant receptors (for mutant receptor structures, see Table 3.6). 5 µl of yeast cell suspensions (OD: ~0.1) co-expressing the indicated chimeric G proteins and wt or mutant V2 receptors were spotted onto uracil- and histidine-deficient SC medium in the absence or presence of AVP (final concentration: 0.4 µM). To suppress background growth, the plates were supplemented with 10 mM AT, a competitive inhibitor of the *HIS3* gene product (for details, see "Materials and Methods"). Plates were incubated at 30°C for 3 days.

transformants were replica-plated and screened for their ability to grow in an AVP-dependent fashion on the selection medium. Based on the observed mutagenesis rate (about six nucleotide changes per clone), this number of clones should be more than sufficient to include any possible nucleotide substitution within the mutagenized receptor region.

The initial screen yielded eleven colonies that were able to grow on plates containing the selection medium (after replica plating). To confirm that their growth phenotype was dependent on a plasmid-borne V2 receptor mutation, receptor plasmids were recovered from these clones, amplified in *E. coli*, and retransformed into the

MPy578q5 parental yeast strain. Colony assays of the resulting transformants showed that four of the recovered receptor plasmids reproducibly conferred on the MPy578q5 strain the ability to grow, in an AVP-dependent fashion, on plates containing the proper selection media (Fig. 3.22). The mutations found in the encoded mutant V2 receptors (RM1-RM4) are given in Table 3.5. Three of the four recovered mutant receptors had three or more mutations within the i2 loop (RM2-RM4), whereas RM1 contained a single amino acid substitution (Met145Trp). Interestingly, all four mutant receptors contained a modification at position Met145, either an amino acid substitution (RM1-RM3) or a deletion (RM4). In fact, the Met145Δ/Leu146Trp mutation in RM4 is identical with a Met145Trp/Leu146Δ mutation (Table 3.5).

Table 3.5

Mutant V2 vasopressin receptors recovered in a yeast genetic screen that gained coupling to the hybrid G protein, Gq5

About 30,000 mutant V2 receptors, generated by random mutagenesis of the region coding for the i2 loop, were screened for their ability to gain coupling to a mutant version of Gpa1 (Gq5) containing C-terminal α_q sequence (for G protein structure, see Fig. 3.14), as described under "Materials and Methods". The four recovered mutant receptors, RM1-RM4, conferred on the MPy578q5 yeast strain (Fig. 3.14) the ability to grow, in an AVP-dependent fashion, on plates lacking uracil and histidine (see text for details).

Recovered V2 mutant receptor	Mutations identified via sequencing
RM1	Met145Trp
RM2	Met145Leu, Leu146Arg, Ala147Thr, Ser152Arg
RM3	Met145Leu, Tyr148Phe, His155Leu
RM4	Met145Δ, Leu146Trp, His150Arg, Ser152Arg (=Met145Trp, Leu146Δ, His150Arg, Ser152Arg)

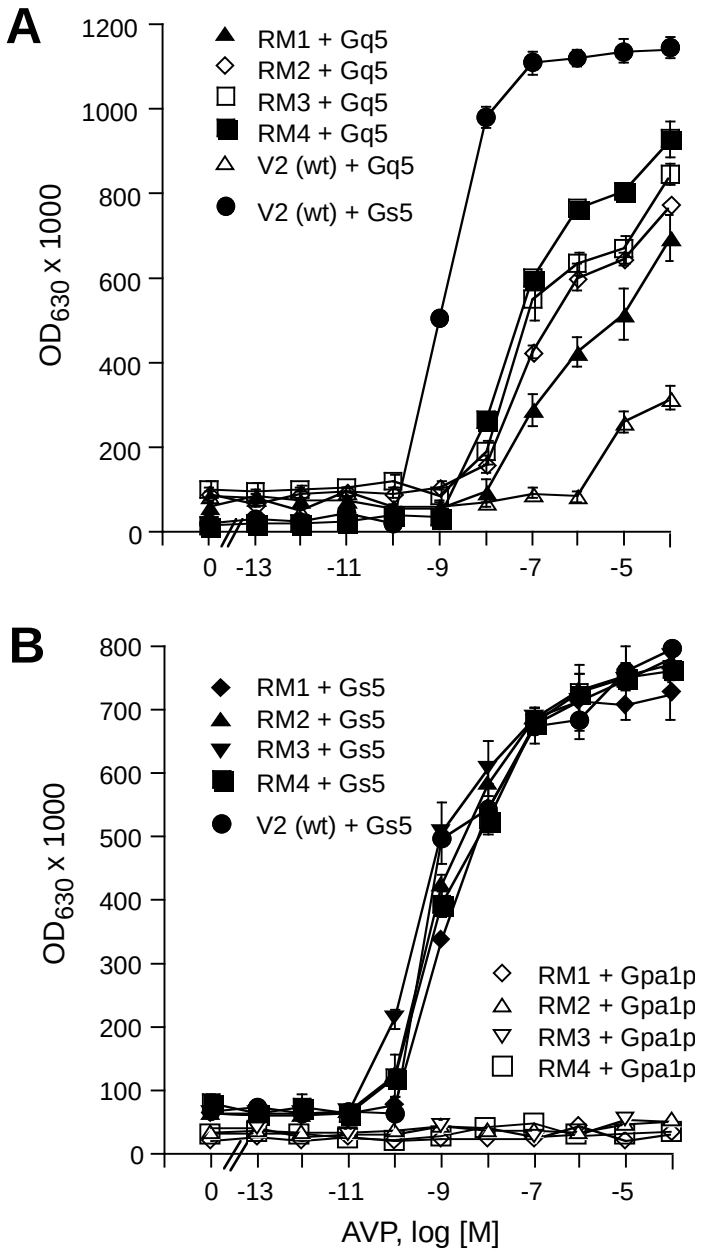


Fig. 3.23 AVP-induced yeast growth mediated by coupling of the RM1-RM4 mutant V2 vasopressin receptors to co-expressed hybrid G proteins. Liquid bioassays were carried out with yeast strains co-expressing the RM1-RM4 mutant V2 vasopressin receptors and the indicated G protein α subunits (for G protein structures, see Fig. 3.14), as described under "Materials and Methods". *A*, Coupling to Gq5. The yeast strain co-expressing the wt V2 receptor and Gs5 was included as a control. *B*, Coupling to Gs5 or Gpa1p. Yeast growth was measured by determining the absorbance at 630 nm (for AVP EC_{50} values, see Table 3.6). Results from one representative experiment, carried out with three independent transformants, are shown. Data are given as means ± S.E. Two additional experiments gave similar results.

3.2.3.5 Characterization of the G protein coupling properties of the RM1-RM4 mutant V2 receptors in yeast liquid bioassays

To examine the G protein coupling properties of the recovered RM1-RM4 mutant receptors in greater detail, liquid bioassays were carried out to examine the ability of these mutant receptors to stimulate Gq5, Gs5, or yeast Gpa1p. As expected based on the colony screen, all four mutant receptors gained considerable coupling to Gq5 (E_{max}~60-80%), as compared to the wt V2 receptor which conferred only a residual growth response on Gq5-expressing yeast cells (Fig. 3.23A, Table 3.6). However, similar to the wt receptor, the RM1-RM4 mutant receptors retained the ability to interact with Gs5 in a highly efficient manner and showed little or no coupling to yeast Gpa1p ((Fig. 3.23B, Table 3.6).

3.2.3.6 Substitutions at position Met145 have pronounced effects on the G protein coupling profile of the V2 vasopressin receptor

One of the four mutant receptors (RM1) recovered from the initial colony screen harbored a single Met145Trp point mutation and two of the recovered mutant receptors (RM2 and RM3) contained an Met145Leu substitution, in addition to other point mutations (Table 3.5). To exclude the possibility that RM1 harbored additional mutations outside the region that was subjected to random mutagenesis and sequencing (i2 loop and adjacent TM domains), a V2(Met145Trp) mutant receptor was recreated via site-directed mutagenesis. In addition, to examine whether the Met145Leu substitution was sufficient to confer on the V2 receptor the ability to interact with Gq5, a mutant V2 receptor construct was generated which contained the Met145Leu substitution as the only mutation. The V2(Met145Trp) and V2(Met145Leu) receptor constructs were transformed into the MPy578q5 strain and then functionally analyzed in yeast liquid bioassays. As shown in Fig. 3.24A, both mutant receptors gained significant coupling to Gq5 (for AVP EC_{50} and E_{max} values, see Table 3.6), as compared to the wt receptor which recognized Gq5 only poorly.

Table 3.6

Amino acid composition and G protein coupling profile of wt and mutant V2 vasopressin receptors expressed in yeast

Numbers refer to amino acid positions in the wt human V2 vasopressin receptor sequence (Birnbaumer *et al.*, 1992). Liquid bioassays were carried out as described under "Materials and Methods". Yeast growth was measured in the presence of increasing concentrations of AVP by determining the absorbance at 630 nm. All receptors were expressed in three different yeast strains (MPy578fc, MPy578s5, and MPy578q5) differing only in the structure of the expressed G protein α subunit. MPy578fc expresses wt yeast Gpa1p, whereas MPy578q5 and MPy578s5 carry mutant versions coding for hybrid Gα subunits (Gq5 and Gs5) in which the last five amino acids of Gpa1p were replaced with the corresponding mammalian $G\alpha_q$ and $G\alpha_s$ sequences, respectively (Fig. 3.14). EC_{50} values were calculated from AVP concentration-response curves using the computer program Kaleidagraph (Synergy Software). Data are given as means ± S.E. of two to seven independent experiments, each carried out in triplicate.

Receptors isolated in the initial screen	V2 receptor mutation	AVP EC_{50}			E_{max} [a]		
		Gq5	Gs5	Gpa1p	Gq5	Gs5	Gpa1p
		nM	*nM*	*nM*	*%*	*%*	*%*
	V2 (wt)	-	5.8 ± 2.3	-	-[b]	100	-
RM1	Met145Trp	1700 ± 400	1.4 ± 0.2	-	62 ± 2	98 ± 3	-
RM2	Met145Leu, Leu146Arg, Ala147Thr, Ser152Arg	490 ± 250	1.8 ± 0.8	-	67 ± 2	105 ± 4	-
RM3	Met145Leu, Tyr148Phe, His155Leu	130 ± 50	1.0 ± 0.5	-	77 ± 1	105 ± 3	-
	Met145Trp[c]	3500 ± 300	1.1 ± 0.5	-	62 ± 2	101 ± 3	-
	Met145Leu	3700 ± 1900	1.8 ± 0.8	-	76 ± 3	99 ± 1	-
	Met145Gly	-	8.0 ± 5.0	-	-	103 ± 3	-
	Met145Ala	-	3.7 ± 2.7	-	-	100 ± 8	-

Table 3.5 continued

Receptors isolated in the initial screen	V2 receptor mutation	AVP EC_{50}			E_{max} [a]		
		Gq5	Gs5	Gpa1p	Gq5	Gs5	Gpa1p
		nM	*nM*	*nM*	*%*	*%*	*%*
RM4[d]	Met145Δ, Leu146Trp, His150Arg, Ser152Arg	32 ± 9	1.4 ± 0.4	-	83 ± 5	95 ± 1	-
	Leu146Trp, His150Arg, Ser152Arg	-	6.0 ± 0.5	-	-	109 ± 3	-
	His150Arg, Ser152Arg	-	6.5 ± 0.6	-	-	102 ± 1	-
	Met145Δ,[e] His150Arg, Ser152Arg	25 ± 3	5.5 ± 4.5	-	83 ± 4	98 ± 8	-
	Met145Δ, His150Arg	120 ± 60	2.4 ± 0.6	-	78 ± 4	102 ± 8	-
	Met145Δ, Ser152Arg	79 ± 15	1.2 ± 0.5	190 ± 75	74 ± 4	98 ± 1	41 ± 2
	Met145Δ	160 ± 74	1.9 ± 0.7	180 ± 46	63 ± 6	111 ± 8	53 ± 3
	Ile141Δ	-	-	-	-	-	-
	Cys142Δ	-	-	-	-	-	-
	Arg143Δ	-	-	-	-	-	-
	Pro144Δ	-	-	-	-	-	-
	Leu146Δ	-	3.4 ± 2.0	-	-	104 ± 3	-
	Ala147Δ	-	4.3 ± 1.0	-	-	110 ± 9	-
	Tyr148Δ	-	3.4 ± 0.8	-	-	103 ± 2	-
	Arg149Δ	-	8.0 ± 3.0	-	-	105 ± 1	-

[a] E_{max} values were expressed as growth responses relative to the response induced by 0.1 mM AVP in the yeast strain coexpressing the wt V2 receptor and Gs5 (E_{max} = 100%).

