GEORG SPEYER HAUS

Role in routing to the plasma membrane of the L_0 domain of the multidrug resistance protein MRP1

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften

vorgelegt beim Fachbereich 14
der Johann Wolfgang Goethe-Universität
in Frankfurt am Main

von
Giulia Calenda
aus Salzburg

Frankfurt am Main im März 2003
(DF1)

vom Fachbereich 14 (chemisch und pharmazeutische Wissenschaften) der Johann Wolfgang Goethe-Universität als Dissertation angenommen.

Dekan:…….

Gutachter:……

Datum der Disputation:……...

Zusammenfassung

Die Mehrfache Chemotherapieresistenz (Multidrug Resistance) wird als die Fähigkeit definiert , die von mehreren Zelltypen erworben werden kann, um sich gegen die tödliche Wirkung von zytotoxischen Substanzen zu wehren. Dies beruht auf vermehrtem Transport von Xenobiotika aus der Zelle, was zu einer dramatischen Verringerung der intrazellulären Konzentration von chemotherapeutischen Substanzen führt. Dieser Effekt wird durch die Expression von transmembranen Transporter-Proteinen, die vor allem der ABC-Familie angehören, verursacht. Zu dieser Familie gehören z.B. das P-Glykoprotein (P-gp, auch MDR1, multidrug resistance protein genannt) und MRP1, die eine große Vielfalt an Substraten transportieren können.

Diese Transporter benötigen bestimmte strukturelle Elemente, die die Funktionsweise der Transporter vermitteln. die Anwesenheit von mindestens zwei transmembranen Domänen (TMD), jeweils gefolgt von einem ATP-Bindungsmodul (ABC). MRP1 ist ein 190 kDa Glykoprotein mit einer vermuteten Topologie, die eine zusätzlich zum typischen P-gp ähnlichen Kern eine amino-proximale transmembrane Domäne aufweist, die für die meisten MRP-Proteine charakteristisch ist.

Diese Domäne besteht aus fünf transmembranen Alpha-Helices und besitzt einen extrazellulären N-Terminus (Bàkos et al., 1996; Hipfner et al., 1997; Kast, Gros, 1997). Sie ist durch einen cytoplasmatischen Verbindungs-Loop (L_0) mit dem P-gp ähnlichen Kern verbunden. Wenn MRP1 in polarisierten Zellen exprimiert wird, wird es zu der basolateralen Membran geleitet.

MRP1 besitzt typische Eigenschaften einer GS-X Pumpe, manchmal auch MOAT (multispecific organic anion transporter) genannt. GS-X Pumpen haben eine relativ große Substrat-Spezifizierung. Sie transportieren typischerweise anionische Glutathion-, Glucuronat- oder Sulfat-Konjugate. Außerdem ist MRP1 fähig, unmodifizierte hydrophobische Substanzen wie natürlich produzierte Chemotherapeutika gleichzeitig mit reduziertem Gluthathion (GSH) zu transportieren. Funktionale Studien haben bewiesen, dass auch beim Transport von Vincristin (Loe et

al., 1998; Renes et al, 1999) und Daunorubicin (Salerno und Garnier-Suillerot, 2001) ebenfalls GSH von MRP1 ko-transportiert wird.

Um die Rolle der N-terminalen Regionen in der Funktion des MRP1 zu untersuchen, haben Bàkos et al.,1998, verkürzte MRP1 Varianten hergestellt. Diese Mutanten bestehen entweder 1) nur aus dem P-gp ähnlichen Kern (ΔMRP1) oder aus dem Kern plus dem Verbindungs-Loop L_0 (L_0ΔMRP1). Die Konstrukte wurden in baculovirus-infizierten Insektenzellen und in polarisierte MDCKII (Madin-Darby canine kidney)-Zellen exprimiert. Es wurde untersucht, ob die Fähigkeit dieser Deletionsmutanten MRP1-Substrate zu transportieren, sowie deren subzellulare Lokalisation in den MDCKII-Zellen untersucht. Der P-gp ähnliche Kern (ΔMRP1) war inaktiv. Durch indirekte Immunofluoreszenz wurde gezeigt, dass die Expression der ΔMRP1 in MDCKII-Zellen auf intrazellulare Bereiche beschränkt war.

Entgegen der Vermutung verhielt sich L_0ΔMRP1 wie das wild-type MRP1 in Transport-Experimenten und es wurde korrekt zu der basolateralen plasmatischen Membrane in der MDCKII-Zelle geführt. Dies könnte andeuten, dass die erste transmembrane Domäne (TMD_0) weder für die Funktion des MRP1 noch für das Routing des Proteins benötigt wird. Diese Ergebnisse sind in Fig. 1.5 in der Dissertation zusammengefasst.

Diese Ergebnisse erlauben die Annahme, dass der zytoplasmatische Verbindungs-Loop L_0 eine funktionale Domäne bildet, die für die durch MRP-1 vermittelte Transportaktivität und für die Lokalisation des Proteins in polarisierten Zellen notwendig und ausreichend ist. Bemerkenswert ist, dass in allen bis heute identifizierten Mitgliedern der MRP Familie L_0 konserviert ist.

In der vorliegenden Arbeit sollte nun die Funktion des amino-terminalen Bereichs von MRP1, der aus der ersten transmembranen Domäne TMD_0 und dem cytoplasmischen Verbindungs-Loop L_0 besteht, untersucht werden. Wir haben durch Expression und Koexpression von diversen MRP1 Mutanten in polarisierten MDCKII Zellen nach Bereichen gesucht, die für das Routing des MRP1 in der basolateralen plasmatischen Membrane verantwortlich sind.

In Bàkos et al., 2000, wird beschrieben, wie gekürzte und mutierte MRP1 Moleküle konstruiert worden sind und ihr Verhalten wird charakterisiert. Dabei wurden sie in den folgenden zwei Systemen exprimiert: 1) in baculovirus-infizierten Insektenzellen und 2) in MDCKII - Zellen.

Im ersten Teil dieser Arbeit wurden die Mutanten, die in MDCKII-Zellen exprimiert worden sind, zunächst näher charakterisiert.

Charakterisierung der MDCKII-MRP1Δ(223-232) Zelllinie

Durch die Vorhersage der sekundären Struktur wurde die Anwesenheit einer amphipathischen Helix innerhalb der L_0 Region vermutet. Es wurde gezeigt, dass in der L_0 Region aller Mitglieder der MRP Familie solch eine Helix vorhanden ist. Um festzustellen, ob diese Helix für die Transportaktivität und/oder dem Routing wesentlich ist, haben wir eine 10-Aminosäure-Deletion Δ(223-232) innerhalb dem vollständigen MRP1 und eine stabile MDCKII-MRP1 Δ(223-232) Zelllinie durch retrovirale Transduktion hergestellt. Das Protein wurde korrekt N-glycosyliert (Fig. 4.4 in der Dissertation). Durch Immunolokalisation wurde gezeigt, dass das Routing nicht durch die Mutation beeinflusst wurde (Fig. 4.5 in der Dissertation).

Signifikant dabei ist, dass die Mutante nicht fähig war, MRP1 Substrate zu transportieren. Dies deutet darauf hin, dass die fehlende Helix für die Funktionalität der MRP1 notwendig ist. *In-Vitro*- Experimente im Baculo-System (Bàkos et al., 2000) haben gezeigt, dass die amphipatische Helix an der Anbindung des MRP1 an der Membrane teilnimmt.

Koexpression des cytoplasmatischen Verbindungs-Loop L_0 mit ΔMRP1 in MDCKII Zellen

Um weitere Untersuchungen über die Rolle des zytoplasmatischen Verbindungs-Loops L_0 anzustellen, haben wir L_0 zusammen mit dem inaktiven C-terminalen Kern von MRP1, ΔMRP1, in MDCKII - Zellen koexprimiert (Fig. 4.8 in der Dissertation). Durch Immunolokalisation wurde festgestellt, dass ein Routing des Peptids zu der

plasmatischen Membrane in polarisierten Zellen stattfindet (Fig. 4.10 in der Dissertation).

Es war uns nicht möglich, einzelne MDCKII-Klone zu isolieren. Wahrscheinliche Gründe sind die Instabilität des Peptids und seine rapide Degradierung. Daher konnte die Transportaktivität der koexprimierten Mutanten in den MDCKII-Zellen nicht gemessen werden.

Dennoch wurde im Baculo-System gezeigt, dass die Koexpression der zwei Konstrukte in einer MRP1-ähnlichen Aktivität resultiert (Bàkos et al., 2002).

Diese Ergebnisse zusammengenommen suggerieren verstärkt, dass das isolierte L_0-Peptid in der Lage ist, sich mit ΔMRP1 zu assoziieren. Dadurch erlangt das Protein wieder seine Funktion und lokalisiert sich in der basolateralen Membrane.

Charakterisierung des N-terminalen Bereichs des MRP1 (TMD_0L_0) in MDCKII polarisierten Zellen.

Um die Schwierigkeiten bei der Isolierung von MDCKII-L_0 Klonen zu umgehen, wurde eine MDCKII-Zelllinie, die das Polypeptid TMD_0L_0 (Aminosäure 1-281) exprimiert, hergestellt. Das Polypeptid TMD_0L_0 besteht aus der ersten transmembranen Domäne $TMD_{0,}$ die um den intrazellularen Verbindungs-Loop L_0 erweitert wurde (Fig. 4.11, Paneel A, in der Dissertation). Durch indirekte Immunofluoreszenz (Fig. 4.12 in der Dissertation) und durch die Methode der Biotinylierung der Zelloberfläche (Fig. 4.15 in der Dissertation) wurde festgestellt, dass dieses Protein sich teilweise in der basolaterale Membrane befindet.

Koexpression von TMD_0L_0 und ΔMRP1 in MDCKII polarisierten Zellen.

Die Mutanten TMD_0L_0 und ΔMRP1 wurden in MDCKII-Zellen koexprimiert (Fig. 4.16, in der Dissertation). Die beiden Peptide ergeben zusammen ein vollständiges MRP1-Molekül.

Die Anwesenheit von TMD_0L_0 genügte, um die Glycosilierung des ΔMRP1 (Fig. 4.17 in der Dissertation) und die Lokalisierung in der basolateralen Membrane des ΔMRP1 zu ermöglichen (Fig. 4.18 in der Dissertation).

Außerdem resultierte die Koexpression der zwei komplementären MRP1-Fragmente in einer wild-type-ähnlichen Transportaktivität (Fig. 4.19 in der Dissertation). Es wurde mittels einer Koimmunoprezipitation gezeigt, dass die beiden Fragmente des MRP1 interagieren (Fig. 4.21 in der Dissertation).

Charakterisierung des chimerischen Konstruktes TMD_0(MRP1)L_0(MRP2) in MDCKII-Zellen

MRP2 ist ein Homolog von MRP1, welches in der apikalen Membrane von polarisierten Zellen lokalisiert ist.

Um zu untersuchen, ob das Signal für die basolaterale Lokalisation von MRP1 in L_0 enthalten ist, wurde ein chimerisches Protein hergestellt, welches aus TMD_0 von MRP1 und L_0 von MRP2 besteht und in MDCKII und MDCKII-ΔMRP1 Zellen exprimiert.

Da dieses chimerische Protein durch Endoglycosidase H deglycosiliert werden konnte, wurde festgestellt, dass das unvollständig glycosiliert ist (Fig. 4.24 in der Dissertation) und dass es sich im endoplasmatischen Reticulum lokalisiert. Die intrazelullare Lokalisation der Chimera in An- und Abwesendheit von ΔMRP1 wurde durch Immunolokalisation bestätigt (Fig. 4.25 in der Dissertation).

Schlussfolgerungen

In dieser Arbeit wurden durch Expression und Koexpression diverser MRP1 Mutanten in polarisierten MDCKII-Zellen Bereiche gesucht, die für das Routing von MRP1 wichtig sind. Signale für die basolaterale Lokalisation von transmembranen Proteinen sind sehr unterschiedlich und diverse molekulare Signale nehmen an der Bestimmung des Routings teil. Im Falle von MRP1 wurde durch die Studien in dieser

Arbeit erkannt, dass die zytoplasmatische Verbindungs-Loop L_0 von MRP1 ein Signal enthalten könnte, welches zur basolateralen Lokalisation des Proteins beiträgt. Es war nicht möglich, eine exakte Sequenz zu bestimmen. In MRP1 sind keine der bisher kodifizierten Motive vorhanden. Eine extensive Untersuchung mittels weiterer Mutanten von MRP1, ergänzt durch Studien über MRP1/MRP2 chimerische Proteine sind notwendig, um die Determinanten für das Routing von MRP1 festzustellen.

Es wurde eine Alpha-Helix innerhalb des Verbindungs-Loops L_0 identifiziert, die für die Funktionalität von MRP1 als Transporter wichtig ist. Gleichzeitig wurde gezeigt, dass das Routing des Proteins nicht durch die Mutation beeinträchtigt wurde. Das bedeutet, dass ein korrektes Routing keine ausreichende Bedingung für die Funktionalität des MRP1 ist.

Die Interaktion von zwei komplementären MRP1-Fragmenten TMD_0L_0 und ΔMRP1 wurde nachgewiesen. Sie stabilisieren sich gegenseitig und bilden ein funktionsfähiges MRP1 Molekül. Da TMD_0L_0 dazu beiträgt, das basolaterale Routing von ΔMRP1 zu ermöglichen, wird die Hypothese zusätzlich bestätigt, dass L_0 eine wichtige Funktion in der korrekten Lokalisation des MRP1 in der plasmatischen Zellmembrane übernimmt.

Die in dieser Arbeit gewonnen Erkenntnisse können somit dazu beitragen, die Funktionsweise des systems der Mehrfachen Chemotherapieresistenz in Säugerzellen besser zu verstehen, so dass bei der Therapie diverser Erkrankungen, bei denen der Transport von Chemotherapeutika aus den Zielzellen unterbunden werden soll, das Transportsystem entsprechend beeinflusst werden kann.