[b] Only residual activity at the highest AVP concentration used (E_{max}<10%).

[c] Recreated by site-directed mutagenesis.

[d] Note that RM4 is also equivalent to: Met145Trp, Leu146Δ, His150Arg, Ser152Arg.

[e] Note that Met145Δ is equivalent to the Met145Leu/ Leu146Δ double mutation.

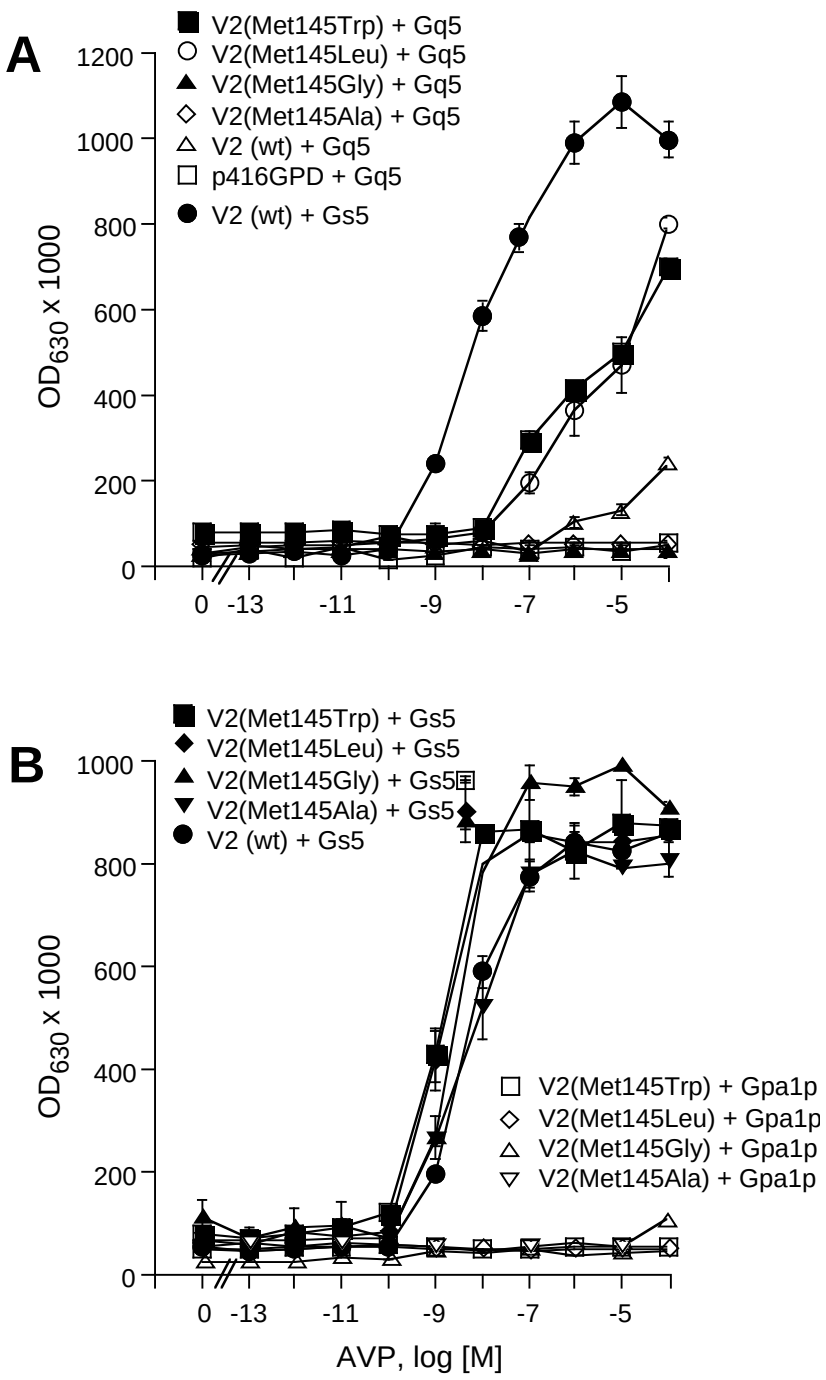


Fig. 3.24 Effect of substitutions at position Met145 on the ability of the V2 vasopressin receptor to couple to hybrid G proteins in yeast. Liquid bioassays were carried out with yeast strains co-expressing the indicated mutant V2 vasopressin receptors and G protein α subunits (for G protein structures, see Fig. 3.14), as described under "Materials and Methods". *A*, Coupling to Gq5. The yeast strain co-expressing the wt V2 receptor and Gs5 was included as a control. *B*, Coupling to Gs5 or Gpa1p. Yeast growth was measured by determining the absorbance at 630 nm (for AVP EC_{50} values, see Table 3.6). Results from one representative experiment, carried out with three independent transformants, are shown. Data are given as means ± S.E. Two additional experiments gave similar results.

[^{3}H]-SR121463 saturating binding studies showed that the V2(Met145Leu) construct yielded B_{max} values that were not significantly different from the corresponding wt receptor values (Table 3.7), indicating that the observed gain-of-function phenotype was not caused simply by overexpression of the two mutant receptors.

Interestingly, replacement of Met145 with either glycine or alanine, two small amino acids lacking large hydrophobic side chains, led to mutant V2 receptors (V2(Met145Gly) and V2(Met145Ala), respectively) that were unable to activate Gq5 (Fig. 3.24A). However, the V2(Met145Gly), and V2(Met145Ala) mutant receptors, like the V2(Met145Trp) and V2(Met145Leu) constructs, retained the ability to productively interact with Gs5, in a fashion similar to the wt V2 receptor (Fig. 3.24B, Table 3.6). This observation excludes the possibility that the inability of the V2(Met145Gly) and V2(Met145Ala) mutant receptors to stimulate Gq5 was due to improper folding of the mutant receptor proteins.

Fig. 3.24B also shows that none of the four mutant receptors (V2(Met145Trp), V2(Met145Leu), V2(Met145Gly), and V2(Met145Ala)) gained the ability to couple to yeast Gpa1p. Taken together, these data indicate that proper recognition of the C-terminus of $G\alpha_q$ is dependent on the chemical nature of the amino acid present at position 145 in the i2 loop of the vasopressin receptor.

3.2.3.7 Functional analysis of RM4-based mutant V2 vasopressin receptors

Sequence analysis showed that Met145 was deleted in one of the four mutant receptors (RM4) that were recovered in the initial colony screen (Table 3.5). However, it should be noted that the Met145Δ/Leu146Trp mutation contained in RM4 (besides the His150Arg and Ser152Arg substitutions) is identical with a Met145Trp/Leu146Δ mutation (Table 3.5). To assess the functional impact of the individual mutations present in RM4, several RM4-based mutant receptors were generated which contained the Met145Δ deletion as the only mutation (note that the Met145Δ deletion is equivalent to a Met145Leu/Leu146Δ double mutation), or which contained the mutations present in RM4 in various different combinations (see Table 3.6, center).

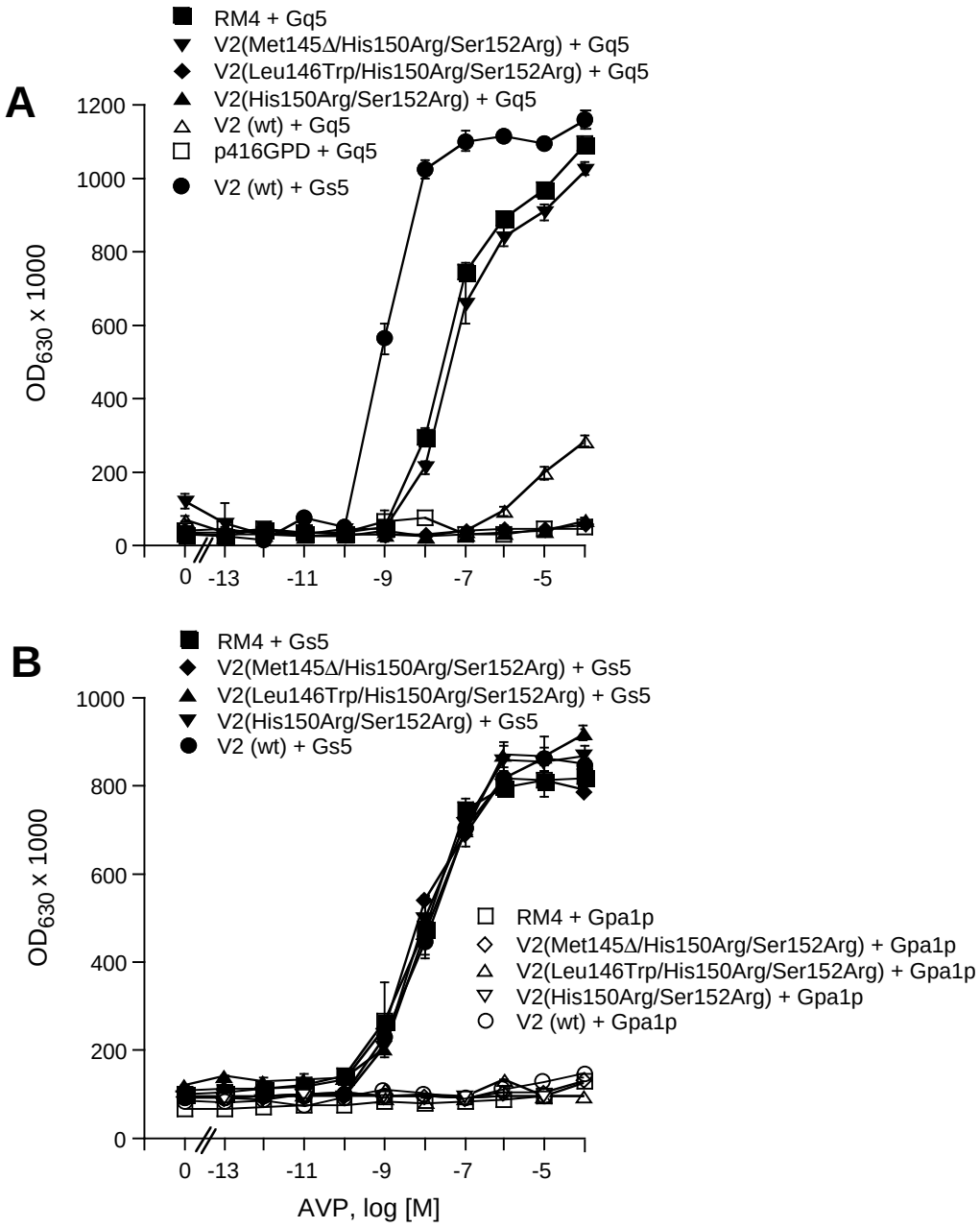


Fig. 3.25 AVP-induced yeast growth mediated by coupling of RM4-derived mutant V2 vasopressin receptors to co-expressed hybrid G proteins. Liquid bioassays were carried out with yeast strains co-expressing the RM4-derived mutant V2 vasopressin receptors and the indicated G protein α subunits (for G protein structures, see Fig. 3.14), as described under "Materials and Methods". *A*, Coupling to Gq5. The yeast strain co-expressing the wt V2 receptor and Gs5 was included as a control. *B*, Coupling to Gs5 or Gpa1p. Yeast growth was measured by determining the absorbance at 630 nm (for AVP EC_{50} values, see Table 3.6). Results from one representative experiment, carried out with three independent transformants, are shown. Data are given as means ± S.E. Two additional experiments gave similar results.

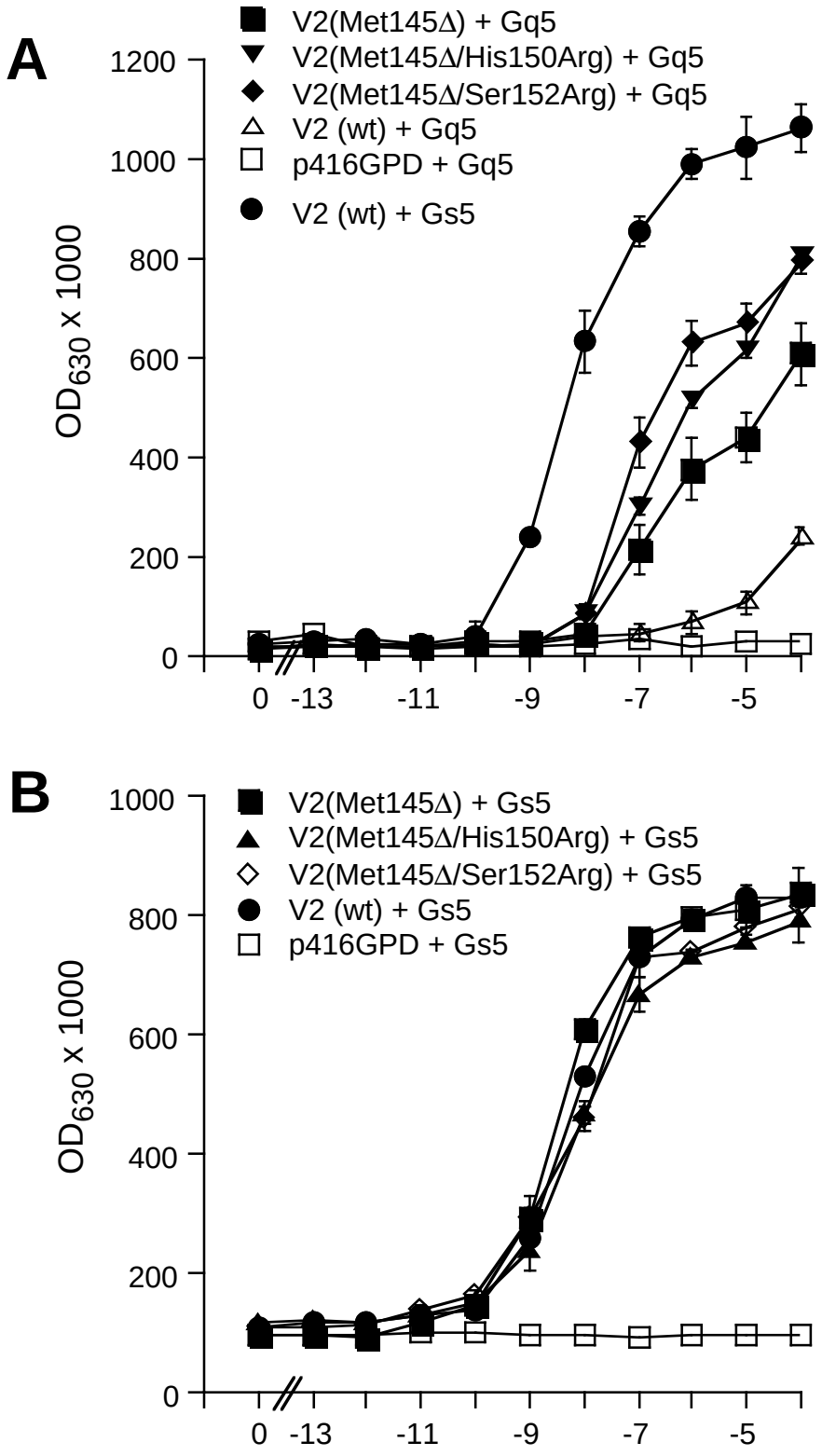
Table 3.7

Ligand binding properties of wt and mutant V2 vasopressin receptors expressed in yeast (MPy578q5)

Yeast membrane homogenates were incubated with eight different concentrations (0.1 - 15 nM) of the V2 vasopressin receptor antagonist, [^{3}H]-SR121463, as described under "Materials and Methods". K_D and B_{max} values were calculated from saturation binding curves using the program LIGAND (Munson and Rodbard, 1980). Because of the limited supply of the radioligand, only several representative mutant V2 receptors could be analyzed in [^{3}H]-SR121463 binding studies. Data are given as means ± S.E. of two or three independent experiments, each carried out in duplicate.

Receptor	[^{3}H]-SR121463 binding	
	K_D	Receptor density B_{max}
	nM	*fmol/mg*
V2 (wt)	8.0 ± 2.5	322 ± 102
V2(Met145Trp)	10.1 ± 0.9	476 ± 164
V2(Met145Leu)	8.5 ± 1.6	363 ± 51
V2(His150Arg, Ser152Arg)	7.2 ± 0.8	318 ± 136
V2(Met145Δ, His150Arg, Ser152Arg)	7.0 ± 1.4	527 ± 145
V2(Met145Δ)	5.4 ± 0.4	592 ± 51
V2(Ile141Δ)	ND[a]	-
V2(Cys142Δ)	ND	-
V2(Arg143Δ)	ND	-
V2(Pro144Δ)	ND	-

[a] ND, no detectable specific [^{3}H]-SR121463 binding activity.


A
V2(Met145Δ) + Gq5
V2(Met145Δ/His150Arg) + Gq5
V2(Met145Δ/Ser152Arg) + Gq5
V2 (wt) + Gq5
p416GPD + Gq5
V2 (wt) + Gs5
OD630 x 1000
1200
1000
800
600
400
200
0
0 -13 -11 -9 -7 -5
B
V2(Met145Δ) + Gs5
V2(Met145Δ/His150Arg) + Gs5
V2(Met145Δ/Ser152Arg) + Gs5
V2 (wt) + Gs5
p416GPD + Gs5
OD630 x 1000
1000
800
600
400
200
0
0 -13 -11 -9 -7 -5

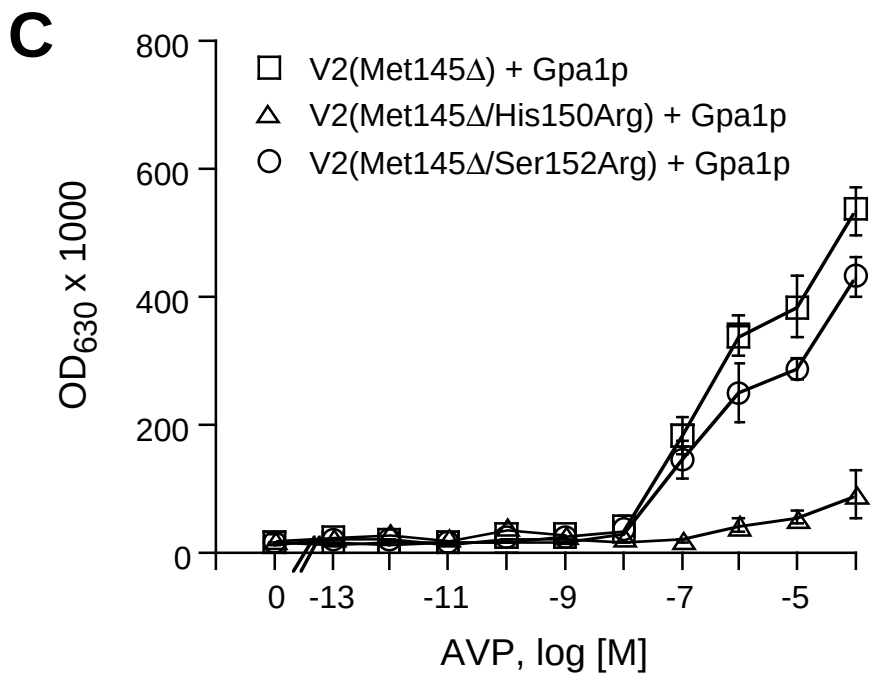


Fig. 3.26 AVP-induced yeast growth mediated by Met145Δ-derived mutant V2 vasopressin receptors to co-expressed wt and hybrid G proteins. Liquid bioassays were carried out with yeast strains co-expressing the indicated Met145Δ-derived mutant V2 vasopressin receptors and G protein α subunits (for G protein structures, see Fig. 3.14), as described under "Materials and Methods". *A*, Coupling to Gq5. The yeast strain co-expressing the wt V2 receptor and Gs5 was included as a control. *B*, Coupling to Gs5. *C*, Coupling to Gpa1p. Yeast growth was measured by determining the absorbance at 630 nm (for AVP EC_{50} values, see Table 3.6). Results from one representative experiment, carried out with three independent transformants, are shown. Data are given as means ± S.E. Two additional experiments gave similar results.

Liquid bioassays showed that all mutant receptors that contained the Met145Δ deletion gained coupling to Gq5, as compared to the wt receptor (Figs. 3.25A and 3.26A; Table 3.6). On the other hand, all RM4-based mutant receptors that lacked the Met145Δ deletion were unable to interact with Gq5 (Figs. 3.25A and 3.26A; Table 3.6). [^{3}H]-SR121463 saturating binding studies showed that the V2(Met145Δ) and V2(Met145Δ/His150Arg/Ser152Arg) mutant receptors (because of the limited supply of the [^{3}H]-SR121463 radioligand, we could only study two representative RM4-based mutant receptors which gained coupling to Gq5) were expressed at levels similar to those found with the wt V2 receptor (Table 3.7), excluding the possibility that the

Met145Δ deletion enhances receptor/Gq5 interactions due to overexpression of the mutant receptor protein.