Contents

1 INTRODUCTION

1.1 Multidrug resistance

Cells exposed to toxic compounds can develop drug resistance by a number of mechanisms including decreased uptake, resistance against apoptotic signals, increased detoxification, alteration of target proteins, or increased excretion. Some of these mechanisms can lead to multidrug resistance (MDR) in which cells gain resistance against a whole range of drugs in addition to the drug to which they were originally exposed, a feature first described by Kessel et al. in 1968 (Kessel et al., 1968*;* Biedler and Riehm, 1970). One form of MDR is called classical multidrug resistance. Classical MDR was defined as the phenomenon that mammalian cells could become resistant to a range of cytotoxic drugs that do not share a common target or structure, after selection with a single drug. Whereas the capacity of cells to become MDR is conserved throughout evolution, in this thesis I will concentrate on transport proteins, which overproduction can result in drug resistance in mammalian cancer cells. MDR cell lines show an increased efflux of cytotoxic drugs from the cell by the overexpression of transporter molecules, resulting in a decreased intracellular drug concentration. The transporters responsible for the active extrusion of drugs are members of the ATP-binding cassette (ABC) transporter superfamily. The ABC-transporters represent the largest family of transmembrane proteins. Proteins are classified as ABC-transporters based on the sequence and organization of their ATP-binding domains, also known as nucleotide binding folds (NBFs). The NBFs contain characteristic motifs called Walker A, C and B, separated over 90-120 amino acids (Walker et al., 1982). Functional transporters typically contain two cytoplasmically localized NBFs and at least two transmembrane domains. In humans currently 51 ABC-proteins have been identified (Müller, http://nutrigene.4t.com/humanabc.htm), at least six of which have been associated with drug transport. All human and mouse ABC genes have a standard nomenclature and are subdivided in seven classes (ABCA-ABCG; Dean et al., 2002). Synonymous gene names are still used, however. The first ABC-transporter identified

whose overexpression caused MDR to a range of drugs in tumor cells was MDR1 P-glycoprotein (Pgp) or ABCB1 (Juliano and Ling, 1976; Chen et al., 1986; Roninson et al., 1986). MDR1 Pgp is an approximately 170-kDa glycosylated transmembrane protein that consists of two similar halves each containing six transmembrane spanning domains and a cytosolically localized C-terminal ATP-binding domain. MDR1 Pgp has to be localized in the plasma membrane in order to cause drug resistance. MDR1 Pgp has been shown to be a promiscuous transporter transporting a wide range of hydrophobic amphipathic compounds. Examples of cytotoxic drugs transported by MDR1 Pgp are anthracyclines, *Vinca* alkaloids, colchicine, cyclosporine A and taxol, drugs with different cellular targets and chemical structures.

As not all multidrug resistant cells showed overexpression of Pgp, a search for other efflux pumps was initiated, leading to the identification of the multidrug resistance protein MRP1 (ABCC1; Cole et al., 1992). Subsequently it was shown that MRP1 is member of a larger family (Kool et al., 1997) some of which have been shown to be able to transport at least some drugs. Since MRP proteins are the subject of this thesis, they will be discussed extensively in paragraph 1.3. Other ABC proteins that have been associated with drug resistance are: (i) The breast cancer resistance protein (BCRP, MXR, ABCP or ABCG2), which was cloned from cells resistant to mitoxantrone (Miyake et al., 1999; Ross et al., 1999). (ii) A distant homolog of MDR1 Pgp, sister of Pgp or the bile salt export protein (BSEP or ABCB11) confers low level resistance to paclitaxel in liver cells (Childs et al., 1998).

Identification of the upregulated genes in different resistant tumors and detailed knowledge of the causes and the mechanisms of drug resistance will allow predicting the response of patients to chemotherapy.

1.2 The human ABC gene family

1.2.1 Structure / activity relationship

Chang and Roth (2001) elucidated for the first time the high resolution structure by x-ray crystallography of a complete ABC transporter, the MsbA lipid A transporter from *Escherichia coli*. By sequence homology MsbA is more closely related to the mammalian MDR1 Pgp than any other bacterial ABC-transporter (Tomii and Kanehisa, 1998).

The msbA gene encodes a half transporter that consists of six membrane spanning regions and a nucleotide-binding domain located at the cytoplasmic side of the cell membrane. MsbA is assembled as a homodimer with a total molecular mass of 129 kDa (Doerrler et al., 2001; Karow and Georgopoulos, 1993). MsbA transports lipid A, a major component of the bacterial outer cell membrane (Zhou et al., 1998).

The crystal structure confirmed the prediction that the half transporter is organized as a homodimer in the cell, with each subunit containing two domains, a TMD composed of six α-helices and one NBD. The chamber is formed between two angled blocks of six α-helices that correspond to the two TMDs and is closed at the extracellular face of the membrane. The chamber surface facing the inner leaflet is rich in positively charged amino acids, while the surface facing the outer leaflet is much more hydrophobic. The chamber has two large side entrances between the TMDs that would be accessible from the inner leaflet of the bilayer. These side entrances are consistent with evidence that Pgp extracts substrates from the inner leaflet of the lipid bilayer and transports them directly in the extracellular aqueous medium**.**

Figure 1.1 indicates the structural organization of Eco-MsbA hypothesized by Chang and Roth.

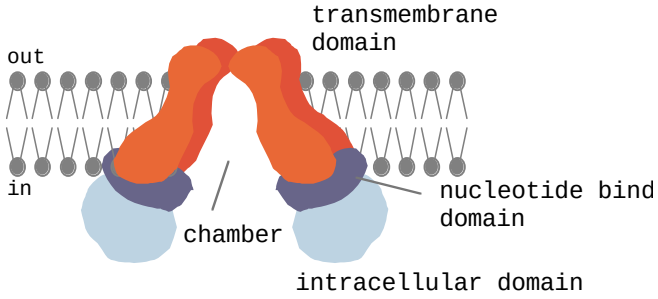


Fig. 1.1. Structure of Eco-msbA.

Representation of the dimer forming the chamber opening. The transmembrane domain, the nucleotide binding domain, and intracellular domain are colored red, dark blue and light blue respectively.

Chang and Roth propose a possible flipping mechanism for the transport of lipid A based on the structure of MsbA that could be generally valid for the members of the MDR-ABC transporter group. The entrance of lipid A from the inner leaflet of the plasma membrane into the chamber induces some conformational changes in the TMDs that are transferred to the NBDs via the intracellular domain. This change initiates the hydrolysis of ATP, with as a consequence closing of the chamber. The hydrophobic substrate in the chamber is in an energetically unfavorable environment as it is in close proximity to the positively charged residues of the transmembrane domains. To switch to a more favorable condition, the substrate flips to the outer leaflet of the lipid bilayer or directly into the aqueous space.

This model is not completely consistent with the low resolution structure of Pgp and of MRP1, which chambers seem to be widely open at the extracellular side of the plasma membrane (Rosenberg et al., 1997, 2001a). It has been shown for the bacterial transporter LmrA, a phosphatidylethanolamine transporter, that the substrate passes from the inner leaflet of the lipid bilayer directly to the outside of the cell (Bolhuis et al., 1997); consequently the chamber should be open to the extracellular side. Additionally this model would not explain how hydrophilic substances, like *e.g.* sugars, or in the case of the channel CFTR (or ABCC7), the cystic fibrosis transmembrane conductance regulator, ions, could pass the membrane.

There are questions that still remain open, for example, how exactly ATP hydrolysis and the conformational changes in the transmembrane domains are coupled, and not much is known about the TMDs, the substrate binding sites, and their conformational changes during the catalytic cycle. There is evidence indicating that the NBDs work in alternation, but it is not known whether they interact directly (Higgins and Linton, 2001). Rosenberg et al., (Rosenberg et al., 2001b) brought some light into these questions by generation two-dimensional crystals of Pgp blocked in different steps of the catalytic cycle. They observed that the membrane spanning domains undergo structural reorganization after ATP binds to the NBDs. Consequently, the affinity for drug binding decreases suggesting that the binding of ATP provides the initial energy for the translocation. After ATP hydrolysis, ADP and phosphate are released, and the transporter returns to the initial configuration. Higgins and Linton (2001) proposed different possible working models for ABC transporters, which are summarized in fig. 1.2.

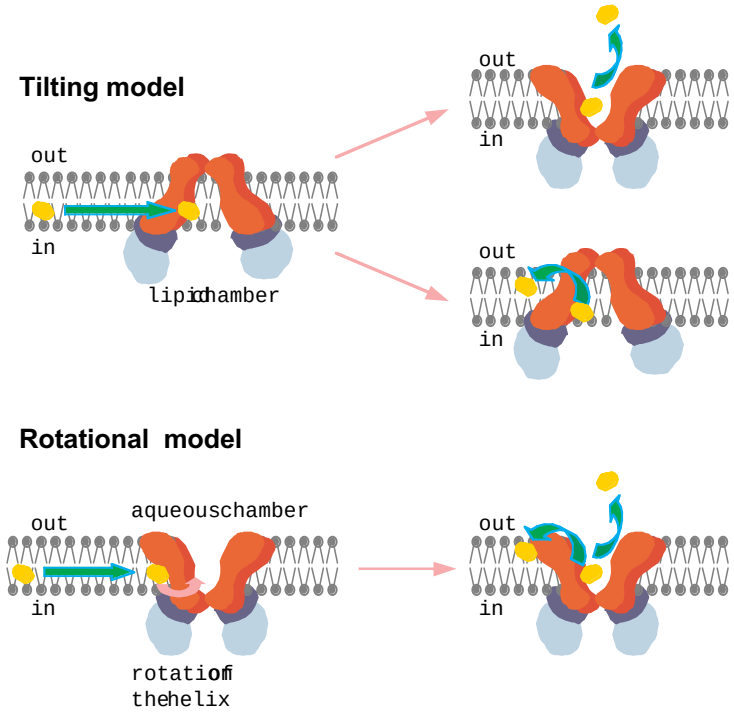


Fig. 1.2. Conformational changes induced during the transport cycle in an ABC transporter proposed by Higgins and Linton, 2001.

The authors suggest two models for how a substrate-binding site may be reorientated to allow export of a substrate from the cell. According to the “tilting” model, shown in the top figure, the substrate enters the inner leaflet of the membrane and from there the chamber. Following ATP-binding and hydrolysis a conformational change occurs in the transporter and resulting in export of the substrate out of the cell. For MsbA a variation of the “tilting model” is suggested: the chamber is lipid-filled so that it does not open to the external aqueous phase but reorientates so that the substrate is directed towards the outer leaflet of the bilayer. In the “rotational model” shown in the bottom of the picture, twisting of the transmembrane α-helix reorientates the substrate-binding site from the inner leaflet of the membrane bilayer, where the substrate can bind, to the aqueous chamber. From there the solute is released and can either exit the cell or partition into the outer leaflet of the bilayer.

1.2.2 Physiological role of ABC transporters

Although several human ABC transporters have been demonstrated to be involved in multidrug resistance in tumor cells, they are widely distributed in normal tissues and transport a wide range of (endogenous) substrates (Gottesman et al., 2002). An important function is controlling the permeability of the central nervous system. The blood-brain barrier (BBB), which protects the central nervous system from toxins, is composed of endothelial cells on the blood capillaries. MDR1 Pgp is localized in the apical (luminal) membrane of the endothelial cells, preventing toxins from entering the brain (Schinkel et al., 1994, 1996; Xie et al., 1999). MRP1 is localized in the choroid plexus (Rao et al., 1999); mouse Mrp1 has been found in the testicular tissue as well as Pgp (Wijnholds et al., 1998; Melaine et al., 2002). In placenta, MDR1 Pgp is localized on the apical syncytiothrophoblast, where it protects the fetus against toxic cationic xenobiotics. Also MRP1 and the half transporter ABCG2 are localized in placenta (Maliepaard et al., 2001).

ABC transporters expressed in liver, kidney and the gastrointestinal tract protect the organism against toxic agents. In the canalicular membrane of hepatocytes MDR1

Pgp transports toxins from the liver into bile (Schinkel et al., 1997), while MRP2 transports bilirubin glucuronide and other organic anions into the bile (König et al., 1999a). Other ABC proteins expressed in hepatocytes at relevant levels are ABCB11 (also known as BSEP) (Strautnieks et al., 1998), and MDR2 (Smit et al., 1993; Ruetz and Gros, 1994). Gastrointestinal mucosal cells contain MDR1 Pgp in the apical membrane that faces the intestinal lumen, establishing a barrier against absorption of toxins, and limiting oral bioavailability of some drugs (Greiner et al., 1999; Smit et al., 1998; Sparreboom et al.1997; Mayer et al., 1997; van Asperen et al., 1999; Kruijtzer et al., 2002). In general MDR1 Pgp seems to have a protecting function against toxic compounds and metabolites in specific organs like the blood brain barrier, intestine, testis, and placenta.

1.3 The MRP family of transporters

Generally xenobiotics are metabolized to more hydrophilic compounds to facilitate their excretion. Hepatic enzymes induce two metabolism pathways. In phase I substances are modified by members of the cytochrome P450 family with a hydroxylic group via oxidation, reduction, hydrolysis or hydration. Metabolites or parent compounds can also be conjugated to glutathione, glucuronide or sulfate in phase II. Such chemical modifications allow elimination of the drug but they also interfere with its biological activity, decreasing or sometimes increasing toxic effects. The resulting conjugates are too hydrophilic to diffuse out of the cell and require dedicated transporters to extrude them. It was postulated that ABC transporters would be responsive for such transport activity, but since the substrates of MDR1 Pgp are typically non-anionic it was concluded that other pumps able to transport anionic conjugates should exist (Ishikawa, 1992).

MRP1 was shown to mediate the ATP-dependent unidirectional transport of lipophilic substances conjugated to glutathione, glucuronate or sulfate (Jedlitschky et al., 1996). In addition a number of unconjugated amphiphilic anions are transported, like the acetoxymethyl ester and the free anion forms of the fluorescent dye calcein, the

organic anion anticancer agent methotrexate and several other cytotoxic drugs (Zaman et al., 1994; Hollo et al., 1996; Bàkos et al., 2000b). The discovery of MRP1 initiated a search for homologues that resulted in the identification of eight additional proteins that showed homology to MRP1. Phylogenetic analyses confirmed that the MRP1-like proteins belonged to a novel branch of the ABC superfamily. In 1996 the gene of cMOAT (now MRP2; ABCC2) was cloned from rat liver cDNA libraries (Paulusma et al., 1996; König et al., 1996); in 1997 MRP3 (ABCC3), 4 (ABCC4) and 5 (ABCC5) followed (Kool et al. 1997). Recent work has added four more members to the MRP family: MRP6 (ABCC6; Kool et al., 1999a), MRP7 (ABCC10; Hopper et al., 2001), and MRP8 (ABCC11) and 9 (ABCC12; Tammur et al., 2001).