Growth assays also showed that all RM4-based mutant receptors retained the ability to stimulate Gs5 with high efficiency, in a fashion similar to the wt V2 receptor (Figs. 3.25B and 3.26B; Table 3.6**,** center). Likewise, as observed with the wt receptor, most RM4-based mutant receptors showed little or no interaction with yeast Gpa1p (Figs. 3.25B and 3.26C; Table 3.6). Surprisingly, two RM4-derived mutant receptors, V2(Met145Δ) and V2(Met145Δ/Ser152Arg), displayed substantial coupling to Gpa1p (AVP EC_{50} ~200 nM; E_{max} ~ 40-50%) (Fig. 3.26C; Table 3.6). Thus, these two mutant receptors were able to interact with all three G proteins used in the present study. Interestingly, in the presence of an additional His150Arg point mutation, the V2(Met145Δ) and V2(Met145Δ/Ser152Arg) mutant receptors lost the ability to activate Gpa1p, but retained coupling to Gs5 and Gq5 (Figs. 3.25B and 3.26C; Table 3.6).

3.2.3.8 Amino acids at the beginning of the i2 loop are critical for proper folding of the V2 vasopressin receptor

As outlined in the previous paragraph, deletion of Met145 (which is equivalent to a Met145Leu/Leu146Δ double mutation) led to a mutant V2 receptor that displayed promiscuous G protein coupling properties. To examine whether simple shortening of the i2 loop by a single amino acid might be responsible for this phenomenon, eight additional mutant V2 receptors were generated which contained single amino acid deletions at positions immediately N-terminal (Ile141Δ, Cys142Δ, Arg143Δ, and Pro144Δ) and immediately C-terminal of Met145 (Leu146Δ, Ala147Δ, Tyr148Δ, and Arg149Δ) (Fig. 3.21). Liquid bioassays indicated that the four mutant receptors harboring deletions C-terminal of Met145 showed essentially the same coupling profile as the wt V2 receptor (Fig. 3.27 and Table 3.6). On the other hand, the four mutant receptors lacking single amino acids N-terminal of Met145 were completely devoid of functional activity. These mutant receptors were unable to interact with either Gs5 (Fig. 3.27), Gq5 (Table 3.6), or yeast Gpa1p (Table 3.6).

Western blotting studies showed that the four coupling-defective deletion mutants (Ile141Δ, Cys142Δ, Arg143Δ, and Pro144Δ) were expressed at high levels in yeast which even exceeded the corresponding wt V2 receptor levels (Fig. 3.15, lanes 1-4). However, in contrast to the wt receptor, none of the four coupling-defective mutant receptors was able to bind significant amounts of the [^{3}H]-SR121463 radioligand (Table 3.7). Taken together, these data strongly suggest that the Ile141Δ, Cys142Δ, Arg143Δ, and Pro144Δ deletions disrupt proper folding of the V2 receptor protein.

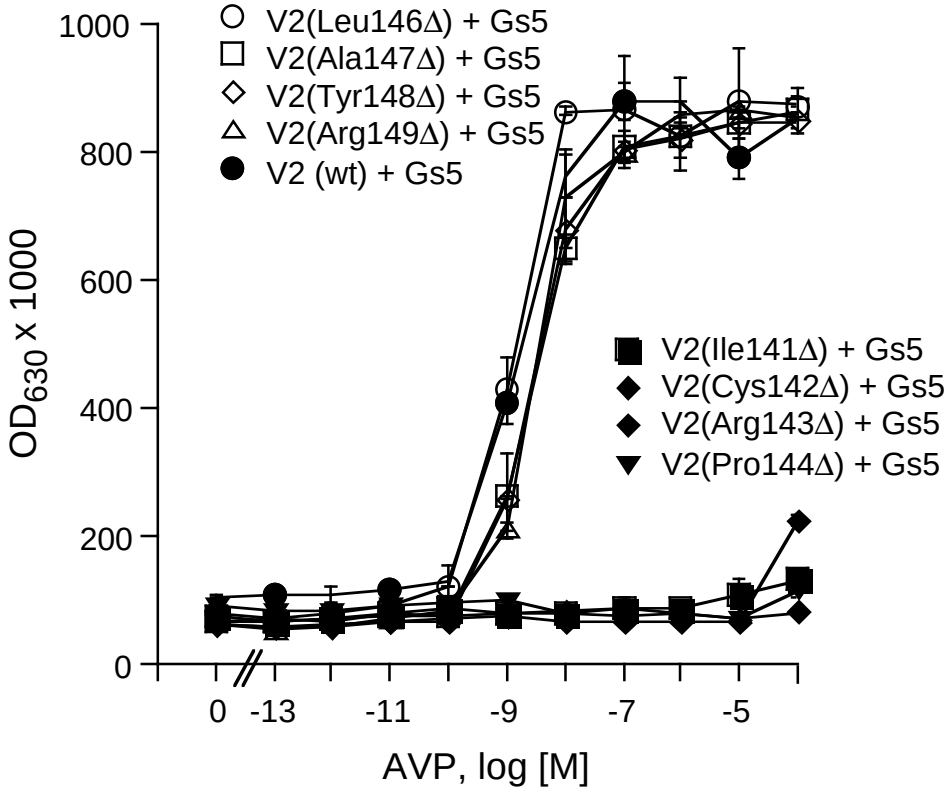


Fig. 3.27 Effect of single amino deletions in the i2 loop on the ability of the V2 vasopressin receptor to couple to the Gs5 hybrid G protein. Liquid bioassays were carried out with yeast strains co-expressing the indicated V2 vasopressin receptor deletion mutants and the hybrid Gα subunit, Gs5 (Fig. 3.14), as described under "Materials and Methods". Yeast growth was measured by determining the absorbance at 630 nm (for AVP EC_{50} values, see Table 3.6). Results from one representative experiment, carried out with three independent transformants, are shown. Data are given as means ± S.E. Two additional experiments gave similar results.

3.2.4 Discussion

To facilitate structure-function relationship studies of the V2 vasopressin receptor, the human V2 receptor was expressed in yeast strains that required productive receptor/G protein coupling for growth (Pausch *et al.*, 1998; Price *et al.*, 1995, 1996). The yeast strains that were employed for these studies only differed in the nature of the G protein α subunit that they expressed (Fig. 3.14). Growth assays showed that the V2 vasopressin receptor, which preferentially activates G_s in mammalian cells (Birnbaumer *et al.*, 1992; Lolait *et al.*, 1992; Burbach *et al.*, 1995; Liu and Wess, 1996; Thibonnier *et al.*, 1998), was unable to couple to the endogenous yeast G protein, Gpa1p, or a mutant version of Gpa1p in which the last five amino acids of Gpa1p were replaced with the corresponding mammalian $G\alpha_q$ sequence (Gq5). On the other hand, the V2 receptor was able to productively interact with a hybrid Gpa1p/α_s subunit (Gs5) in which the last five amino acids were derived from mammalian $G\alpha_s$ (Fig. 3.16). In yeast growth assays, V2 receptor/Gs5 coupling was characterized by remarkably high agonist (AVP) potency (EC_{50} ~6 nM). The AVP EC_{50} value found in yeast was in the same range as AVP EC_{50} values determined previously in mammalian systems measuring V2 receptor-mediated stimulation of adenylyl cyclase (Birnbaumer *et al.*, 1992; Burbach *et al.*, 1995; Liu and Wess, 1996; Innamorati *et al.*, 1996; Yun *et al.*, 2000).

Interestingly, the $G_{q/11}$-coupled M_1, M_3, and M_5 muscarinic receptors, when expressed in the same yeast strains, were able to activate Gq5 with much higher efficiency (agonist potency) than Gs5, thus displaying a G profile coupling profile that is reverse of that observed with the V2 vasopressin receptor. These observations indicate that GPCRs maintain their proper G protein coupling selectivity in yeast, as studied with yeast Gpa1p/mammalian Gα hybrid G proteins. It should be noted that the use of hybrid Gα subunits for the studies described here was prompted by previous work demonstrating that the last five amino acids of mammalian Gα subunits play a key role in proper receptor recognition (Conklin *et al.*, 1993; Liu *et al.*, 1995). Most importantly, studies with hybrid mammalian Gα subunits have shown that the character of the last five

Gα residues largely determines by which class of GPCRs a specific G protein can be activated (reviewed in Wess, 1998).

The specific goal of this study was to identify mutant V2 receptors that would gain the ability to couple to Gq5, a hybrid G protein containing C-terminal α_q sequence (Fig. 3.14). Studies with V1a/V2 hybrid receptors previously showed (Liu and Wess, 1996) that the ability of the V1a vasopressin receptor to preferentially activate G proteins of the G_q family is critically dependent on the structural integrity of the i2 loop. Based on this observation, the i2 loop of the V2 vasopressin receptor was subjected to random mutagenesis in an attempt to generate and recover mutant receptors that would gain coupling to Gq5 in the yeast expression system.

A yeast genetic screen of about 30,000 mutant receptors (this number is more than sufficient to mutate every amino acid in the i2 loop multiple times) led to the identification of four mutant receptors, RM1-RM4 (Table 3.5), which, in contrast to the wt receptor, showed pronounced coupling to Gq5 (Fig. 3.23). Interestingly, all recovered mutant receptors contained a mutation at position 145, either an amino acid substitution (Met145Trp or Met145Leu) or a deletion (as discussed in more detail below, a Met145Δ deletion is equivalent to a Met145Leu/Leu146Δ double mutation). Functional analysis of mutant V2 receptors containing the Met145Trp or Met145Leu amino acid replacements as the only mutations showed that either substitution was able to confer on the V2 receptor the ability to interact with Gq5 (Fig. 3.24A, Table 3.6). On the other hand, this activity was not observed after replacement of Met145 with glycine or alanine. These data suggest that the presence of a relatively large hydrophobic amino acid side chain at position 145 favors recognition of the C-terminus of $G\alpha_q$. This conclusion appears to be inconsistent with our observation that mutant V2 receptors in which Met145 was deleted also gained coupling to Gq5. However, as shown in Fig. 3.21, Met145 is immediately followed by a leucine residue (Leu146) in the wt V2 receptor sequence. As a result, deletion of Met145 in the wt receptor corresponds to a Met145Leu substitution (which promotes interaction with Gq5), combined with a Leu146Δ deletion which does not disrupt V2 receptor function (see below; Fig. 3.27, Table 3.6). Similarly, the RM4 mutant receptor which lacks Met145 and contains a Leu146Trp substitution

(besides two additional point mutations; Table 3.5) is equivalent to a mutant V2 receptor carrying a Met145Trp substitution (which, as discussed above, promotes coupling to Gq5) and a Leu146Δ deletion (which does not interfere with V2 receptor function; Fig. 3.27, Table 3.6).

Whereas the V2 vasopressin receptor is preferentially linked to G_s (Birnbaumer *et al.*, 1992; Lolait *et al.*, 1992; Burbach *et al.*, 1995; Liu and Wess, 1996; Thibonnier *et al.*, 1998), all other members of the vasopressin/oxytocin receptor family (oxytocin and V1a and V1b vasopressin receptors) are selectively coupled to $G_{q/11}$ (Burbach *et al.*, 1995; Liu and Wess, 1996; Thibonnier *et al.*, 1998). Interestingly, consistent with the observation that a Met145Leu substitution allows mutant V2 receptors to interact with Gq5, a leucine residue is present in the oxytocin and V1a and V1b vasopressin receptors at the position corresponding to Met145 in the V2 receptor (Burbach *et al.*, 1995, Liu and Wess, 1996; shown for the V1a receptor in Fig. 3.21).

Taken together, the above observations strongly support the concept that the presence of a leucine or tryptophan residue at position Met145 in the V2 receptor facilitates recognition of the C-terminus of $G\alpha_q$. One possible scenario is that these relatively large hydrophobic residues can directly contact the C-terminus of $G\alpha_q$ which, like the C-termini of other $G\alpha$ subunits, contain a cluster of hydrophobic amino acids predicted to be involved in receptor recognition (Conklin and Bourne, 1993; Rens-Domiano and Hamm, 1995; Wess, 1998). Consistent with this concept, co-expression studies with hybrid M_2/M_3 muscarinic receptors and hybrid α_s/α_q G proteins suggested that residues within the i2 loop of the $G_{q/11}$-coupled M_3 muscarinic receptor may participate in interactions with the C-terminus of $G\alpha_q$ (Kostenis *et al.*, 1997c). However, it is likely that additional amino acids located on receptor domains other than the i2 loop, such as the C-terminal portion of the i3 loop and the adjacent cytoplasmic end of TM VI, can also contact C-terminal $G\alpha$ residues (Liu *et al.*, 1995; Kostenis *et al.*, 1997a; Acharya *et al.*, 1997).

A sequence comparison shows that most GPCRs of the rhodopsin family contain a relatively bulky lipophilic amino acid (primarily leucine, isoleucine, phenylalanine, or valine) at the position corresponding to Met145 in the V2 vasopressin receptor (Moro *et*

al., 1993; Watson and Arkinstall, 1994). Replacement of this amino acid with hydrophilic amino acids or alanine in the M_1 and M_3 muscarinic receptors (coupled to $G_{q/11}$; Moro *et al.*, 1993), the β_2-adrenergic receptor (coupled to G_s; Moro *et al.*, 1993), the GnRH receptor (coupled to $G_{q/11}$; Arora *et al.*, 1995), or the H2 histamine receptor (coupled to G_s; Smit *et al.*, 1996) showed that the resulting mutant receptors were greatly impaired in their ability to activate G proteins. These loss-of-function mutagenesis data suggested that the targeted i2 loop residue is generally important for productive receptor/G protein coupling (Moro *et al.*, 1993; Arora *et al.*, 1995; Smit *et al.*, 1996). The mutagenesis data presented in this study however indicate that substitution of Met145 with small amino acids such as alanine or glycine does not interrupt G protein coupling in general, as demonstrated by the ability of the resulting mutant V2 receptors to productively interact with Gs5 (Fig. 3.24). Importantly, the gain-of-function data suggest that the precise chemical nature of the amino acid present at position 145 (V2 receptor sequence) plays a key role in regulating receptor/G protein coupling selectivity.

The high-resolution X-ray structure of bovine rhodopsin indicates that the i2 loop exhibits an L-like structure when viewed parallel to the membrane plane but lacks regular secondary structure (Palczewski *et al.*, 2000). Like the other intracellular loops, the i2 loop does not fold over the area defined by the intracellular ends of TM I-VII (Palczewski *et al.*, 2000) (see Fig. 1.3B). Since the cytoplasmic extension of TM III and the N-terminal segment of the i2 loop show considerable sequence homology among GPCRs of the rhodopsin family (Moro *et al.*, 1993; Watson and Arkinstall, 1994; Fig. 3.21), it is likely that the i2 loop of the V2 receptor adopts a structure similar to that observed in rhodopsin (Fig. 3.28). If this assumption is correct, Met145 (highlighted in magenta in Fig. 3.28) is predicted to be located just N-terminal of the bend of the L-like structure that is a characteristic feature of the i2 loop (Palczewski *et al.*, 2000), where it is easily accessible for interactions with G proteins.

Interestingly, the V2(Met145Δ) and V2(Met145Δ/Ser152Arg) mutant receptors did not only gain coupling to Gq5 but were also able to interact with yeast Gpa1p (Fig. 3.26C, Table 3.6). However, coupling of these two mutant receptors to Gpa1p could be

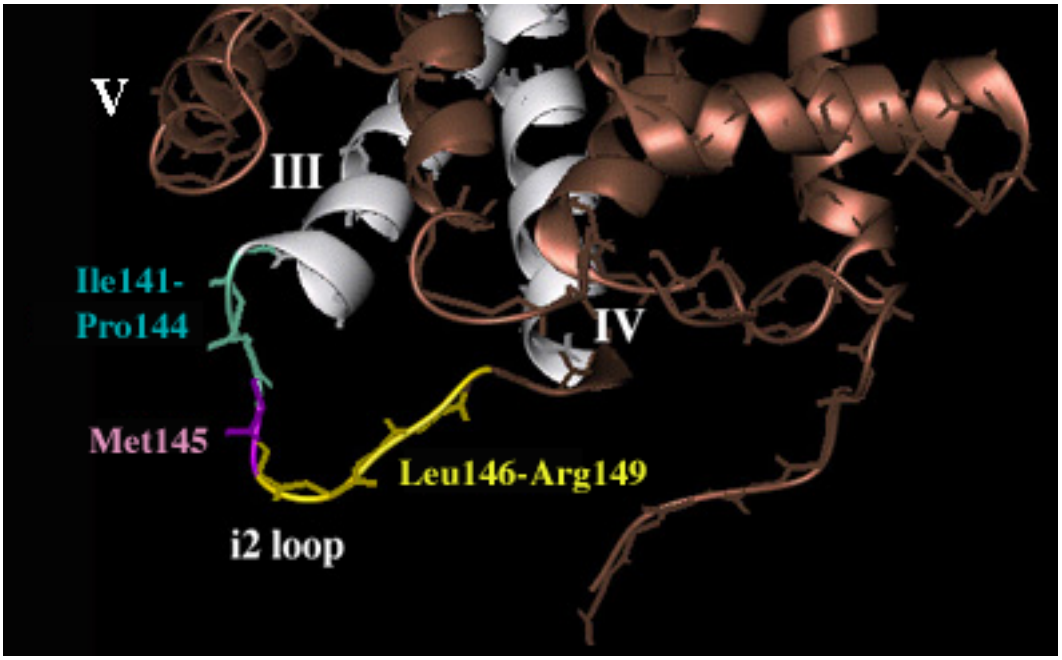


Fig. 3.28 Model of the three-dimensional structure of the i2 loop of the V2 vasopressin receptor. The structure depicted here is based on the high resolution X-ray structure of bovine rhodopsin (Palczewski *et al.*, 2000). The intracellular receptor surface is viewed from the cytoplasm. Met145 (highlighted in magenta) is predicted to be located close to the bend of the L-like structure that is a characteristic feature of the i2 loop in bovine rhodopsin (Palczewski *et al.*, 2000). Deletion of any of the residues highlighted in light blue (Ile141-Pro144) completely abolished V2 receptor function, probably by interfering with proper receptor folding. On the other hand, deletion of any of the residues highlighted in yellow (Leu146-Arg149) had no effect on the G protein coupling properties of the V2 receptor.

selectively disrupted by an additional His150Arg point mutation (Figs. 3.25B and 3.26C, Table 3.6). This finding further highlights the fact that minor changes in the structure of the i2 loop can lead to pronounced changes in the G protein coupling profile of the V2 vasopressin receptor and probably other GPCRs.

Prompted by the observation that the Met145Δ deletion (which is identical with a Met145Leu/Leu146Δ double mutation) had profound effects on the functional properties of the V2 receptor, the G protein coupling properties of additional mutant receptors, which lacked single amino acids immediately N-terminal or C-terminal of Met145, was

studied. It was found that deletion of single amino acids immediately N-terminal of Met145 (Ile141Δ, Cys142Δ, Arg143Δ, or Pro144Δ) resulted in misfolded receptor proteins that were unable to bind ligands and to couple to G proteins (Tables 3.6 and 3.7). Fig. 3.28 shows that amino acids Ile141-Pro144 are predicted to be located at the very beginning of the i2 loop structure, just adjacent to the cytoplasmic extension of TM III which also contains the highly conserved DRY(H) motif (Fig. 3.21). One possibility therefore is that residues within the Ile141-Pro144 segment are in contact with the adjacent cytoplasmic ends of TM III and/or TM V, thus stabilizing the proper arrangement of the transmembrane receptor core. However, the possibility cannot be ruled out that deletions at the beginning of the i2 loop cause major structural rearrangements of the entire i2 loop which may disrupt proper receptor folding by mechanisms that are difficult to predict. On the other hand, deletion of single amino acids located C-terminal of Met145 (Leu146Δ, Ala147Δ, Tyr148Δ, or Arg149Δ) did not interfere with proper V2 receptor folding and function (Fig. 3.27, Table 3.6). These residues are located within the central portion of the i2 loop (Fig. 3.28) and are therefore unlikely to be involved in contacts with the transmembrane receptor core.

It should be noted that all mutant receptors analyzed in this study (except for the Leu146Δ, Ala147Δ, Tyr148Δ, and Arg149Δ deletion mutants which were completely devoid of functional activity) retained highly efficient coupling to Gs5, in a fashion similar to the wt receptor (range of AVP EC_{50} values: ~1-8 nM; Table 3.6). This observation is consistent with previous findings (Liu and Wess, 1996) that replacement of the i2 loop of the V2 vasopressin receptor with the corresponding V1a vasopressin or SS4 somatostatin receptor sequences (the SS4 receptor is preferentially linked to G_i-type proteins) had little effect on the ability of the V2 receptor to productively interact with G_s. As shown in the first part of this thesis, efficient coupling of the V2 vasopressin receptor to G_s is determined primarily by sequence elements within the i3 loop and the membrane-proximal portion of the C-terminal tail. The ability of essentially all analyzed mutant V2 receptors to activate Gs5 with similarly high efficiency (AVP potency) provides a suitable internal control indicating that the lack of coupling to Gq5 displayed by many of

the mutant receptors analyzed in this study was not due to generally impaired G protein coupling.

Two recent studies (Baranski *et al.*, 1999; Geva *et al.*, 2000) have applied a strategy similar to that described here to identify amino acids that are essential for the proper function of the chemoattractant C5a receptor, another member of the rhodopsin family of GPCRs. In these studies, transmembrane domains of the C5a receptor were subjected to random mutagenesis followed by a yeast genetic screen that allowed the isolation of functional mutant receptors. Sequence analysis of the recovered mutant receptors suggested possible molecular mechanisms involved in GPCR activation (Baranski *et al.*, 1999) and the existence of a potential receptor oligomerization domain (Geva *et al.*, 2000).

In conclusion, the results presented in this study indicate that receptor random mutagenesis, complemented with yeast expression technology, represents a powerful new approach to study the molecular basis underlying receptor/G protein coupling selectivity. The approach described here, which is applicable to other receptor regions and other classes of GPCRs, should eventually provide a more comprehensive picture of the structural elements regulating the selectivity of receptor/G protein interactions.

4. Summary

G protein-coupled receptors (GPCRs) represent one of the largest protein families found in nature (Watson and Arkinstall, 1994). GPCRs are cell surface receptors, which interact with cellular effector systems via heterotrimeric G proteins to activate a wide range of signal transduction pathways.

Despite the structural diversity of their activating ligands (Schwartz, 1994), most GPCRs interact only with a limited set of the many structurally similar G proteins expressed within a cell (Hedin *et al.*, 1993; Conklin and Bourne, 1993). Elucidating how this receptor/G protein coupling selectivity is achieved at a molecular level is of fundamental importance for understanding cellular signal transduction.