MRP RNAs exhibit different patterns of tissue distribution and most MRP proteins are encoded by genes located on different chromosomes (Table 1.1). While MRP1 and MRP5 are ubiquitous in the human body, the tissue expression patterns of MRP3 and MRP6 are similar to the tissue distribution of MRP2. The MRP6 gene is located on chromosome 16 next to the MRP1 gene (Kool et al., 1999a). Recently, MRP8 and MRP9 were identified adjacent to each other on chromosome 16q12.1 (Tammur et al., 2001; Bera et al. 2002).

	Chromosomal localization	Tissue distribution	Subcellular localization
MRP1	16p13.1	Ubiquitous	basolateral
MRP2	10q24	Liver, kidney, gut	apical
MRP3	17q21.3	Pancreas, kidney, gut, liver, adrenal	basolateral
MRP4	13q32	Prostate, testis, ovary, gut, pancreas, lung, muscle	still unclear
MRP5	3q27	Ubiquitous	basolateral
MRP6	16p13.1	Liver, kidney	basolateral
MRP7	6p21	Colon, skin, testis	unknown
MRP8	16q12.1	Breast, testis, liver, brain, placenta	unknown
MRP9	16q12.1	Brain, testis, ovary, skeletal muscle, pancreas	unknown

Table 1.1. Chromosomal localization, tissue distribution and subcellular localization of MRP proteins

The MRP proteins differ from MDR1 Pgp in their membrane topology. MRP1, 2, 3, 6 and 7 contain an additional amino-proximal membrane-spanning domain predicted to consist of 5 transmembrane α-helices. MRP4, 5, 8 and 9 lack this first domain but contain an intracellular amino-terminal loop extending the typical secondary structure of most human full-length ABC proteins at the N-terminus. The predicted membrane topology of the various MRP family members is shown in figure 1.3.

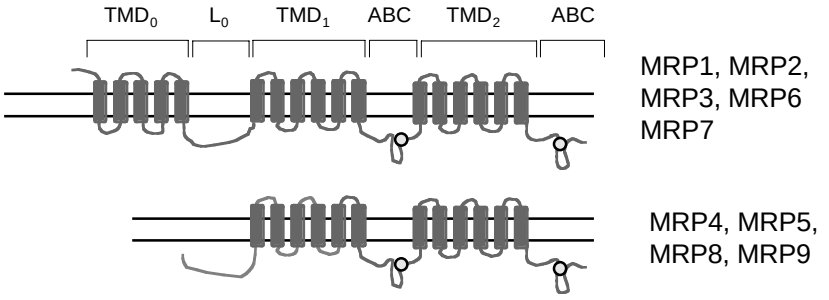


Fig. 1.3. Membrane topology models of the MRP family..

TMD: transmembrane domain; L_0: linker; ABC: ATP binding cassette

Like many other ABC-transporters that are localized in the plasma membrane, in polarized cells they are routed to either the apical or basolateral plasma membrane. Therefore the transported substrates accumulate in a specific compartment. While P-glycoprotein and MRP2 are localized in the apical plasma membrane, MRP1, MRP3, MRP5 and MRP6 are localized in the basolateral membrane (Evers et al., 1996; Paulusma et al. , 1996; Buchler et al., 1996; König et al., 1999b; Kool et al. 1999b; Wijnholds et al., 2000b; Madon et al., 2000). Currently nothing is known about the subcellular distribution of the remaining MRP family members MRP7-9 and that of MRP4 is still controversial

1.3.1 An inventory of the MRP family members

1.3.1.1 MRP1

The multidrug resistance protein, MRP1, was discovered in 1992 in H69AR cells, a small cell lung cancer cell line that displayed a multidrug resistance phenotype in absence of Pgp. These cells showed relatively high levels of resistance to the *Vinca* alkaloids, epipodophyllotoxins, doxorubicin, mitoxantrone, daunorubicin, epirubicin and colchicine (Mirski et al.,1987; Cole, 1992). MRP1 mRNA was overexpressed 100-200 fold in comparison to the parental H69 cell line from which H69AR is derived (Cole et al., 1992). In addition MRP1 was found to be overexpressed in a doxorubicin-selected multidrug resistant HeLa cell line that did not overexpress Pgp. In both cell lines the *MRP1* gene was amplified (Slovak, 1993). By sequence analysis the 1531 amino acid protein was identified as a member of the ABC superfamily.

Newly synthesized MRP1 migrates on SDS-polyacrylamide denaturing gels as a protein with an apparent molecular weight of about 170 kDa. This immature polypeptide is processed to a 190-kDa mature form by addition of N-linked sugar residues (Almquist et al., 1995). Sequence analysis of MRP1 indicates that there are 14 potential N-glycosylation sites, distributed over both halves of the molecule. Mutation studies (Hipfner et al., 1997) demonstrated that Asn 19 and Asn 23 are glycosylated, in accord with the extracellular localization of the N-terminus. Asn 1006 in TMD2 was identified as a third functional N-glycosylation site. Several MRP-related proteins and CFTR contain glycosylation sequons at similar locations.

The topology of MRP1 was studied using epitope insertion methodology. Hemagglutinin (HA) epitope tags were inserted at various locations and their orientation was determined in permeabilized and non-permeabilized cells expressing the MRP1 mutants. Based on these studies a topological model for MRP1 was proposed. MRP1 was predicted to consist of three transmembrane domains, the first containing five membrane spanning α- helices. The other two transmembrane domains each contain six

helices, followed by a nucleotide binding domain (Kast and Gros , 1997, 1998; Hipfner et al. 1997).

1.3.1.1.1 Transport activity of MRP1

MRP1 is able to transport glutathione conjugates, such as dinitrophenyl glutathione (DNP-GS) and leukotriene C_4 (LTC_4)(Jedlitschky et al., 1994; Leier et al., 1994; Müller et al., 1994). Transporters with this characteristic are called GS-X pumps, or multispecific organic anion transporter (MOAT). GS-X pumps have a relatively broad substrate specificity; they typically transport glutathione, glucuronate or sulfate conjugates. ATP-dependent uptake of numerous endogenous and exogenous conjugated organic anions into inside-out membrane vesicles isolated from several cell types overexpressing MRP1 was reported (Müller et al., 1994; Leier et al., 1994; Keppler , 1997; Suzuki , 1998).

It was observed that overexpression of MRP1 cDNA in human lung carcinoma cells caused an increased efflux of reduced GSH in the presence of arsenite (Zaman et al., 1995); Probably arsenite is transported together with glutathione as a short lived complex. Interestingly, the transport of at least three authentic organic anions, glucuronosyl-etoposide (Sakamoto et al., 1999), estrone-3-sulfate (Qian et al., 2001), and the glucuronosyl derivative of a nicotine-derived carcinogen, NNAL-O-glucuronide (Leslie et al., 2001), is greatly enhanced by physiological concentrations of GSH. A role of GSH in the export of compounds by MRP1 is supported by studies using buthionine sulfoximine (BSO). BSO is a potent inhibitor of γ-glutamylcysteine synthase, the enzyme catalyzing the first rate limiting step of the biosynthesis of GSH. Treatment of cells with BSO results in a drastic reduction of the intracellular GSH pool. In several studies with MRP1 overexpressing cells BSO treatment resulted in an increased drug accumulation and toxicity, suggesting that the modulation of MRP1 activity by BSO is a direct result of GSH depletion (Vanhoefer et al., 1997; Versantvoort et al., 1995; Zaman et al., 1995; Draper et al. 1997). Given that MRP1 is a GS-X pump, it was not expected to transport chemotherapeutic drugs like anthracyclines and *Vinca* alkaloids,

compounds not known to be conjugated to negatively charged ligands. It was speculated that MRP1 confers drug resistance by elimination of conjugation products from cells (Cole and Deeley, 1998). There are some problems associated with this model of MRP1-mediated drug resistance. Metabolic conjugation to GSH is a detoxification pathway producing more water-soluble and often less toxic metabolites. This implicates that increased elimination of such drug metabolites may result in drug resistance only if their transport out of the cell is the rate limiting step. In addition, most of the known substrates of MRP1 are not primarily metabolized by conjugation to GSH. Phase II metabolism occurs mainly in the liver and intestine and not in several of the tissues in which MRP1 overexpression confers drug transport. MRP1-mediated resistance to natural product drugs is not dependent upon phase II metabolism, as only unconjugated daunorubicin and vincristine are found in the culture medium of MRP1-transfected resistant cells (Zaman et al., 1995). All these considerations indicated that the transport of MRP1 could not be limited to that of conjugated compounds.

In vesicle uptake experiments drugs like doxorubicin, daunorubicin, vincristine, vinblastine, VP-16 or paclitaxel are not able to inhibit transport of LTC_4, even at high concentrations (Müller et al., 1994; Loe et al., 1996). However, *Vinca* alkaloids can competitively inhibit transport of LTC_4 if relatively high concentrations of reduced GSH are present (Loe et al., 1996). Subsequently it was demonstrated that transport of unmodified vincristine is both ATP- and GSH-dependent, being the transport rate in the absence of GSH very low and not affected by the presence of ATP (Loe et al., 1996, 1998). Functional studies demonstrated that GSH is also substrate of MRP1 if vincristine (Loe et al., 1998; Renes et al., 1999) and daunorubicin (Salerno and Garnier-Suillerot, 2001) are transported. Fig. 1.4 shows that MRP1 transports xeno- and endobiotics following two different mechanisms. It can act as a GS-X pump in synergy with glutathione-S-transferases, or transport unmodified compounds in association with unmodified glutathione.

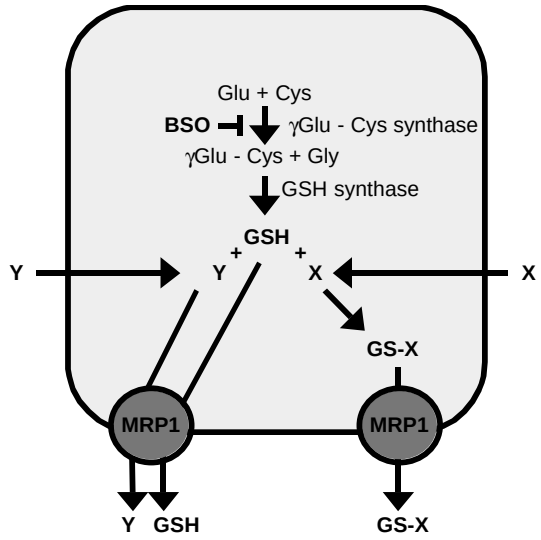


Fig. 1.4. Model of the transport activity of MRP1.

After entering the cell, compound X is conjugated to glutathione by a glutathione S-transferase, resulting in a GS-X conjugate that is actively extruded from the cell by MRP1. Compound Y is not metabolized by the cell to a negatively charged conjugate and is transported by MRP1 unmodified. Export of unconjugated compounds by MRP1 is associated with the transport of reduced glutathione. In both cases transport is depending on the intracellular amount of glutathione.

1.3.1.1.2 Physiological role of MRP1

The MOAT-like transport activity of MRP1 has been well characterized before its molecular identity was known. A well-characterized substrate of MRP1 is the cysteinyl leukotriene LTC_4 (Ishikawa et al., 1990; Quian et al., 2001; Haimeur et al., 2002; Ren et al., 2002). This compound is an important chemical mediator in certain inflammatory responses. LTC_4 is a derivative of arachidonic acid, which is conjugated to glutathione after a series of reactions. Vesicle transport experiments with membranes derived from MRP1 expressing cells, confirmed that MRP1 is the major high affinity transporter of LTC_4 (Leier et al., 1994; Müller et al., 1994). Subsequently it was discovered that more endogenous substrates of MOAT are efficiently transported by MRP1. Examples are glucuronides and sulfate conjugated bile salts (Jedlitschky et al., 1996), bilirubin glucuronides (Jedlitschky et al., 1997), glutathione disulfide (GSSG)

(Heijn et al., 1997), the 17β estradiol 17-(β-D-glucuronide)(Loe et al., 1996), prostaglandine A2 conjugated with GSH (Evers et al., 1997) and the glutathione conjugate of 4-hydroxinonenal (4HNE-GS) (Renes et al., 2000).

Based on the ubiquitous expression of MRP1 and its capacity to transport anionic conjugates, it was proposed that the physiological function of MRP1 is the extrusion of detoxification products resulting from phase II metabolism (Müller et al., 1996). MRP1 could also play a role in oxidative stress. GSSG and 4HNE-GS are detoxification products generated under conditions of an altered intracellular redox state (Wang and Ballatori, 1998; Esterbauer et al., 1991), which would suggest that MRP1 takes part in the cellular defense system against oxidative stress (Leier et al., 1996; Renes et al., 2000). MRP1 would help to prevent the damaging effects of the rising GSSG/GSH ratio in cultured rat astrocytes exposed to oxidative stress, as GSSG is transported more efficiently than GSH (Hirrlinger et al., 2001). Thus, extrusion of these metabolites by MRP1 may be required to prevent cellular damage.

The transport of LTC_4 and PGA_2, molecules involved in inflammation, could be necessary to react against inflammatory stimuli. Mice lacking Mrp1 show a reduced response to inflammation induced by a nonspecific inflammatory stimulus (Wijnholds et al., 1997). These mice are also defective in the trafficking of antigen-bearing dendritic cells from the epidermis to lymph nodes (Robbiani et al., 2000). Nevertheless, no pathology has been reported that can be attributed to this effect on dendritic cells and Mrp1 (-/-) mice are healthy in a protected environment (Wijnholds et al., 1997; Lorico et al. 1997).

MRP1 is located in intracellular vesicles of undefined nature in $e.g.$ macrophages (Flens et al., 1996) and in the basolateral membrane of polarized cells (Evers et al., 1996; Paulusma et al. , 1996), supporting the possibility of MRP1 being a system of cellular defense rather than one of total organism defense like Pgp and MRP2.