Structure-function analysis of different classes of biogenic amine GPCRs, including the muscarinic acetylcholine (Wess, 1996) and adrenergic receptors (Dohlman *et al.*, 1991; Savarese and Fraser, 1992; Strader *et al.*, 1994), has led to considerable insight into the structural elements that control the G protein coupling selectivity of this class of receptors. In contrast, the molecular mechanisms governing the coupling selectivity of GPCRs activated by peptide ligands are not well understood at present.

Thus, the goal of this thesis was to elucidate the structural elements regulating the G protein coupling preference of peptide GPCRs. The vasopressin receptor family differs from virtually all other peptide receptor subfamilies in that its individual members clearly differ in their G protein coupling profile. Whereas the V1a and V1b receptors are selectively coupled to G proteins of the $G_{q/11}$ class, thus stimulating phospholipase Cβ, the V2 receptor preferentially interacts with the G protein G_s, resulting in the activation of adenylyl cyclase (Birnbaumer *et al.*, 1992; Morel *et al.*, 1992; Thibonnier *et al.*, 1994). Therefore, the vasopressin receptor subtypes constitute an attractive model system to study peptide receptor domains that are critical for G protein recognition.

Taking advantage of the different coupling preferences of the V1a and V2 vasopressin receptor subtypes, the focus of this thesis was to use the V2 vasopressin receptor as a model system to explore the structural basis underlying peptide receptor/G protein coupling selectivity, employing classical mutagenesis strategies and yeast expression technology.

4.1 Molecular basis of V2 vasopressin receptor/G_s coupling selectivity

A former study (Liu and Wess, 1996) found that efficient coupling to $G_{q/11}$ by the wild type (wt) V1a or hybrid V1a/V2 receptors was critically dependent on the presence of V1a receptor sequence in the second intracellular (i2) loop, whereas efficient recognition of G_s by the wt V2 receptor or V1a/V2 mutant receptors required the presence of V2 receptor sequence in the third intracellular (i3) loop. To explore the structural elements underlying the ability of the V2 receptor to selectively recognize G_s in greater detail, a gain-of-function mutagenesis approach was employed. Distinct V2 receptor segments (or single amino acids) were substituted into the V1a receptor and the resulting hybrid receptors were studied for their ability to gain coupling to hormone-dependent cAMP production. This strategy appeared particularly attractive since hormone stimulation of the V1a receptor has virtually no effect on intracellular cAMP levels.

As mentioned above, the i3 loop of the V2 receptor plays a key role in selective recognition of G_s. However, the maximal response mediated by a mutant V1a receptor, containing V2 receptor sequence in its i3 loop, was smaller than that observed with the wt V2 receptor, suggesting that other V2 receptor domains are also critical for optimal G_s coupling efficiency (Liu and Wess, 1996). Based on this observation, a series of V1a/V2 chimeric receptors was created to examine the contribution of the second and fourth intracellular V2 receptor domains to V2 receptor/G_s coupling selectivity. Functional analysis of the various hybrid receptors transiently expressed in mammalian cells (COS-7) indicated that a short sequence at the N-terminus of the cytoplasmic tail makes an important contribution to V2 receptor/G_s coupling selectivity. A mutant V1a receptor that contained V2 receptor sequence in both its i3 loop and the N-terminal segment of the cytoplasmic tail showed a functional profile very similar to the wt V2 receptor.

Next, structural elements within the i3 loop critical for V2 receptor-mediated activation of G_s were studied in more detail. Functional analysis revealed that the major structural determinants regulating V2 receptor/G_s coupling selectivity are contained within the N-terminus of the i3 loop. A series of mutant V1a receptors were created in

which short segments in the N-terminus of the i3 loop were replaced with corresponding V2 receptor sequence. Functional analysis of these mutant receptors showed that an amino acid pair (Gln225, Val226) and triplet (Phe229, Arg230, Glu231) at the N-terminus of the i3 loop of the V2 receptor make important contributions to G_s coupling efficiency. More detailed mutational analysis of this receptor region identified two polar V2 receptor residues, Gln225 and Glu231, that play key roles in G_s recognition. Studies with other classes of GPCRs (Dohlman *et al.*, 1991; Savarese and Fraser, 1992; Strader *et al.*, 1994; Wess, 1996) have also demonstrated that the N-terminus of the i3 loop contributes to regulating receptor/G protein coupling selectivity. These studies reported the involvement of primarily hydrophobic or noncharged residues in proper G protein recognition. Interestingly, the functionally critical V2 receptor residues, Gln225 and Glu231, are highly polar and charged, indicating that receptor/G protein coupling selectivity is not limited to hydrophobic contacts.

Interestingly, additional mutagenesis studies showed that the efficiency of V2 receptor/G_s coupling can also be modulated by the length of the central portion of the i3 loop (rather than the specific amino acid sequence within this domain). The i3 loops of the V1a and V2 receptors differ in length by 13 amino acids. A mutant V1a receptor that contains V2 receptor sequence at the N-terminus of the i3 loop gained efficient coupling to G_s simply by shortening the central portion of the i3 loop by eleven amino acids. Likewise, when the central portion of the i3 loop of hybrid V1a/V2 receptors, which recognized G_s with high efficiency, was extended in length, the resulting hybrid receptors displayed a pronounced loss in G_s coupling.

Taken together, these results suggest that the central portion of the i3 loop, although predicted not to be directly involved in receptor/G protein interactions, can modulate receptor/G protein coupling selectivity by regulating G protein access (*e.g.* via steric hindrance) to functionally important recognition sites on the receptor protein (such as the TM V/i3 loop junction).

4.2 Single amino acid substitutions and deletions that alter the G protein coupling properties of the V2 vasopressin receptor identified in yeast by receptor random mutagenesis

To facilitate structure-function relationship studies of the V2 vasopressin receptor and overcome the limitations of classical mutagenesis strategies, yeast expression technology was employed. The V2 receptor and various chimeric yeast/mammalian Gα proteins were co-expressed in genetically engineered yeast strains which required ligand-activated receptor/G protein coupling for cell growth (Pausch *et al.*, 1998). In this system, productive receptor/G protein coupling triggers the activation of the yeast MAP kinase/pheromone pathway. This eventually leads to transcription of the *FUS1-HIS3* reportergene and the expression of His3p, allowing growth of the auxotrophic (*his3*) yeast strains on histidine-deficient media. Heterologously expressed V2 receptors were unable to productively interact with the endogenous yeast G protein α subunit, Gpa1p, or a mutant Gpa1p subunit containing C-terminal $G\alpha_q$ sequence (Gq5). In contrast, the V2 receptor efficiently coupled to a Gpa1p/$G\alpha_s$ hybrid subunit containing C-terminal $G\alpha_s$ sequence (Gs5), indicating that the V2 receptor retained proper G protein coupling selectivity in yeast. Studies with the three $G_{q/11}$-coupled muscarinic receptors (M_1, M_3, and M_5) and a number of mutant vasopressin and M_3 muscarinic receptors with altered functional properties showed that wt and mutant GPCRs displayed similar phenotypes in yeast and mammalian cells.

To understand why the V2 receptor does not couple to G proteins of the $G_{q/11}$ class efficiently, random mutagenesis techniques were applied to recover mutant V2 receptors with altered G protein coupling properties. In this study, the i2 loop was target for mutagenesis since this domain had been shown to be critical for activation of $G_{q/11}$ by the V1a receptor (Liu and Wess, 1996). Receptor saturation random mutagenesis generated a yeast library expressing mutant V2 receptors containing mutations within the i2 loop (mutagenesis rate: about 10% at the nucleotide level). A subsequent yeast genetic screen of about 30,000 mutant receptors yielded four mutant receptors which, in contrast to the wt receptor, showed substantial coupling to Gq5 (but did not couple to yeast Gpa1).

Functional analysis of these mutant receptors, followed by more detailed site-directed mutagenesis studies, indicated that single amino acid substitutions at position Met145 in the central portion of the i2 loop of the V2 receptor had pronounced effects on receptor/G protein coupling selectivity. The ability of the V2 receptor to productively interact with the Gq5 protein was largely dependent on the presence of a relatively spacious hydrophobic amino acid such as leucine or tryptophane at this position. Substitution of Met145 with small amino acids such as glycine or alanine prevented V2 receptor/Gq5 interactions. Interestingly, all mutant V2 receptors carrying Met145 single point mutations stimulated the Gs5 protein to the same extent as the wt V2 receptor, indicating that the lack of Gq5 interactions observed with the Met145Gly and Met145Ala mutant V2 receptors was not due to a folding deficit of the mutant receptor proteins. Likewise, the ability of the V2(Met145Leu) and V2(Met145Trp) receptors to gain significant coupling to Gq5 was not due to overexpression of receptor proteins, as indicated by radioligand binding studies. Taken together, these results suggest that the precise chemical nature of the amino acid at position 145 plays a key role in regulating V2 receptor/G protein coupling selectivity. Interestingly, all other members of the vasopressin/oxytocin receptor family (oxytocin, V1a and V1b receptors), which are selectively coupled to G proteins of the $G_{q/11}$ class, contain a leucine residue at the position that corresponds to Met145 in the V2 receptor.

One of the mutant V2 receptors, which were recovered from the initial screen, contained a Met145Δ deletion, among several other mutations. Interestingly, more detailed site-directed mutagenesis studies yielded two mutant vasopressin receptors, V2(Met145Δ) and V2(Met145Δ/Ser152Arg) that were able to activate all three G proteins used in this study. To examine whether shortening of the i2 loop by a single amino acid generally results in receptor/G protein coupling promiscuity, additional mutant receptors were created which lacked single amino acids immediately N-terminal or C-terminal of Met145. Functional analysis revealed that deletion of single amino acids N-terminal of Met145 (Ile141Δ, Cys142Δ, Arg143Δ, or Pro144Δ) resulted in mutant V2 receptors that lacked the ability to couple to G proteins. In addition, radioligand binding studies showed that these coupling-defective mutant receptors were unable to bind V2

receptor ligands, indicating that deletion of single amino acids immediately N-terminal of Met145 resulted in misfolded receptor proteins. Amino acids Ile141-Pro144 are predicted to be located at the very beginning of the i2 loop structure, just adjacent to the cytoplasmic extension of TM III which also contains the highly conserved DRY(H)-motif. It is conceivable that residues within this Ile141-Pro144 segment are in contact with cytoplasmic ends of TM III and/or TM V, thus stabilizing the proper arrangement of the transmembrane receptor core. Therefore, deletions of single residues within this stretch of amino acids may destabilize the receptor structure and disrupt proper receptor folding. In contrast, deletions of single amino acids located immediately C-terminal of Met145 (Leu146Δ, Ala147Δ, Tyr148Δ, or Arg149Δ) had no effect on V2 receptor function. These residues are located within the central portion of the i2 loop and are therefore unlikely to be involved in contacts with the transmembrane receptor core.

4.3 Conclusion

In conclusion, the structural elements governing V2 vasopressin peptide receptor/G_s coupling selectivity were studied in molecular detail. V2 receptor coupling selectivity was found to depend on several different structural features, some of which had not been observed previously in studies with other classes of GPCRs. Also, the usefulness of combining receptor random mutagenesis and yeast expression technology to study mechanisms governing receptor/G protein coupling selectivity and receptor folding could be demonstrated. The functional approaches described in this thesis, combined with the application of biophysical techniques such as NMR and X-ray crystallography, should eventually lead to a more comprehensive picture of the structural elements regulating the selectivity of receptor/G protein interactions.

The findings obtained with the V2 vasopressin receptor highlight the diversity of mechanisms by which receptor/G protein coupling selectivity can be achieved. Given the high structural homology found among all GPCRs, these results should be of general importance for the entire GPCR superfamily. Detailed information about the structural mechanisms involved in receptor/G protein coupling should eventually lead to novel

therapeutic strategies aimed at inhibiting or enhancing specific GPCR signaling pathways.

5. Zusammenfassung

G-Protein-gekoppelte Rezeptoren (GPCRs) stellen eine der größten in der Natur vorkommenden Proteinfamilien dar (Watson and Arkinstall, 1994). GPCRs sind plasmamembranständige Proteine, die mit heterotrimären G-Proteinen interagieren und eine Vielzahl an Signaltransduktionswegen aktivieren.

Trotz der strukturellen Vielfalt der an GPCRs angreifenden Liganden stimulieren die meisten GPCRs nur eine begrenzte Anzahl strukturell sehr ähnlicher G-Proteine (Hedin *et al.*, 1993; Conklin and Bourne, 1993). Die Aufklärung der molekularen Mechanismen, die dieser Rezeptor/G-Protein-Kopplungsselektivität zugrunde liegen, ist von fundamentaler Wichtigkeit für das Verständnis zellulärer Signaltransduktion.

Ausführliche Struktur-Funktionsanalysen verschiedener Neurotransmitter-rezeptoren, einschließlich der Muskarinrezeptoren (Wess, 1996) und adrenergen Rezeptoren (Dohlman *et al.*, 1991; Savarese and Fraser, 1992; Strader *et al.*, 1994), haben einen beträchtlichen Beitrag zur Identifizierung der strukturellen Elemente, die für die G-Protein-Kopplungsselektivität dieser Rezeptorgruppe verantwortlich sind, geleistet. Im Gegensatz dazu ist bisher noch weitgehend ungeklärt, welche molekularen Mechanismen der Kopplungsselektivität von GPCRs, die durch Peptidliganden aktiviert werden, zugrunde liegen.

Das Ziel dieser Arbeit war daher, molekulare Grundlagen der G-Protein-Kopplungsselektivität von Peptid-GPCRs näher zu untersuchen und aufzuklären. Die Vasopressinrezeptorfamilie unterscheidet sich von nahezu allen anderen Peptid-GPCRs darin, daß die einzelnen Rezeptorsubtypen deutlich unterschiedliche G-Protein-Kopplungspräferenzen aufweisen. Die V1a- und V1b-Vasopressinrezeptoren stimulieren selektiv G-Proteine der $G_{q/11}$-Familie, was zur Aktivierung von Phospholipase-Cβ Isomeren führt. Im Gegensatz dazu koppelt der V2-Vasopressinrezeptor vornehmlich an das G-Protein G_s, was in einem Anstieg an intrazellulärem cAMP resultiert. Daher stellen die Vasopressinrezeptorsubtypen ein attraktives Modellsystem zum Studium der Peptid-GPCR-Rezeptordomänen, die für die selektive G-Protein-Aktivierung verantwortlich sind, dar. Als Modellsystem für diese Arbeit diente primär der V2-Vasopressinrezeptor.

5.1 Molekulare Faktoren, die die G_s-Kopplungsselektivität des V2-Vasopressinrezeptors bestimmen.

Eine frühere Studie zeigte, daß die Gegenwart der V1a-Rezeptorsequenz in der zweiten intrazellulären (i2) Schleife notwendig war, um den Wildtyp V1a und V1a/V2-Rezeptorchimären effizient an $G_{q/11}$-Proteine zu koppeln (Liu and Wess, 1996). Effiziente Interaktionen zwischen Wildtyp V2 oder V1a/V2-Rezeptorchimären und dem G-Protein G_s waren hingegen hauptsächlich von V2-Rezeptorsequenzen in der dritten intrazellulären (i3) Schleife abhängig. Um die molekularen Grundlagen der G_s-Kopplungsselektivität des V2-Rezeptors näher zu untersuchen, wurden zunächst klassische Mutagenesetechniken ("zielgerichtete Mutagenese") angewandt. Definierte V2-Rezeptorsegmente (oder einzelne Aminosäuren) wurden in den V1a-Rezeptor transferiert, und die resultierenden Hybrid-Vasopressinrezeptoren wurden anschließend in funktionellen Studien auf ihre Fähigkeit, hormonabhängig intrazelluläre cAMP-Konzentrationen zu steigern (G_s-vermittelt), getestet. Diese Strategie schien besonders geeignet, da die Aktivierung des V1a-Wildtyprezeptors nahezu keine Auswirkungen auf intrazelluläre cAMP-Spiegel hat.

Wie bereits erwähnt, ist die effiziente Kopplung des V2-Rezeptors an das G_s-Protein vornehmlich von V2-Rezeptorsequenzen in der i3-Schleife abhängig (Liu and Wess, 1996). Eine V1a-Rezeptormutante, deren i3-Schleife durch die homologe V2-Rezeptorsequenz ersetzt worden war, war in der Lage, effizient mit G_s zu interagieren. Die Fähigkeit dieser Rezeptormutante, G_s zu aktivieren, war jedoch im Vergleich zum V2-Wildtyprezeptor vermindert. Diese Beobachtung ließ die Vermutung zu, daß noch andere intrazelluläre V2-Rezeptordomänen zur optimalen G_s-Kopplung notwendig sind. Daher wurde zunächst eine Reihe von V1a/V2-Rezeptorchimären erzeugt, die den Beitrag der zweiten (i2) und vierten intrazellulären (i4) Rezeptordomäne zur V2-Rezeptor/G_s-Kopplungsselektivität klären sollten. Funktionelle Untersuchungen der resultierenden Hybrid-Rezeptormutanten in Säugetierzellen (COS-7) zeigten, daß ein kurzes Segment im N-terminalen Abschnitt der i4-Domäne einen deutlichen Beitrag zur V2-Rezeptor/G_s-Kopplungsselektivität leistet. Eine V1a-Rezeptormutante, welche in der

i3-Schleife und dem N-terminalen Segment der i4-Domäne (Ni4) homologe V2-Rezeptorsequenzen enthielt, zeigte ein funktionelles Profil (EC_{50} und E_{max}), welches mit dem V2-Wildtyprezeptor nahezu deckungsgleich war.

Anschließend wurden strukturelle Elemente innerhalb der i3-Schleife näher untersucht. Funktionelle Analysen zeigten, daß der N-terminale Abschnitt der i3-Schleife weitgehend das G-Protein-Kopplungsprofil des V2-Rezeptors bestimmt. Eine Reihe von V1a-Rezeptormutanten wurde erzeugt, in denen kurze Segmente des N-terminalen Bereichs der i3-Schleife mit der entsprechenden V2-Rezeptorsequenz ausgetauscht wurden. Funktionelle Untersuchungen ergaben, daß ein Aminosäurepaar (Gln225, Val226) und -triplet (Phe229, Arg 230, Glu231) am Beginn der i3-Schleife des V2-Rezeptors für die effiziente Aktivierung von G_s von entscheidender Bedeutung sind. Durch Punktmutationen in diesem Bereich wurden zwei polare Aminosäuren, Gln225 und Glu231, identifiziert, die für die effiziente V2-Rezeptor/G_s-Interaktion essentiell sind. Untersuchungen mit anderen GPCR-Klassen (Dohlman *et al.*, 1991; Savarese and Fraser, 1992; Strader *et al.*, 1994; Wess, 1996) haben ebenfalls gezeigt, daß dem N-Terminus der i3-Schleife eine besondere Rolle im Rezeptor/G-Protein-Kopplungsprozeß zukommt. In diesen Studien wird berichtet, daß vornehmlich hydrophobe und ungeladene Aminosäuren Schlüsselrollen in der rezeptorvermittelten G-Protein-Aktivierung einnehmen. Die hier beschriebenen Untersuchungen hingegen ergaben, daß zwei polare/geladene Aminosäuren, Gln225 und Glu231, für die V2-Rezeptor/G_s-Kopplung von besonderer Wichtigkeit sind und zeigen daher, daß die Rezeptor/G-Protein-Kopplungsselektivität nicht auf ausschließlich hydrophoben Wechselwirkungen beruht.

Desweiteren konnte beobachtet werden, daß die Länge der i3-Schleife die Effizienz, mit der der V2-Rezeptor G-Proteine der G_s-Klasse zu aktivieren vermag, beeinflußen kann. Die V1a- und V2-Rezeptoren weisen unterschiedlich lange i3-Schleifen auf (die i3-Schleife des V2-Rezeptors ist 13 Aminosäuren kürzer als die des V1a-Rezeptors). Eine V1a-Rezeptormutante, deren N-terminaler Abschnitt der i3-Schleife durch homologe V2-Rezeptorsequenz ersetzt wurde, konnte deutlich effizienter mit G_s interagieren, wenn der mittlere Abschnitt der i3-Schleife um elf Aminosäuren verkürzt wurde. Gleichermaßen konnte die effiziente Kopplung bestimmter V1a/V2-

Hybridrezeptoren an G_s durch Einfügen von elf Aminosäuren in den zentralen Bereich der i3-Schleife deutlich gehemmt werden.

Diese Ergebnisse legen nahe, daß der zentrale Bereich der i3-Schleife die Rezeptor/G-Protein-Kopplungsselektivität beeinflussen kann, obgleich diese Rezeptordomäne vermutlich nicht direkt mit dem G-Protein interagiert. Es ist denkbar, daß die Länge der i3-Schleife den Zugang des G-Proteins zu funktionell wichtigen Rezeptordomänen, z.B. Aminosäuren im Bereich der fünften Transmembrandomäne (TM V) und der i3-Schleife, reguliert.

5.2 Identifizierung einzelner Aminosäuresubstitutionen und Aminosäuredeletionen, die die G-Protein-Kopplungsselektivität des V2-Rezeptors beeinflussen: Einsatz von Hefeexpressionstechnologie und zufallsgerichteter Mutagenese ("random mutagenesis")

Im zweiten Teil dieser Arbeit wurden Hefe-(*Saccharomyces cerevisiae*)-Expressionstechnologien angewandt, um Struktur-Funktionsanalysen des V2-Rezeptors zu erleichtern und Beschränkungen klassischer Mutagenesetechniken zu überwinden. Der V2-Wildtyprezeptor und verschiedene G-Proteinchimären aus Hefe- und Säugetier-Gα-Untereinheiten wurden in genetisch modifizierten Hefelinien, deren Zellwachstum von effizienter Rezeptor/G-Protein-Kopplung abhängig war, co-exprimiert. In diesem System aktiviert produktive Rezeptor/G-Protein-Kopplung den Hefe-MAP-Kinase/Pheromon-Signaltransduktionsweg. Dies führt zur Transkription des *FUS1-HIS3*-Reportergens und somit zur Expression von His3-Protein, was den Histidin-auxotrophen (*his3*) Hefelinien ermöglicht, in histidinfreiem Medium zu wachsen (Pausch *et al.*, 1998). Es konnte gezeigt werden, daß heterolog exprimierte V2-Rezeptoren weder mit der Hefe-G-Protein-α-Untereinheit (Gpa1p) noch mit einem mutierten Gpa1-Protein, in dem die C-terminalen fünf Aminosäuren gegen homologe $G\alpha_q$-Sequenz ausgetauscht worden waren (Gq5), effizient interagierten. Im Gegensatz dazu erwies sich die Interaktion zwischen dem V2-Rezeptor und einem mutierten Gpa1-Protein, dessen C-terminale fünf Aminosäuren die homologe $G\alpha_s$-Sequenz enthielten (Gs5), als hocheffizient. Diese Beobachtungen

zeigten, daß der V2-Rezeptor im Hefesystem sein physiologisches Kopplungsprofil beibehielt. Zur weiteren Validierung des Hefeexpressionssystems wurden die $G_{q/11}$-gekoppelten M_1-, M_3- und M_5-Muskarinrezeptoren und verschiedene mutierte Vasopressin- und M_3-Muskarinrezeptoren mit veränderten funktionellen Eigenschaften heterolog in Hefe exprimiert. Funktionelle Analysen zeigten, daß die Wildtyprezeptoren und die verschiedenen Rezeptormutanten in Hefe und Säugetierzellen ähnliche Phänotypen aufwiesen.