It has been shown that MRP1 is involved in the protection of vital body cavities, such as the contents of the testicular tubules (Wijnholds et al., 1998) and the cerebrospinal fluid (Wijnholds et al., 2000a). MRP1 is localized in the basolateral membrane of the choroid plexus in the brain, where it pumps compounds from the

cerebrospinal fluid into the blood (Rao et al., 1999; de Lange et al., 2000). Wijnholds et al., showed that the lack of Mrp1 protein in Mrp1/Mdr1a/Mdr1b triple-knockout mice causes etoposide levels to increase about 10-fold in the CSF after intravenous administration of the drug, suggesting that Mrp1 contributes to the blood-CSF drug-permeability barrier (Wijnholds et al., 2000a).

MRP1 may be part of the blood-testis barrier and cooperate with Pgp to defend testicular tissue. Pgp is localized in the apical membrane of endothelial cells in the testis (Melaine et al., 2002), while high amounts of Mrp1 have been found in the basolateral membrane in mouse Sertoli cells, protecting sperm within the testicular tubules. Its absence in Mrp1(-/-) mice leads to an increased drug-induced destruction of sperm cells (Wijnholds et al., 1998).

MRP1 is also localized in placenta (St-Pierre et al., 2000; Pascolo et al., 2000) where it participates in the protection of fetal blood against toxic organic anions and eliminates glutathione/glucuronide conjugates resulting from metabolism (St-Pierre et al., 2000).

1.3.1.1.3 Inhibitors of MRP1

No potent and specific MRP1 inhibitors are available. So far non-specific inhibitors of organic anions transport like sulfinpyrazone, probenecid (Evers et al., 1996) and benzbromarone (Berger et al., 1997) are used to reverse resistance in MRP1 expressing cells. Indomethacin is a nonsteroidal anti-inflammatory drug (NSAID) (Draper et al., 1997). It is rapidly conjugated to glucuronide in cells, and it is possible that this conjugate is transported by MRP1 rather than the free drug. It was shown that other NSAIDs have a similar effect (Duffy et al., 1998). It has been suggested that NSAIDs might be used in patients to reverse MRP-mediated drug resistance.

Hooijberg et al. (1999) were able to reverse decreased daunorubicin accumulation in MRP1 cells with several flavonoids. Nevertheless the cytotoxicity of these compounds is relatively high; quercetin can reverse vincristine resistance only

partially and other flavonoids are completely inactive at non-cytotoxic concentrations (Leslie et al., 2001). It seems unlikely that flavonoids can be modified to yield MRP inhibitors with sufficient specificity and therapeutic window to be useful in clinical practice.

Another clinically tested compound, the antagonist of the leukotriene D_4 receptor, MK571, is a good MRP1 inhibitor in vesicular transport experiments, but has only a limited effect on MRP1 in intact cells even at a sub-toxic dose (Gekeler et al., 1995).

The structural elements that contribute to the affinity of a molecule for MRP1 are not clearly defined, but recognition is partly determined by the number and spatial distribution of anionic residues (Seelig et al., 2000). Glutathione conjugates have been used as MRP1 inhibitors of in inside-out membrane vesicles (Furuta et al., 1999). As the glutathione (GSH) conjugate of ethacrynic acid (EA), GS-EA, is a good substrate of MRP1 (Zaman et al., 1996), GS-EA derivatives are expected to be good inhibitors of MRP1. GS-EA analogs demonstrated to inhibit MRP1-mediated [^{3}H]GS-EA and [^{3}H]17β estradiol 17-(β-D-glucuronide) E_2-17βG transport in Sf9 vesicles. One of these compounds inhibits MRP1-mediated efflux of calcein from 2008 ovarian carcinoma cells overexpressing MRP1. Also resistance of these cells toward methotrexate (MTX) could be partially reversed by co-incubation with this compound (Burg et al., 2002).

In addition, certain tricyclic isoxazoles have been found to inhibit ATP-dependent, MRP1-mediated LTC_4 uptake into membrane vesicles and to reverse drug resistance to MRP1 substrates, such as doxorubicin, in intact HeLa-T5 cells at micromolar concentration. How these compound work is still not clear, but they might become a useful tool for research on MRP1 (Norman et al., 2002). Recently an azido tricyclic isoxazole analog (LY475776) demonstrated to be a potent MRP1-specific reversing agent, showing GSH dependent inhibition of LTC_4 transport. Conversely ATP hydrolysis can reduce the affinity of MRP1 for LY475776. Recent studies contributed to understand the structural requirements of this kind of inhibition, characterizing its GSH dependence, and identifying a potential binding site for the inhibitor (Quian et al., 2002; Mao et al., 2002).

1.3.1.2 MRP2

Prior to the cloning of MRP2 (ABCC2), biochemical and genetic studies indicated that a multispecific organic anion transporter was present in the canalicular membranes of hepatocytes (König et al., 1999b). Mutant rat strains (TR(-) and EHBR) lacking this transporter have a hyperbilirubinemic phenotype and impaired secretion of bilirubin glucuronide, sulfated bile salts, GS-conjugates and GSH into the bile (Suzuki and Sugiyama, 1998; Oude Elferink et al., 1993). Dubin–Johnson syndrome patients suffer a form of congenital hyperbilirubinemia caused by mutations in their MRP2 gene (Paulusma et al., 1997; Trauner et al., 1997; Jedlitschky et al., 1997; Toh et al., 1999; Buchler et al., 1996) indicating that this protein is required for the excretion of a wide range of conjugated organic anions including bilirubin glucuronides into the bile (Paulusma and Oude Elferink, 1997; Kartenbeck et al., 1996; Suzuki and Sugiyama, 1998).

MRP2 shows close resemblance to MRP1 in terms of structure and substrate specificity. Using intact cell and in vitro models it was demonstrated that MRP2 performs an ATP-dependent, primary active transport of the glutathione GS-conjugate leukotriene C4 and of various glutathione, sulfate, and glucuronide conjugates (Jedlitschky et al., 1996, 1997). It has also been demonstrated to transport unconjugated xenobiotics, such as vinblastine and sulfinpyrazone in association with GSH (Evers et al., 1998, 2000; Van Bladeren, 2000; Glatt, 2000; Ritter, 2000; Paumi et al., 2001). It has been suggested that MRP2, like MRP1 is capable of transporting GSH, either alone or together with other substrates. Paulusma et al. (1999) demonstrated a role for MRP2 in the excretion of GSH both in vivo and in vitro. In rats heterozygous for a MRP2 mutation (TR/tr) biliary excretion of GSH was decreased to 63% of controls, and in MDCKII cells stably expressing MRP2, GSH efflux was more than 10-fold higher than in parental MDCKII cells. Competitive inhibition of ATP-dependent DNP-GS transport in membrane that vesicles isolated from MDCKII-MRP2 cells was observed by high concentrations of GSH, suggesting that MRP2 mediates low-affinity transport of GSH (Paulusma et al., 1999). This observation is in accord with the finding that suppression

of MRP2 levels by antisense in HepG2 cells elevates the intracellular concentration of GSH two-fold (Koike et al., 1997).

Compared to MRP1, MRP2 has a more limited tissue distribution. In humans MRP2 is mainly expressed in liver, kidney and small intestine (Kool et al.,1997). MRP2 is found in the canalicular membrane of hepatocytes (Paulusma et al., 1996; Buchler et al., 1996). In human and rat kidney it is expressed in the apical domain of the proximal tubule epithelia where it presumably mediates the export of conjugated organic anions into the urine (Schaub et al., 1999). Studies of rat and rabbit Mrp2 expression in small intestine showed that Mrp2 is found in the brush border membrane surface of villi (Mottino et al., 2000; van Aubel et al., 2000). Villi have an absorptive function and thus Mrp2 could prevent or modulate the passage of certain xenobiotics or their metabolites from the gut into the portal blood.

Based on its localization and substrate specificity, it is proposed that the physiological function of MRP2 is the transport of xenobiotics into bile and the lumen of excretory organs.

1.3.1.3 MRP3

MRP3 (ABCC3) is an organic anion transporter mostly expressed in liver cholangiocytes, kidney and gut (Kool et al., 1997; Scheffer et al., 2002). In addition, MRP3 is present in pancreas, adrenal gland and prostate. Within the MRP family MRP3 shares the highest amino acid identity with MRP1 (58%) and MRP2 (49%). Like MRP1, MRP3 is localized in the basolateral membranes in polarized cells (König et al., 1999b; Kool et al., 1999b).

While under normal conditions MRP3 expression is hard to detect in hepatocytes, it is strongly induced under cholestatic conditions in hepatocytes from MRP2-deficient Dubin–Johnson syndrome patients and in TR(-) rats (Kool et al., 1999b; König et al., 1999b; Hirohashi et al., 1998; Ortiz et al., 1999). It has been suggested that MRP2 and MRP3 expression may be inversely regulated in a coordinated manner (Stockel et al., 2000). MRP3 mRNA is induced by bilirubin (Ogawa et al.,

2000). It has been proposed for human (Kool et al., 1999b) and rat (Ogawa et al., 2000) that MRP3 mediates the efflux of organic anions from the hepatocyte into the blood when secretion into the bile is not sufficient due to reduced MRP2 expression. Rat Mrp3 has been demonstrated to transport some bile salts in conjugated and unconjugated form (Hirohashi, 2000); it therefore may also be involved in the recirculation of bile salts into the blood. Results obtained by Zelcer et al. (2003) supported the assumption that MRP3 may have a role in the removal of bile acids from the liver under cholestatic conditions. MRP3 transports both taurocholate and glycocholate and sulfated bile salts showed to be high-affinity competitive inhibitors of etoposide glucuronide transport by MRP3 (Zelcer et al., 2003b).

Little is known about additional factors increasing human hepatic MRP3 expression. It has been observed that MRP3 expression was induced in livers of patients treated with omeprazole and by beta-naphthoflavone in HepG2 cells (Hitzl et al., 2003).

MRP3 is expressed in the endothelial cells of human term placenta tissue, like MRP1 (St.Pierre et al., 2000). Thus MRP1 and MRP3 in this tissue may serve to prevent or limit entry of organic anions into the fetal circulation while allowing excretion of conjugated waste products into the maternal circulation.

MRP3 differs from MRP1 and MRP2 in that it appears to be unable to transport GSH (Kool et al. 1999b, Zelcer et al., 2001). This may explain why cells expressing MRP3 are not resistant to most of the anticancer drugs that are postulated to be cotransported with GSH by MRP1 and MRP2. Exceptions are the epipodophyllotoxins, etoposide and teniposide. MRP3-mediated resistance against these drugs does not require intracellular GSH, and etoposide appears to be transported by MRP3 in unmodified form (Kool et al., 1999b; Zelcer et al., 2001; Zeng et al., 1999; König et al. 1999b).

MRP3 is present in cancer cell lines from many tissues but initial studies did not suggest any association between MRP3 levels and resistance (Kool, 1997). Recently a strong correlation was found between MRP3 and doxorubicin resistance in lung cancer lines (Young et al., 1999, 2001). As MRP3 does not cause anthracycline resistance in transfected cell lines, the clinical significance of this correlation is doubtful.

1.3.1.4 MRP4 and MRP5

MRP4 and MRP5 are „short“ MRPs that lack the TMD0 N-terminal domain present in MRP1 (fig. 1.3). They share only 36% sequence identity, even although they transport similar substrates.

The tissue distribution of MRP4 and MRP5 is not well defined at the moment. Recent studies suggest that MRP4 is highly expressed in kidney and prostate (Lee et al., 2000). Subcellular localization of MRP4 is still controversial Lee et al. (2000) analyzed transfected cells and prostate tissue immunohistochemically to determine the subcellular localization of MRP4. They detected MRP4 in the basolateral membrane of the acinar cells in the prostate. Recently, van Aubel et al. (2002) reported that MRP4 is localized in the apical membrane of the proximal tubule of the human kidney. In contrast to this observation, Lai and Tan (2002) recently localized MRP4 by immunocytochemistry in the basolateral membrane of polarized MDCKII cells. MRP5 is ubiquitously expressed, with highest levels in skeletal muscle and brain (Kool et al., 1997; McAleer et al., 1999; Belinsky et al., 1998; Zhang et al., 2000). In polarized MDCKII cells, MRP5 is localized in the basolateral membrane (Wijnholds et al., 2000b).

Overexpression of MRP4 and MRP5 has not been detected in drug resistant cell lines suggesting that their role in ‘classical’ drug resistance is limited (Kool et al., 1997, 1999a). They confer a unique drug resistant phenotype transporting cyclic nucleotides and nucleotide analogs, a class of amphipathic conjugates apparently not transported by MRP1–3 or 6. The rate of cyclic nucleotide transport by these transporters is low and the physiological role of this transport remains to be defined.

MRP4 overexpression was demonstrated to confer resistance to the antiretroviral compound 9-(2-phosphonyl methoxyethyl) adenine (PMEA) and to azidothymidine monophosphate (AZT-MP) but much less to other nucleoside analogs used in antiviral therapy (Schuetz et al., 1999). It has been proposed that MRP4 modulates the cellular response to ganciclovir (GCV) (Adachi et al., 2002). MRP4 transports methotrexate

(MTX) as well as the physiological folates folic acid (FA) and N(5)-formyltetrahydrofolic acid (leucovorin)(Chen et al., 2002). It has been shown by Zelcer et al. (2003a) that bile salts, and cholestatic estrogens are substrates of MRP4, suggesting an involvement of MRP4 in transport of conjugated steroids and bile acids. The authors found also that MRP4 is able to transport dehydroepiandrosterone-3-sulfate (DHEAS), the most abundant circulating steroid in humans, made in the adrenal gland.

Both MRP4 and MRP5 transport cGMP and cAMP, with the difference that MRP4 has a higher affinity for cAMP and MRP5 for cGMP (Jedlitscky, 2000; Hopper et al., 2001). cGMP and cAMP are important second messengers activating cellular signal transduction pathways, and it has been speculated that MRP4 and MRP5 might play a regulatory role in signal transduction pathways by removing cyclic nucleotides from the cell. The observation that Silfenadil (Viagra), trequinsin, and zaprinast, which inhibit the degradation of intracellular cGMP by phosphodiesterases, have been shown to reduce MRP5-mediated transport (Jedlitscky, 2000) could support this hypothesis.