Um zu untersuchen, weshalb der V2-Rezeptor nicht effizient an G-Proteine der $G_{q/11}$-Familie koppelt, sollte der in Hefe exprimierte V2-Rezeptor zufallsgerichteter Mutagenese ("random mutagenesis") unterzogen und Mutanten mit veränderten G-Protein-Kopplungeigenschaften isoliert werden. Im speziellen wurde die i2-Schleife untersucht, da eine frühere Studie gezeigt hatte, daß vornehmlich die i2-Schleife des V1a-Rezeptors für die V1a-Rezeptor/$G_{q/11}$-Kopplungsselektivität verantwortlich ist (Liu and Wess, 1996). Mittels zufallsgerichteter Mutagenesetechnik wurde in Hefe eine Bibliothek von V2-Rezeptormutanten erzeugt, deren i2-Schleife Mutationen mit einer Mutageneserate von ungefähr 10% (auf der Nukleotidebene) enthielt. Anschließend wurden in einem Selektionsverfahren ("screen") 30 000 V2-Rezeptormutanten auf ihre Fähigkeit, mit Gq5 zu interagieren, überprüft. Es konnten vier V2-Rezeptormutanten isoliert werden, welche effizient an Gq5 (jedoch nicht an Hefe-Gpa1p) koppelten. Funktionelle Untersuchungen mit diesen und anderen mittels zielgerichteter Mutagenese erzeugter V2-Rezeptormutanten zeigten, daß die Substitution einer einzigen Aminosäure (Met145) im zentralen Bereich der i2-Schleife beträchtliche Auswirkungen auf die Rezeptor/G-Protein-Kopplungsselektivität hatte. Die Fähigkeit des V2-Rezeptors, produktiv mit Gq5 zu interagieren, war von der Anwesenheit relativ großer, hydrophober Aminosäuren wie Leucin und Tryptophan abhängig. Austausch von Met145 mit kleinen Aminosäuren wie Glycin oder Alanin erlaubte dem V2-Rezeptor nicht, Gq5 zu aktivieren. Interessanterweise interagierten alle V2-Rezeptormutanten, die eine Met145-Punktmutation aufwiesen, mit Gs5 ähnlich effizient wie der V2-Wildtyprezeptor. Die Unfähigkeit der V2(Met145Gly)- und V2(Met145Ala)-Rezeptoren, Gq5 zu aktivieren, beruht daher nicht auf einem Faltungsdefizit. Gleichermaßen basierte die Fähigkeit der

V2(Met145Trp)- und V2(Met145Leu)-Rezeptoren, produktiv an Gq5 zu koppeln, nicht auf der Überexpression von Rezeptorprotein. Diese Ergebnisse zeigen, daß die chemische Eigenschaft der Aminosäure an Position 145 die V2-Rezeptor/G-Protein-Kopplungsselektivität reguliert. Interessanterweise befindet sich in allen anderen Subtypen der Vasopressin/Oxytocin-Rezeptorfamilie (V1a-, V1b-, und Oxytocin-Rezeptoren), welche selektiv an G-Proteine der $G_{q/11}$-Klasse gekoppelt sind, ein Leucin an der Stelle, die zu Met145 (V2-Rezeptorsequenz) homolog ist.

Eine der vier ursprünglich isolierten V2-Rezeptormutanten enthielt neben verschiedenen Punktmutationen eine Deletion in Position Met145. In detaillierteren zielgerichteten Mutagenese-Studien wurden zwei V2-Rezeptormutanten erzeugt, die alle drei G-Proteine (Gq5, Gs5 und Gpa1p) aktivieren konnten. Um zu untersuchen, ob ein generelles Verkürzen der i2-Schleife um eine Aminosäure der Grund für die beobachtete Rezeptor/G-Protein-Promiskuität ist, wurden verschiedene V2-Rezeptormutanten erzeugt, in denen einzelne Aminosäuren unmittelbar N- und C-terminal von Met145 deletiert worden waren. Funktionelle Untersuchungen ergaben, daß die Deletion einzelner Aminosäuren N-terminal von Met145 (Ile141Δ, Cys142Δ, Arg143Δ oder Pro144Δ) in V2-Rezeptormutanten resultierte, die nicht mit G-Proteinen interagieren konnten. Radioligand-Bindungsstudien zeigten, daß diese V2-Rezeptormutanten keine V2-Liganden binden konnten, was darauf schließen läßt, daß Deletionen einzelner Aminosäuren N-terminal von Met145 zu mißgefalteten Rezeptoren führen. Die Aminosäuren Ile141-Pro144 befinden sich am Beginn der i2-Schleife, unmittelbar neben der α-helikalen zytoplasmatischen Verlängerung der dritten Transmembrandomäne (TM III) in der Nähe des hochkonservierten DRY(H)-Motivs. Es ist denkbar, daß Aminosäuren innerhalb des Ile141-Pro144-Segments mit den zytoplasmatischen Abschnitten von TM III und/oder TM V interagieren und diese Wechselwirkungen die Rezeptorstruktur stabilisieren. Im Gegensatz dazu hatten Deletionen unmittelbar C-terminal von Met145 (Leu146Δ, Ala147Δ, Tyr148Δ oder Arg149Δ) keinerlei Auswirkungen auf die Funktion des V2-Rezeptors. Diese Aminosäuren befinden sich im zentralen Bereich der i2-Schleife, der nicht mit den transmembranären Domänen des Rezeptorproteins interagieren kann.

5.3 Schlußfolgerung

Die strukturellen Faktoren, die der V2-Vasopressinrezeptor/G_s-Kopplungsselektivität zugrunde liegen, wurden auf molekularer Ebene untersucht. Die V2-Rezeptor-Kopplungsselektivität scheint auf mehreren unterschiedlichen strukturellen Elementen zu beruhen. Einige dieser strukturellen Faktoren sind bisher noch nicht in Studien mit anderen GPCR-Klassen beschrieben worden. Desweiteren konnte in dieser Arbeit die Nützlichkeit eines Hefeexpressionssystems und die Anwendbarkeit von zufallsgerichteter Mutagenese-Technik zum Studium von Rezeptor/G-Protein-Kopplungsselektivitäten gezeigt werden

Die Beobachtungen, die in dieser Arbeit anhand des V2-Rezeptors gemacht werden konnten, unterstreichen die Vielfalt der Mechanismen, die für die Rezeptor/G-Protein-Kopplungsselektivität wichtig sind. Da alle GPCRs eine ähnliche molekulare Struktur aufweisen, sollten die am Beispiel des V2-Vasopressinrezeptors erzielten Resultate auch auf andere Mitglieder dieser Rezeptor-"Superfamilie" übertragbar sein. Die Aufklärung der strukturellen Mechanismen, die die Rezeptor/G-Protein-Kopplungsselektivität bestimmen, sollte zu neuen Therapieansätzen führen, welche gezielt spezifische GPCR-Signaltransduktionswege hemmen oder aktivieren können.

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7. List of Abbreviations

[^{3}H]AVP	[^{3}H]-8-arginine-vasopressin
[^{3}H]NMS	[^{3}H]-N-methylscopolamine
[^{35}S]GTPγS	[^{35}S]-guanosine-5'-O-(3-thiotriphosphate)
ACTH	adrenocortiotropic hormone
AQP(2-4)	aquaporin-(2-4)
AT	amino-1,2,4-triazole
ATP	adenosine 5'triphosphate
AVP	8-D-arginine-vasopressin
B_{max}	maximum number of binding sites
bp	basepair
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'-monophosphate
CCK	cholecystokinin
CDI	central/hypothalamic diabetes insipidus
CG	choriogonadotropin
cpm	counts per minute
cGMP	cyclic guanosine 5' monophophate
Ci(1-3)	C-terminal domain of the (first – third) intracellular loop
Ci4	C-terminal domain of the cytoplamic tail
COS-7	African green monkey kidney cell
CR(1-33)	chimeric receptor (1-33)
CYC1	gene encoding cytochrome-c oxidase
DAG	diacylgycerol
dDAVP	deamino-8-D-Arginine-vasopressin, desmopressin
DEAE-dextran	diethylaminoethyl dextran
DI	diabetes insipidus
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
e(1-3) loop	(first – third) extracellular loop
EC_{50}	concentration of drug at which half-maximal effect occurs
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
E_{max}	maximally possible effect
ET	endothelin
FCS	fetal calf serum
FSH	follicle-stimulating hormone
G protein	guanine nucleotide binding protein
GABA	γ-aminobutyric acid
GDP	guanosine 5'-diphophate

GPCR	G protein-coupled receptor
GPD	gene encoding glyceraldehyde-3-phosphate dehydrogenase
GRK	G protein-coupled receptor kinase
GTP	guanosine 5'-triphosphate
GTPase	guanosine 5'-triphosphatase
HA	hemagglutinin
HBSS	Hank's balanced salt solution
i(1-3) loop	(first – third) intracellular loop
IBMX	3-isobutyl-1-methylxanthin
IP_3	phosphatidylinositol-1,4,5-trisphophate
K_D	equilibrium dissociation constant; ligand concentration at which 50% of binding sites are occupied
LH	luteinizing hormone
LVP	8-lysine-vasopressin
MAD	multiwavelength anomalous diffraction
MAP kinase	mitogen-activated protein kinase
MGluR	metabotropic glutamate receptor
mRNA	messenger ribonucleic acid
MSH	melanocyte-stimulating hormone
NDI	nephrogenic diabetes insipidus
Ni(1-3)	N-terminal portion of the (first-third) intracellular loop
Ni4	N-terminal portion of the cytoplasmic tail
NMR	nuclear magnetic resonance
OT	oxytocin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethyleneglycol
PG	prostaglandin
PI	phosphoinositide
PIP_2	phophatidyl-4,5-bisphosphate
PKA, C	protein kinase A, C
PLC	phospholipase C
PMSF	phenylmethylsulfonylfluoride
PTX	pertussis toxin
RGS proteins	regulator of G protein signaling proteins
RM(1-4)	mutant receptor isolated in yeast genetic Screen
RNA	ribonucleic acid
SC	synthetic complete
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
SV40	Simian virus 40

TCA	trichloroacetic acid
TM	transmembrane
TSH	thyroid-stimulating hormone
UTP	uridine 5'-triphosphate
XNDI	X-linked nephrogenic diabetes insipidus
wt	wild type
YNB	yeast nitrogen base

8. Bibliography and Curriculum Vitae

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