MRP4 and 5 could possibly mediate resistance to nucleobases and nucleoside analogs that are used in anticancer and antiretroviral therapies. Both MRP4 and MRP5-overexpressing cells showed low-level resistance against 6-mercaptopurine (6MP) and 6-thioguanine (TG). MRP4 and MRP5 may be involved in thiopurine resistance and pharmacokinetics in leukemia patients (Wielinga et al., 2002). No human disease has been associated with alterations in MRP5, and the Mrp5 (-/-) mice have no obvious phenotype (Wijnholds et al., 2000b).

1.3.1.5 MRP6

Human MRP6 is mainly expressed in liver and kidney (196, 294, 295). In the rat liver Mrp6 is predominantly found in the basolateral membrane of hepatocytes (Madon et al., 2000; Borst , Oude Elferink , 2002). In contrast to some other MRPs, the expression level of Mrp6 is stable. The overexpression of MRP6 in drug resistant cells is associated with co-amplification of MRP1. This may be related to the localization of the two genes closed on the same chromosome (Kool et al., 1999a).

The substrate specificity of MRP6 is still not well defined, little is known about its functional characteristics and its potential role in drug resistance. The first substrate identified by vesicular transport experiments was BQ-123, an anionic cyclopentapeptide antagonist of the endothelin A receptor (Madon et al., 2000). It has recently been shown that expression of MRP6 is also associated with MgATP-dependent transport of glutathione *S*-conjugates like leukotriene C4, S-(2,4-dinitrophenyl) glutathione and N-ethylmaleimide S-glutathione (NEM-GS) but not glucuronide conjugates (Ilias et al., 2001). Additionally, MRP6-transfected cells showed low levels of resistance to natural product drugs like etoposide, teniposide, doxorubicin, and daunorubicin (Belinsky et al., 2002).

The 3' end of the MRP6 gene is amplified in leukemic cells resistant to epirubicin (Longhurst et al., 1996; O'Neill et al., 1998; Kuss et al., 1998). Resistance was initially thought to be due to a new resistance protein, called ARA, the anthracycline resistance gene. Subsequent work has shown, however, that the epirubicin resistance of cell lines with ARA gene amplification can be explained by the coamplification of the MRP1 gene together with the MRP6 gene (Kool et al., 1999a; Belinsky and Kruh, 1999).

The physiological role of MRP6 remains to be clarified. Mutations of the *MRP6* gene are associated with pseudoxanthoma elasticum (PXE), a heritable connective tissue disorder characterized by calcification of elastic fibers in skin, arteries, and retina. How mutations in MRP6 are associated with this disease is still unclear (Ringpfeil et al., 2000).

1.4 Work preceding this thesis

1.4.1 Characterization of truncation mutants of MRP1

MDR1 Pgp (ABCB1) and MRP1 (ABCC1) share a similar core structure, containing a tandem repeat of transmembrane domains (TMDs) and cytoplasmic ATP-

binding cassette (ABC) regions. However, MRP1 and several homologs form a subfamily of ABC-transporters, characterized by a triple membrane-bound domain structure. These include an MDR1-like core region, containing two ABC units and two membrane-bound domains (TMD1 and –2), and an N-terminal polypeptide region of about 280 amino acids, forming a cytoplasmic loop and a membrane bound domain (L_0 and TMD_0, respectively). To examine the role of the N-terminal regions in the function of MRP1, Bàkos et al. analyzed MRP1 variants (containing either the MDR-like core alone, called ΔMRP1, or the core plus linker L_0 called L_0ΔMRP1). It was examined whether the mutants were able to transport substrates of MRP1 by vesicle uptake experiments with the MRP1 substrates leukotriene C4 (LTC_4) and N-ethylmaleimide glutathione (NEM-GS). It was found in these studies that the mutant ΔMRP1 was inactive. By indirect immunofluorescence it was demonstrated that the expression of ΔMRP1 in polarized MDCKII was restricted to an intracellular compartment. Unexpectedly, a truncated MRP1 mutant lacking only the entire first transmembrane domain, TMD_0, but still containing L_0 (L_0ΔMRP1) behaved like wild-type MRP1 in vesicle uptake and nucleotide trapping experiments. L_0ΔMRP1 was properly routed to the lateral plasma membrane in MDCKII cells where it transported DNP-GS and daunorubicin (fig. 1.5). These results suggested that the loop L_0 forms a functional domain that is sufficient and essential for MRP1-mediated transport activity and routing to the lateral membrane in polarized cells. Interestingly, L_0 is conserved among all the members of the MRP family identified to date. It was speculated that L_0 could be required for substrate recognition, for lateral routing and/or for proper folding of the molecule. The N-terminal region TMD_0 did not demonstrate any apparent function, which is unexpected as this domain is present in other MRP homologs (MRP2, MRP3, MRP6 and MRP7). Although this region shows a low overall amino acid identity among these proteins, it is structurally conserved in evolution.

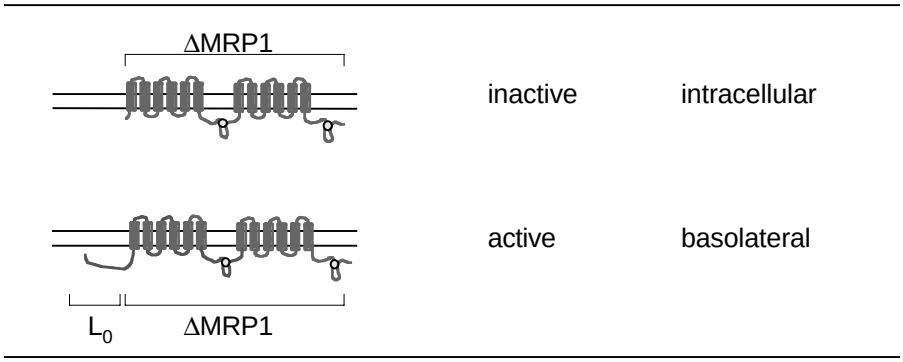


Fig. 1. 5. Schematic representation of truncated mutants of MRP1 and their characterization

To investigate whether fusing L_0 to the N-terminus of MDR1 would make MDR1 Pgp an organic anion transporter, Bàkos et al., (2000a) constructed chimeric MRP1-MDR1 mutants. All MRP1-MDR1 chimeric proteins expressed in Sf9 cells preserved the transport characteristics of wild type MDR1 and were routed to the apical membrane in MDCKII cells.

1.4.2 Identification of an amphipathic helix within the loop L_0

If the L_0 loop is co-expressed with ΔMRP1 in Sf9 cells, transport activity is restored (Bàkos et al., 2000a). *In vitro* experiments demonstrated that a significant amount of the L_0 protein was associated with the membrane fraction in vesicles. Only by washing the membranes with urea or Triton-X100 the association between L_0 and the membrane was disrupted. Secondary structure predictions indicated the presence of a conserved amphipathic helix in all currently known MRP homologues. Analysis of this region demonstrated that it is conserved among MRP1-9 and in the yeast cadmium resistance factor Ycf1 (fig. 1.6), an orthologue of human MRP1 (Tommasini et al., 1996)

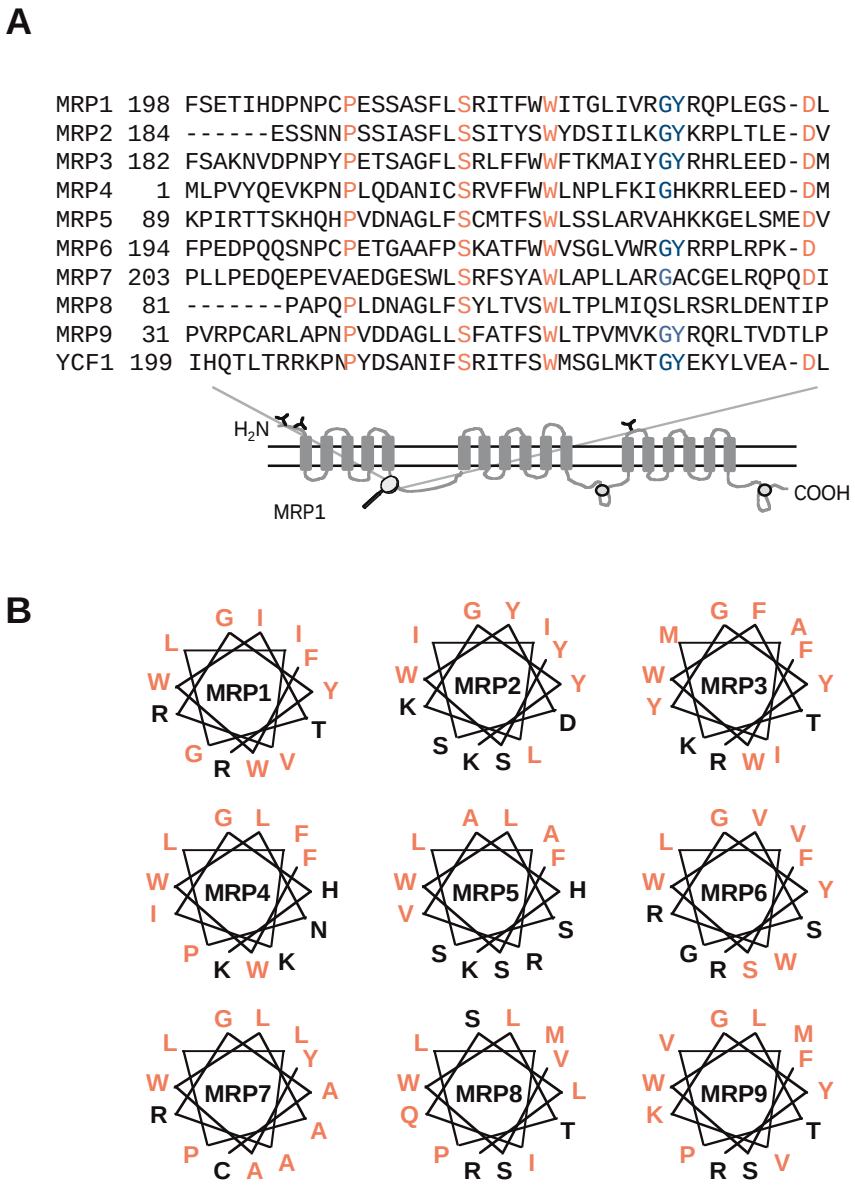


Fig. 1.6. Sequence alignments and predicted secondary structure of L_0 regions corresponding to aa 198-241 in MRP1.

A. Sequence alignments of the L_0 loop of MRP1-6 and of the yeast homologue YCF1.
B. Computer-predicted distribution of the residues in the helix in MRP1-6. Hydrophobic residues are signed in red, hydrophilic in black.

The hydrophobic (in red, fig. 1.6 panel B) and hydrophilic (in black, fig. 1.6 panel B) amino acids are distributed on opposite sides of the helix in all MRP proteins. The amphipathic helix may produce hydrophobic interactions with the lipid bilayer or with hydrophobic regions of other membrane proteins.

2 AIMS OF THIS THESIS

In previous studies by Bàkos et al., (1998) on the N-terminal domain of MRP1 it has been shown that the MDR1-like core of MRP1, called ΔMRP1, is inactive and localized in an intracellular compartment in Madin-Darby Canine Kidney (MDCKII) cells. In baculovirus infected Sf9 cells transport function could be restored by co-expressing the full N-terminal TMD_0L_0 region together with ΔMRP1. It was also found that extending the core with the N-terminal linker L_0 in the absence of the TMD_0 region resulted in an active transporter that was properly routed to the plasma membrane in MDCKII cells. To extend these previous results, this thesis describes the isolation and characterization of MDCKII cell lines stably expressing various MRP1 variants. The final aim was to investigate the role of the intracellular loop L_0 in basolateral routing and to characterize the role of this domain in MRP1-mediated drug transport in polarized MDCKII cells.

Based on computer secondary structure predictions of the loop L_0 a conserved amphipathic α-helix was identified in MRP1. An MRP1 variant was generated in which this region was deleted. An MDCKII-derived cell line stably expressing this mutant was established and the transport and routing characteristics of the mutant were investigated.

To investigate whether L_0 could rescue the transport activity and lateral routing of the intracellularly localized ΔMRP1, MDCKII cells were co-transfected with L_0 and ΔMRP1. By confocal laser microscopy (CLSM) the localization of L_0 in these cells was studied.

To investigate the N-terminal TMD_0L_0 region of MRP1 in further detail, MDCKII-derived cell lines stably expressing TMD_0L_0 alone or together with ΔMRP1 were isolated. By various experimental strategies like immunofluorescence-, Western blotting-, deglycosylation-, vectorial transport-, co-immunoprecipitation- and surface biotinylation experiments, the characteristics of MRP variants in these cell lines were studied.

To determine whether L_0 of MRP2 (an apically localized homolog of MRP1) could substitute for L_0 of MRP1, a chimeric TMD_0L_0 construct was engineered consisting of the TMD_0 of MRP1 and L_0 of MRP2. MDCKII–derived clones stably expressing the chimeric construct were established. In addition the construct was expressed in MDCKII-ΔMRP2 cells. In these cells, the chimeric protein was studied by immunofluorescence and deglysosylation experiments

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plasmids

3.1.1.1 Bacterial expression-vectors

Plasmid	Insert	Reference
pGEM®-7Zf(Promega, Madison, WI)	TMD_0(MRP1)L_0(MRP2) chimeric construct	this thesis

3.1.1.2 Eucaryotic expression-vectors

Plasmid	Insert	Reference
pCMV-neo	MRP1Δ(223-232)	cDNA cloned by Dr. R. Evers as described in Bàkos et al., 2000
pBabe-puro-CMV	L_0 fragment	Bàkos et al., 2000
pCMV-neo	TMD_0L_0 fragment	Bàkos et al., 1998
pBabe-puro-CMV	TMDo(MRP1)Lo(MRP2) chimeric construct	this thesis

3.1.2 Antibodies

3.1.2.1 First antibodies

Antibody	Specificity	Isotype	Provenience
MAb MRPr1	MRP1, internal epitope $G^{238}SDLWSLNKE^{247}$	Rat IgG2a	Dr. R. J. Scheper (Free University Hospital, Amsterdam, The Netherlands).
MAb MRPm6	MRP1, internal epitope $P^{1511}SDLLQQRGL^{1520}$	Mouse IgG1	Dr. R. J. Scheper (Free University Hospital, Amsterdam, The Netherlands).
Mab QCRL3	MRP1,conformational epitope aa 617-932	Mouse IgG2a	ALEXIS Biochemicals Deutschland (Grünberg, D)
MAb M_2I-4	MRP2, internal epitope	Mouse IgG1	Dr. R. J. Scheper (Free University Hospital, Amsterdam, The Netherlands).

3.1.2.2 Secondary antibodies

Antibody		Developed in	Provenience
Anti- mouse IgG	Peroxidase conjugate	goat	Sigma (Saint-Louis, Missouri, USA
Anti- mouse IgG	Peroxidase conjugate	goat	Zymed laboratories (San Francisco, CA, USA)
Anti- mouse IgG	FITC conjugate	sheep	Roche Molecular Biochemicals, (Mannheim, Germany)

Anti- rat IgG	Peroxidase conjugate	goat	Zymed laboratories (San Francisco, CA, USA)
Anti- rat IgG	FITC conjugate	goat	Molecular Probes (Eugene, OR, USA)

3.1.3 Primers

Tos 1

5´AACGGATCCGTGCCCGCCGCCGCCCGCGCCACCGGC3´

Tos2

5´GAATGAAGCTATGGATGATGGATTATTTGATGACTCGTGGATG GTTTTCCGAGAACAGGGGTGAGCGATCTGA3´

Tos3

5´TCAGATCGCTCACCCCTGTTCTCGGAAACCATCCACGAG TCATCAAATAATCCATCATCCATAGCTTCATTC3´

Tos 4

5´ATCGGATCCTTACTGCTGGGAGCTCTTCTCCTGCCGTCTCTG3´

3.1.4 *E. Coli* Competent cells

Strain	Genotype
DH5α	*supE44, delta lacU169 (phi 80 lacZ delta M15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1*

Transformed bacteria were incubated over-night at 37°C and 200 rpm in LB medium with 100µg/ml ampicillin. Bacteria were stored at –80°C in 50% LB medium and 50% glycerin.

3.1.5 Cell lines and media

Cell line	Provenience
MDCKI	Madin Darby canine kidney type II (MDCKII) epithelial cells
Phoenix	HEK293T human embryonic kidney cell line

Cells were cultured at 37°C and 5% CO_2. MDCKII was cultured in DMEM medium (Gibco BRL, Life Technologies, Paisley, PA) containing 2mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% of heat inactivated (30 min 56°C) FCS. Cells were subcultured each 3-4 days by incubation with trypsine (15-30 min, 37 °C) after washing with PBS. Phoenix cells were cultured in ISCOVES Dulbecco modified medium (Gibco BRL, Life Technologies, Paisley, PA)

supplemented as described for DMEM. They were subcultured each 3-4 days by incubation with trypsine (1 min, 37 °C).

To be stored cells were trypsinized and resuspended DMEM medium (0,5 ml/aliquot). An equal amount of freezing medium (60% DMEM, 20% fetal calf serum and 20% DMSO) was added slowly to avoid osmotic shock and to minimize the heating that occurs when DMSO is added to the aqueous solution. Cells were stored for 24 hour at –80°C before being transferred in liquid nitrogen.

3.1.6 Solutions and buffers

DNA-gel sample buffer	Ficoll	25%
	SDS	0.5%
	EDTA pH 8	5 mM
	Tris-Cl pH 8	20 mM
	orange G	0.8%
PBS (1x)	NaCl	137 mM
	KCl	2,7 mM
	Na2 HPO4	4,3 mM
	KH2 PO4	1,4 mM
TE (1x)	Tris-HCl pH 7,4	10 mM
	EDTA	1 mM
TAE (1x)	Tris-Acetat	40 mM
	EDTA	1 mM
DNA-running buffer	TAE	1x
	Ethidium bromide	0,5 mg/l

Ligase Mix (10x)	Tris-HCl pH 7,4	0,5 M
	$MgCl_2$	0,1 M
	DTT	0,1 M
PBS C/M	PBS	1x
	$CaCl_2$	1 mM
	$MgCl_2$	0,5 mM
HBSS (10x)	KCl	0,05 M
	KH_2PO_4	4 mM
	$MgCl_2$. $6H_2O$	5 mM
	$MgSO_4$. $7H_2O$	4 mM
	NaCl	1,3 M
	Na_2HPO_4	0,3 M
	HEPES	0,1M
	Glucose	0,05 M
	$NaHCO_3$	4,2 mM
Lysis buffer	KCl	10 mM
	$MgCl_2$	1,5 mM
	Tris-HCl pH 7,4	10 mM
	SDS	2%
	Leupeptin	1 µg/ml
	Pepstatin	1 µg/ml
	Aprotinin	1 µg/ml
SDS sample buffer (2x)	SDS	4%
	Tris HCl pH 6,8	0,1M
	Glycerin	13%

	mercapto-ethanol	0,6%
	Bromophenol blue	0,6%
SDS running buffer	SDS	0,15%
	Tris	0,05 M
	glycine	0,2 M
Transfer buffer	Tris	0,025 M
	glycine	0,2 M
	methanol	20%
ECL soulution	sodium luminol	1,25 mM
	H_2O_2	9,3%
	Tris-HCl pH 8,6	0,1 M

3.1.7 General materials and chemicals

Basic chemicals were from Roth (Karlsruhe, Germany), Merck & Co. Inc (USA) and Sigma-Aldrich. (Saint-Louis, Missouri, USA). Restriction enzymes as well as further enzymes for molecular biology are from New England BioLabs and MBI Fermentas Molecular Biology, (Heidelberg, Germany). Protein molecular ladders were from Bio-Rad (Hercules, CA, USA) and Gibco BRL Life Technology (Paisley PA). Double well plates for immunohistochemistry and transport experiments were Transwell™ 3414 (Costar) plates. Other plastic materials for cell culture were from Greiner (Sigma-Aldrich, Saint-Louis, Missouri, USA), Corning/Costar (Myriad Industries, San Diego, CA), Becton Dickinson (Franklin Lakes, NJ) or Schleicher & Schuell (Traben Trarbach, Germany). Radioactive inulin, daunorubicin and CDNB were from Amersham Pharmacia Biothec (Buckinghamshire, England)

3.2 Molecular biological methods

3.2.1 Polymerase chain reaction

Specific DNA fragments were amplified by PCR. The polymerase chain reaction was conduced in a total volume of 50 µl containing 10 mM dNTPs, 50 mM $MgSO_4$, 10 pmol/µl primers, 200 ng DNA template, 1-2 U Pfx polymerase (Gibco BRL, Life Technologies, Paisley PA), and 5 µl 10x Pfx buffer provided from the manufacturer. After initial DNA denaturation (94 °C for 5 min) 25 cycles were performed, consisting of 1 min at 94 (denaturation step), 60°C for 1 min (annealing step), 78°C for 1 min (elongation step). At the end cycles were followed by an incubation of 5 min at 78 °C to allow complete elongation of all DNA products. PCR products were isolated by agarose-gel electrophoresis size fractionation, and fragments were purified with a JETSORB Gel Kit (Genomed, Bad Oeynhausen, Germany).

3.2.2 Enzymatic treatments of DNA

3.2.2.1 Enzymatic digestion with restriction-nucleases

0,5 µg DNA was digested by 1-10 U restriction enzymes in the reaction buffer suggested by the manufacturer (New England BioLabs, MBI Fermentas Molecular Biology, Heidelberg, Germany) at at 37°C for 1 hour, as indicated by the manufacturer.

2.2.2.2 Calf Intestinal Alkaline Phosphatase (CIP) reaction

Alkaline phosphatase catalyses the removal of 5' phosphate residues from DNA to prevent self-ligation. DNA was suspended in the reaction buffer provided by the

manufacturer and 0,5-1U CIP (Roche Molecular Biochemicals, Mannheim, Germany) were added. The reaction was incubated 15 minutes at 37°C.

3.2.2.2 Ligation of DNA fragments

For ligating DNA fragments into plasmid vectors 4 to 1 fold molar excess of insert fragment to vector was used. Twenty units T4-DNA-ligase (New England BioLabs) and 1 µl of 10 mM ATP were added to 10 µl of 1x ligase Mix (Tris-HCl pH 7,4 0,5 M, $MgCl_2$ 0,1 M, DTT 0,1M). The reaction was incubated for 12-16 hours at 16°C.

3.2.3 Separation of DNA fragments by agarose gel electrophoresis

Agarose gel electrophoresis was used to size fractionate DNA molecules. 0,7 to 1% agarose is suspended in 1X TAE containing 1 µg/ml ethidium bromide for visualization of nucleic acids. Ethidium bromide is an intercalating dye, which binds to the DNA and fluoresces under UV light. 0,7 to 1% agarose gels can separate DNA fragments from 0,5-6 Kb in size. Electrophoresis occurred in TEA buffer. DNA was visualized under UV light at 366 nm.

3.2.4 Cloning of MRP1 and MRP1/MRP2 variants in expression vectors

For expression of the MRP1 variants MRP1Δ(223-232) and TMD_0L_0 in kidney-derived MDCKII cells, cDNAs were inserted as blunt-end fragments into the retroviral vector pCMV-neo (Bàkos et al., 1998, 2000).

The L_0 fragmente was cloned in the pBabe-puro-CMV vector. This vector was generated by cloning the blunt-ended HindIII fragment (Bàkos et al., 2000).

TMD_0(MRP1)L_0(MRP2) was generated by overlap PCR as described in 3.6.1, and the fragments were verified by sequence analysis. The amplified overlap product was digested with *Bam HI* and was inserted by ligation into the *Bam HI* digested cloning vector pGEM®-7Zf, codifying for resistance to ampicillin, (Promega, Madison, WI).

Competent *E. Coli* cells from the strain DH5α were transformed with the plasmid. The plasmidic DNA was isolated from a positive clone, purified in agarose gel and extracted in significant quantity by midi preparation. From pGEM®-7Zf the construct was cloned into the *Bam*HI digested site of the pBabe-puro-CMV vector.

Plasmids are shown in 2.5.

3.2.5 Transformation of competent cells

E. Coli DH5α cells were transformed with ligation mixtures in order to select and amplify plasmids containing recombinant DNA fragments.

3.2.5.1 Preparation of competent cells (DH5α)

Solution 1	100 mM $RbCl_2$, 50 mM $MnCl_2$, 30 mM KaAc, 10 mM $CaCl_2$, 13% Glycerin (v/v) pH 5,8
Solution 2	10 mM MOPS (pH 7,0), 10 mM $RbCl_2$, 75 mM $CaCl_2$, 13 % Glycerin (v/v) pH 7,0

A single colony of *E. Coli* was inoculated in 5 ml of LB broth without ampicillin and incubated over night at 37°C. The culture was grown to OD_{600} = 0,5. Cells were centrifuged and the pellet was suspended in 200 ml solution 1 and incubated on ice for 2 hours. After the incubation the cells were collected by centrifugation for 10 min, 4°C,

2000 rpm and resuspended in 15 ml of solution 2. Hundred µl cell aliquots were frozen in liquid nitrogen and stored at –80°C.

3.2.5.2 Transformation of competent cells

Ten µg of DNA were added to a 100 µl aliquot of competent bacteria thawed on ice. To allow the DNA to enter the cells, they were incubated on ice for 30 min. The bacteria were heat-shocked for 90 seconds at 42°C and cooled on ice for 30 seconds. Subsequently 1 ml of LB medium was added and the cells were incubated at 37°C for 60 min. A 100 µl aliquot was plated on an LB Amp agar plate (100µg/ml ampicillin) and incubated over night at 37°C.

3.2.6 Isolation of plasmid DNA from *E. Coli*

3.2.6.1 Mini preparation

Buffer P1	50 mM Tris-Cl pH 8, 10mM EDTA, 100g/ml RNase A
Buffer P2	200mM NaOH, 1% SDS
Buffer P3	3M potassium acetate, pH 5,5

Single cultures of transformed bacteria were amplified over night in 5 ml LB containing 100µg/ml ampicillin. The bacterial pellet was collected by centrifugation at 3000 rpm for 10 min. The pellet was resuspended in 100 µl buffer P1 and incubated on ice 200 µl of buffer P2 was added. After incubating for 5 minutes at 4 °C, 100 µl of buffer P3 were added. Lysates were centrifuged at 13000 rpm for 5 minutes at room

temperature and the supernatant was added to 800 µl of ethanol (100%). DNA was precipitated by centrifugation at 13000 rpm for 10 minutes at room temperature. 300 µl of cold ethanol (70%) were added to the pellet and it was centrifuged at 13000 rpm for 5 minutes at room temperature. After drying of the pellet, it was resuspended in water (30 u/µl).

3.2.6.2 Midi preparation

Higher amounts of plasmid DNA were obtained from amplification of 100 ml of bacterial cultures using a JETSTAR Plasmid Kit (Genomed, Bad Oeynhausen, Germany) following the protocol provided by the manufacturer.

3.2.6.3 Determination of DNA concentration

By samples without significant contamination, the absorption of ultra-violet (UV) light by the ring structure of purines and pyrimidines can be used to measure the amount of DNA. A DNA sample was tested in a spectrophotometer for absorbance at wavelengths of 260 nm and 280 nm, where OD_{260} measures nucleic acids and OD_{280} proteins. The concentration can be calculated based on an OD of 1 at 260 nm corresponding to 50 µg/ml for double stranded DNA. The purity of the DNA solution can be estimated using the ratio of OD readings at 260nm and 280nm. Pure preparations of DNA have an OD_{260}/OD_{280} ratio of 1,8.

3.3 Cell culture methods

3.3.1 Transfection of Phoenix packaging cells and collection of the viral supernatant

24 hours before transfection cells were subcultured 1:7 in 10 cm diameter tissue culture plates. Approximately 3 hours before transfection medium was replaced. Cells were transfected with Calcium Phosphate using a kit from Gibco BRL, (Gibco BRL Life Technologies, Paisley PA).

After over night incubation, medium was changed. After 24 hours the medium containing the virus released by the Phoenix cells was removed, centrifuged at 1500 rpm for 5 min, divided into 1 ml aliquots and stored at –80°C. Cells were washed in cold (4°C) PBS and scraped from the plate. After washing two times with PBS the cells were resuspended in lysis buffer containing SDS. Protein lysates were analyzed by Western blotting.

3.3.2 Retroviral transduction of MDCKII cells

MDCKII cells grown to confluence in monolayer were subcultured 1:10 in a T25 flask. The next day 1 ml of viral supernatant, 1,15 ml DMEM and 10 µl DOTAP (Roche Molecular Biochemicals, Mannheim, Germany) were added. After 3-4 hours of incubation at 37°C, 1,5 ml DMEM were added. The day after transduction medium was replaced. The next day cells were diluted 1:8, 1:16, 1:32, 1:64, 1:128 in 10 cm Ø diameter tissue culture plates. Cells were selected in medium containing 3 µg/ml puromycin for 4-6 days or 800 µg/ml gentamycin (G-418) for 7 days. After selection positive clones were isolated and identified by Western blotting.

3.3.3 Analysis of MDCKII monolayers by immunofluorescence

For identification of the proteins by immunocytochemistry cells were grown on microporous polycarbonate filters (3µm pore size, 24,5 mm diameter) at a density of $4*10^5$ cells/well to confluence (for 3 days). Medium was changed every day, and was replaced 1 hour before starting the experiment.

Cells were fixed in 3% formaldehyde in PBS for 10 min at RT. To allow entrance of the monoclonal antibodies, cells were permeabilized with 0,1% Triton X-100 in PBS for 5 min. Cells were incubated for 2 hours at RT with the first mAb diluted 1:100 in 3% BSA in PBS; subsequently with FITC labeled secondary antibody diluted 1:50 in 3% BSA in PBS (anti-rat for MRPr1, anti mouse for MRPm6, QCRL3 and M_2I4) for 1 hour in the dark. After each step cells were washed two times 5 min with PBS C/M. Filters were placed on microscope slides. A drop Vectashield Mounting Medium (H-1000) (VECTORlabs, Burlingame, CA) containing 1µg/ml propidium iodide was added. Propidium iodide is a membrane-impermeant dye that stains by intercalating into nucleic acid molecules. Monolayers were viewed by confocal laser scanning microscopy.

3.3.4 Transport essays with MDCKII cells

3.3.4.1 Transport DNP-GS by MDCKII monolayers

Cells were seeded at a density of $16*10^5$/well on double well plates (Costar) and grown to confluence (for 3 days). Medium was changed every day and was replaced 1 hour before starting the experiment. Export of radioactive dinitro-phenyl-glutathione ([^{14}C]DNP-GS) from cells was determined by incubating cells with 1-chloro-2,4-dinitro[^{14}C]benzene (10 Ci/mol) (CDNB) (Amersham Pharmacia Biothec, Buckinghamshire, England). [^{14}C]CDNB was dissolved in dimethyl sulfoxide to a concentration of 10 mM and diluted in HBSS (Hanks buffered saline solution) to a

concentration of 2 µM just before the start of the experiment. 2 ml of medium containing [^{14}C]CDNB were applied to both the apical and basal compartment of the monolayer and 200 µl aliquots were taken at various time points (t = 1, 3, 6, 12, 20 min). Incubations were done at RT. Samples were immediately extracted with 200 µl of ethyl acetate and centrifuged for 2 min at 12000 rpm. Radioactivity in 160 µl samples of the hydrophobic phase was determined by liquid scintillation counting. The amount of radioactivity was corrected for the decrease in volume of the culture medium. To determine [^{14}C]DNP-GS accumulated in the cell monolayer, cells were washed with cold PBS, filters were cut from the plate and counted directly in liquid scintillation fluid.

3.3.4.2 Transport of [^{3}H]Daunorubicin by MDCKII monolayers

Cells were seeded on microporous polycarbonate membrane filters and grown to confluence (see above). One hour before starting the experiment monolayers were preincubated with DMEM containing 0,1 µM PSC833 to inhibit endogenous canine MDR1 Pgp (Evers et al., 1998). The experiment was started (t = 0) by replacing the medium at either the apical or the basal side of the cell monolayer with 2 ml of complete medium containing 2 µM of [^{3}H]Daunorubicin (4 µCi/ml) (Amersham Pharmacia Biothech Buckinghamshire, England), [^{14}C]-labeled inulin (0.025 µCi/ml, 4.2 µM) (Amersham Pharmacia Biothec, Buckinghamshire, England), and 0,1 µM PSC833. The cells were incubated at 37°C in 5% CO_2 and 50 µl aliquots were taken from each compartment at various time points (t = 1, 2, 3, 4 h). Radioactivity was measured as the fraction of total radioactivity added at the beginning of the experiment. The paracellular flux was monitored by the appearance of inulin [^{14}C]carboxylic acid in the opposite compartment. Radioactivity was determined by liquid scintillation counting.

3.3.5 Cell surface biotinylation essays

3.3.5.1 Selective cell surface biotinylation – detection by confocal laser scanning microscopy

MDCKII cells were seeded and grown to confluence as described in 2.3.3. The monolayers were treated at either the apical or the basolateral compartment with 10mM sodium periodate ($NaIO_4$) (freshly prepared) for 30 min at 4°C allowing oxidation of sugar residues. After washing two times for 5 min with PBS C/M, surface proteins were labeled with 2 mM EZ-Link™ Biotin Hydrazide (Pierce, Rockford, IL) dissolved in sodium acetate pH 5,5 (freshly prepared) for 30 min at 4°C. After washing, cells were fixed with formaldehyde (3% in PBS) for 10 min at RT, washed and labeled for 15 min in the dark with Fluorescein Avidin DCS (VECTOR labs, Burlingame, CA). Filters were placed on microscope slides and mounted as described above.

3.3.5.2 Cell surface biotinylation – precipitation of biotinylated proteins

MDCKII cells were seeded at a density of $16*10^5$ in 6 well plates and grown to confluence for 3 days. The cell surface was incubated with 10mM sodium periodate ($NaIO_4$) (freshly prepared) for 15 min at 4°C to allow oxidation. After washing two times for 5 min with PBS C/M surface proteins were labeled with 2 mM EZ-Link™ Biotin Hydrazide (Pierce, Rockford, IL) in sodium acetate, pH 5,5 (fresh) for 30 min at 4°C. UltraLink Immobilized Monomeric Avidin (Pierce, Rockford, IL) were pretreated 2 mM biotin in PBS for 10 min, washed twice with 0,1 M glycine pH2,8 and two times with PBS. After biotinylation of the cells, plates were scraped with 200 µl of lysis buffer (50mM Tris-Cl pH8, 150mM NaCl, 1% NP 40, pepstatin, aprotinin, leupeptin each 1µg/ml). Insoluble material was pelleted spinning the lysates at 13000 rpm. A 20µl

sample was taken for Western blot analysis. The volume of the supernatant was brought to 950 µl with lysis buffer and 50 µl of the pretreated beads were added. After over-night incubation at 4°C with end-over-end rotation beads were collected by brief centrifugation for 30 seconds at 14000g. After washing the beads, bound material was eluted using 2 mM biotin PBS and analyzed by Western blot.

3.3.6 Membrane isolation from MDCKII cells

MDCKII cells were n grown to confluence, washed in PBS (37°C), scraped and centrifuged at 1200 rpm for 5 min. After washing the pellet 2 times with PBS, it was resuspended in hypotonic lysis buffer (20mM Tris-Hepes pH 7,4, 1 mM PMSF, aprotinin, pepstatin, leupeptin, each 1µg/ml) and left on ice for 30 min The pellet was homogenized in Dounce homogenizer and centrifuged at 2500 rpm, 5 min, 4°C. Cell lysate was loaded on sucrose cushion (38% sucrose in 20 mM Tris-Hepes pH 7,4, 1 mM EDTA) and centrifuged in ultracentrifuge at 30000 rpm for 2h at 4°C. The membrane containing band was removed, diluted in TS buffer (10 mMTris-Cl pH 7,4, 250 mM sucrose) and centrifuged at 30000 rpm, for 30 min, at 4°C. The pellet was resuspended in PBS containing protease inhibitors.

3.4 Protein analysis

3.4.1 Cell lysates

After trypsinization cells were pelleted by centrifugation at 2000 rpm for 5 min and washed two times with PBS. Pellet was resuspended in lysis buffer containing 1mM PMSF (phenyl methyl sulfonyl fluoride), 2% SDS and aprotinin, pepstatin, leupeptin (each 1µg/ml) and mechanically disrupted by sonification.

3.4.2 Determination of protein concentration

The protein concentration in cell lysates was determined using the Roti-Quant (Roth, Karlsruhe, Germany) kit based on the Bradford method. The colorimetric method is based on the proportional binding of Coomassie to proteins. Coomassie absorbs at 595 nm. The protein concentration of a test sample is determined by comparison to that of a series of protein standards (BSA) known to reproducibly exhibit a linear absorbance profile in this assay. Eight hundred µl of the essay reagent and 200 µl of water were mixed to each of four standard solutions of BSA (0, 2, 8, 12, 20 µg/ml) and to 2µl of protein sample (cell lysate). After incubation at room temperature for 5-30 min, the absorbance of each sample at 595 nm was measured.

3.4.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Stacking gel	Polyacrylamide	5%
	Tris-HCl pH 6,8	0,06 M
	APS	3 mM
	SDS	0,1%
	TEMED	0,03%
Resolving gel	Polyacrylamide	7,5/11%
	Tris-HCl pH 8,8	0,375 M
	APS	3 mM
	SDS	0,1%
	TEMED	0,06%

SDS-PAGE permits separation of proteins under denaturing conditions. When an electric current is applied to the gel, protein migration is determined by their molecular weight. Cell lysates were diluted in sample buffer 1:1 and loaded onto a polyacrylamide gel. The separating gel contained 7,5 or 11% polyacrylamide and the stacking gel contained 5% polyacrylamide. Gels were electrophoresed at 150 Volt for 1 hour in SDS-running buffer.

3.4.4 Western blot analysis

Ponceau S	Ponceau	0,5% (w/v)
	acetic acid	1% (v/v)
blocking milk	BSA	10% (w/v)
	milkpowder	10%(w/v)
	Tween 20	0,05% (v/v)
	PBS	1x

The proteins separated by SDS-PAGE were transferred to a solid membrane for Western blot analysis. To obtain protein transfer an electric current is applied to the gel so that the separated proteins move through the gel and onto the membrane in the same pattern as they separate on the SDS-PAGE. After electrophoresis the pre-wet (in transfer buffer) fiber pads and filter paper, the SDS gel and the nitrocellulose membrane were assembled in the transfer cassette. Transfer was conduced over-night, at 4°C, 0,1 Ampere/gel. After transfer the membrane was stained with Ponceau S for 5 minutes to visualize protein bands. The blotted nitrocellulose membrane (Schleicher & Schuell, Traben Trarbach, Germany) was blocked in freshly prepared blocking milk for 1 hour at room temperature with constant agitation. The first antibody was diluted 1:500 in blocking milk and nitrocellulose membrane was placed in the primary antibody solution and incubated for 2 hours at room temperature. The membrane was washed 3 times for

10 min at RT in blocking milk before incubating membrane in the secondary antibody coupled with horseradish peroxidase for 1 hour (dilution 1:1000). The membrane was washed as described and one time with PBS for 10 min at RT. Proteins were detected by enhanced chemiluminescence (ECL). The washed membrane was incubated with 0,125 ml luminol solution per cm^2 with 1% (v/v) enhancer solution ($5*10^{-4}$ M p-hydroxy coumaric acid in DMSO). The peroxidase oxidates luminol in presence of hydrogen peroxide. Following the reaction, luminol reaches an exited state and decays to the ground state via the emission of light that is detected by placing the blot in contact with an X-ray film (Hyperfilm, Amersham Pharmacia Biothec, Buckinghamshire, England).

3.4.5 Co-immunoprecipitation

MDCKII cells were grown to confluency, scraped and resuspended in lysis buffer (150 mM NaCl, 1mM EDTA, 50 mM Tris-Cl pH 7, 1% NP40, 10% glycerol, 1 mM PMSF, aprotinin, pepstatin, leupeptin, each 1µg/ml) on ice. Lysates were centrifuged at 2500 rpm at 4°C to pellet insoluble material After storage of the supernatant on ice for 30 min, it was centrifuged at 14000 rpm for 10 min at 4°C. A sample (10% of the total amount) was taken for Western blot analysis. Cell lysates were diluted in a detergent solution (0,1% CHAPS, 10 mM Tris-Cl, 50 mM NaCl). ΔMRP1 was immunoprecipitated using Dynabeads Protein A (Dynal Biothech, Oslo, Norway) with captured mAb QCRL3, following the procedure described by the package insert. Precipitated proteins were detected by Western blot.

3.4.6 Enzymatic deglycosylation of glycoproteins

3.4.6.1 Deglycosylation with Tunicamycin

MDCKII cells were cultured in medium containing 3µg/ml tunicamycin for 0, 12, 25 or 40 hours. After the incubation time cell lysates were prepared as described in 2.4.1 and analyzed by Western blot.

3.4.6.2 Deglycosylation with N-glycosidase F

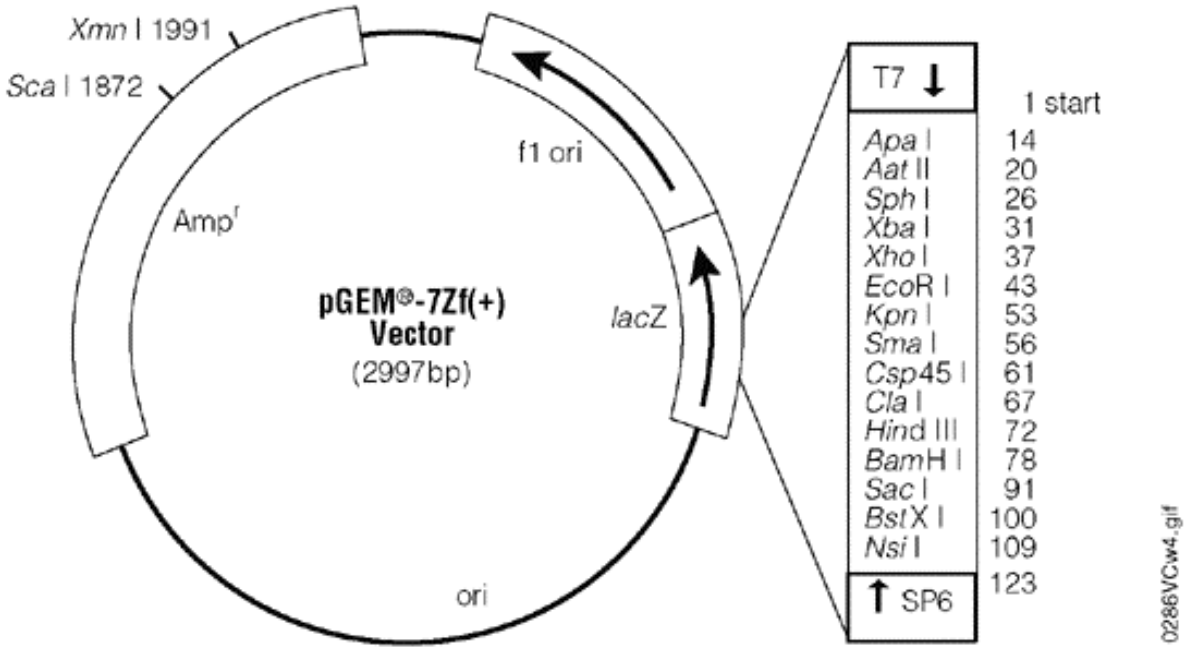
Membranes from MDCKII cells isolated as described in 2.3.6, were treated with N-glycosidase F (Roche Molecular Biochemicals, Mannheim, Germany) as indicated in the kit protocol. Samples were not incubated at 95 °C. Proteins were detected by Western blot.

3.4.6.3 Deglycosylation with Endoglycosdase H

Cell lysates prepared as described 2.4.1 in were treated with Endo H from New England BioLabs, following the procedure indicated in the kit protocol. Proteins were detected by Western blot.

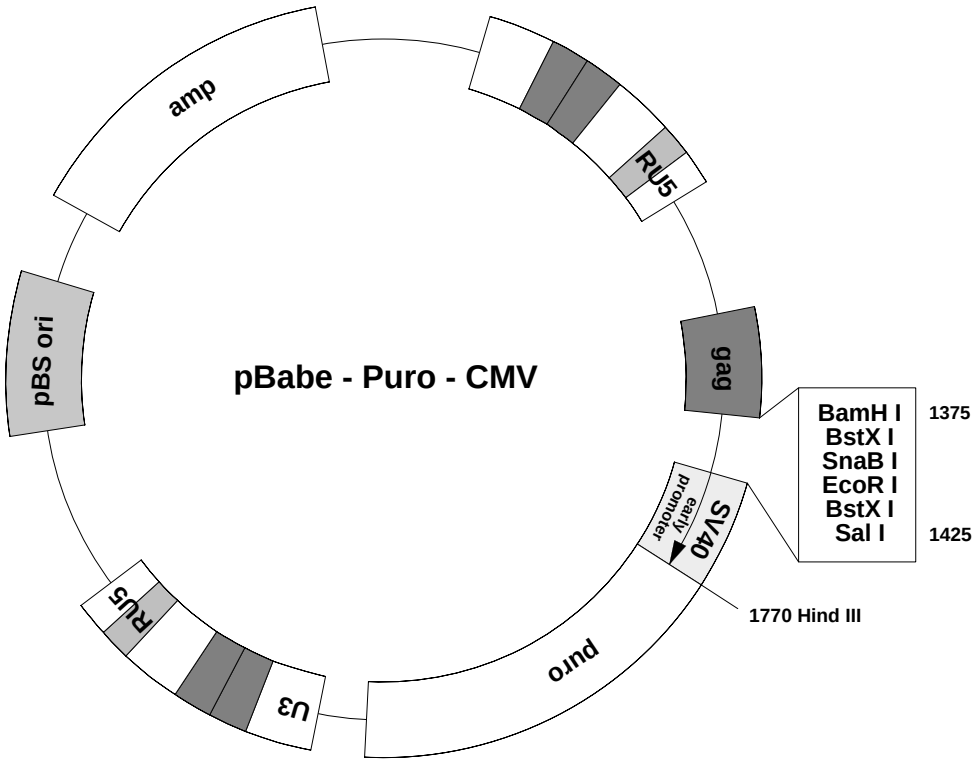
3.5 Plasmids

3.5.1 pGEM®-7Zf



Picture taken from the Promega catalog, www.promega.com

3.5.2 pBabe-puro-CMV



4 RESULTS

4.1 Monoclonal antibodies specifically recognizing human MRP1

In this thesis three different monoclonal antibodies (mAbs) were used for the detection of MRP1 and its mutant forms by Western blotting and immunofluorescence. MAb MRPr1 recognizes an internal epitope between amino acids (aa) 238 and 247 within the loop L_0. MAb MRPm6 recognizes an internal epitope between aa 1511 and 1520 at the very C-terminal part of the protein (Flens et al., 1994; Hipfner et al., 1998). MAb QCRL3Q recognizes a conformation-dependent internal epitope (aa 617-932) of human MRP1 (Hipfner et al., 1994, 1999).

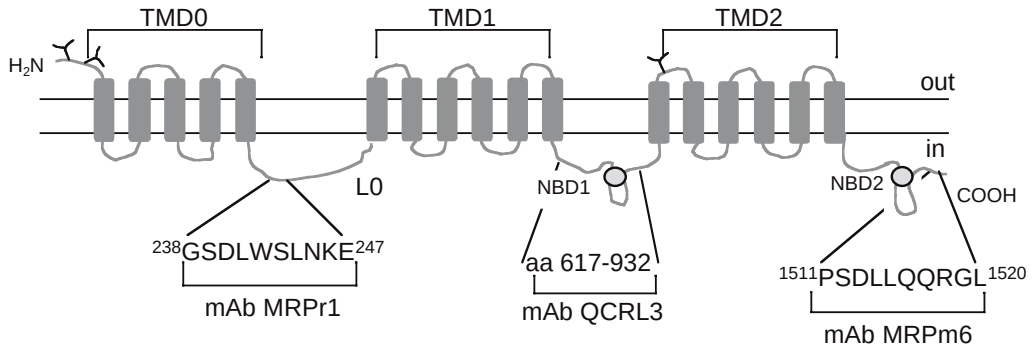


Fig. 4.1. Schematic representation of MRP1.

The epitopes recognized by the antibodies used in this thesis are indicated. Asn residues19, 23 and 1006 are N-glycosylated. Sugar residues are represented by the branched structures.

4.2 Characterization of the MDCKII-MRP1Δ(223-232) cell line

4.2.1 Expression of MRP1Δ(223-232) in MDCKII polarized cells

In order to analyze whether the amphipathic α-helix in the N-terminal linker L_0 (fig. 1.6) was essential for routing and/or function, a 10-aminoacid deletion Δ(223-232) was constructedin full length MRP1. The deletion is shown in fig. 4.2 (panel A). The construct was kindly provided by R. Evers

An MDCKII cell line stably expressing MRP1Δ(223-232) was generated by retroviral transduction. For expression MRP1Δ(223-232) cDNA was inserted as blunt-end fragment into the retroviral vector pCMV-neo (Bàkos et al., 2000).

In fig. 4.2 (panel B), a Western blot with cell lysates from MDCKII-MRP1Δ(223-232) cells is shown. To estimate the expression level of MRP1Δ(223-232), 4 and 20 µg of protein were size fractionated, and compared with the same amounts of protein from lysates from MDCKII-MRP1 and wild-type MDCKII cells. The MRP1Δ(223-232) protein was efficiently produced and detected in similar quantities as the positive control MRP1 (lanes 3 and 4 *versus* lanes 5 and 6). No signal was detected in the non-transfected cells (lanes 1 and 2).

A

MRP1 198 FSETIHDPNPCPESSASFLSRITFWWITGLIVRGYRQPLEGS-DL

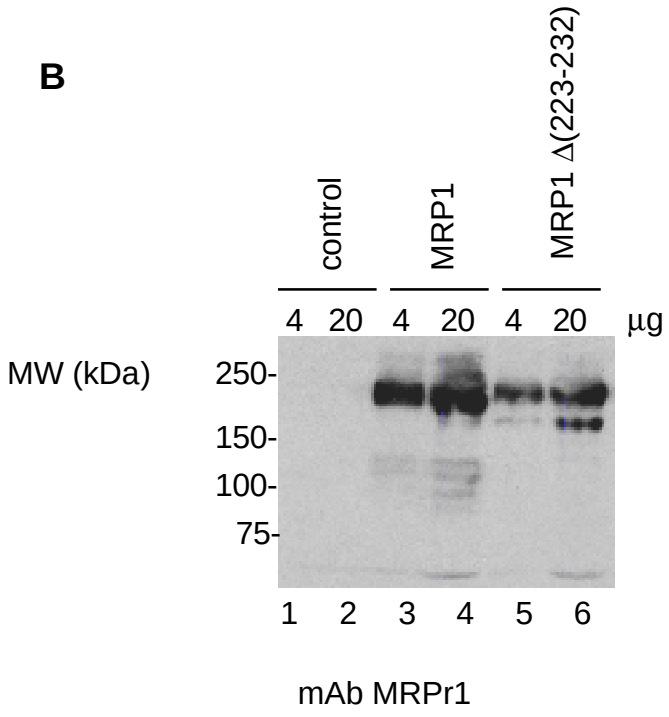


Fig 4.2 The mutation Δ(223-232) in MRP1.

A.The construct MRP1Δ(223-232) lacks the 10-amino acid long sequence between Trp223 and Tyr232 contained into the predicted amphypathic alpha helix .
B. Western blot analysis of lysates from parental MDCKII, MDCKII-MRP1and MDCKII-MRP1Δ(223-232) cells. Four and 20 µg of protein were size-fractionated. The blot was incubated with mAb MRPr1

In lysates from MDCKII-MRP1Δ(223-232) cells two bands were detected: a band with an apparent molecular weight of 190, consistent with the molecular weight of wild-type MRP1, and an additional band of about 170 kDa, which corresponds to the molecular weight of non-glycosylated MRP1 (fig. 4.2, panel B, lanes 2 and 3 *versus* lanes 5 and 6 and see below).

4.2.2 Determination of the glycosylation state of MRP1Δ(223-232) in MDCKII cells

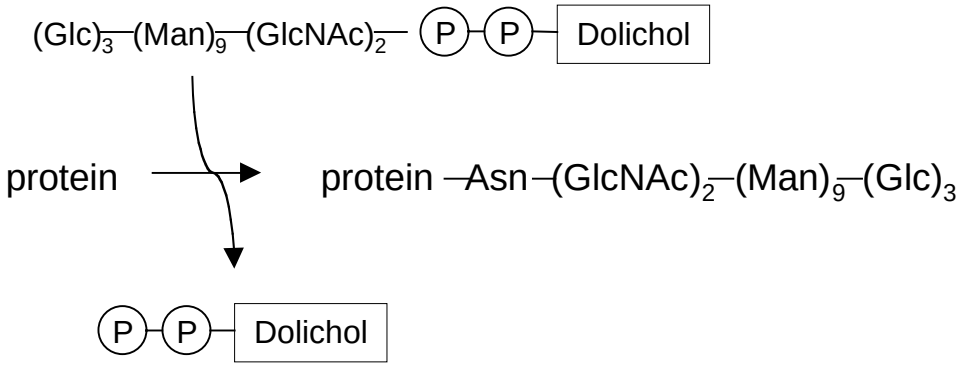
Many membrane proteins are covalently linked to oligosaccharide chains. The carbohydrate units are linked to an asparagine residue by N-glycosidic bonds or to

serine and threonine residues by O-glycosidic bonds. The asparagine residue must be part of an Asn-X-Ser of Asn-X-Thr motif and has to be sterically accessible to the transferase. In MRP1 the following three asparagine residues are known to be glycosylated: Asn 19, Asn 23 and Asn 1006 (Hipfner et al., 1997).

As shown in fig. 4.3 (panel A*)* the synthesis of the glycosidic chain starts from dolichol phosphate, a long phosphorylated complex lipid. Active monosaccharide derivatives of UDP, GDP and dolichol phosphate are added sequentially to dolichol phosphate by transferases until a 14-residue block is built.

The activated oligosaccharide core, consisting of two N-acetylglucosamine, nine mannose and three glucose residues, is attached to the nascent protein at the luminal side of the endoplasmic reticulum. Subsequent modifications will occur in the Golgi apparatus resulting in the typical core of N-linked oligosaccharide chains. The core itself will undergo further changes reaching its final sugar composition. Tunicamycin is an antibiotic inhibitor of N-glycosylation. It is an analog of UDP-N-acetylglucosamine, which competes for the binding of dolichol phosphate at the very first step of the synthesis.

A



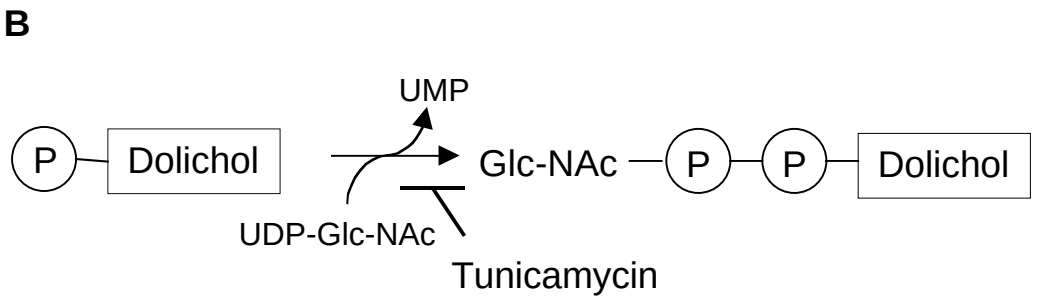


Fig. 4.3. Core glycosylation of proteins at Asn residues.

A. Mechanism of action of tunicamycin: nascent proteins are N-glycosylated by transfer of an activated oligosaccharide to an asparagine residue.
B. The first step of the synthesis of the activated oligosaccharides: the reaction of dolichol phosphate with UDP-N-acetylglucosamine. Tunicamycin is a hydrophobic analog of UDP- N-acetylglucosamine, which prevents glycosylation by acting as a competitive inhibitor.

To determine whether the MRP1Δ(223-232) protein was glycosylated, cell monolayers were treated with tunicamycin for 12, 25 and 40 hours. Fig. 4.4 shows a Western blot with lysates of MDCKII-MRP1 and MDCKII-MRP1Δ(223-232) cells prepared after treatment: a protein with an apparent molecular weight of 170 kDA was detected by Western blot analysis (lanes 2-3 and 6-8). The size of this protein corresponds to the molecular weight of non-glycosylated MRP1.

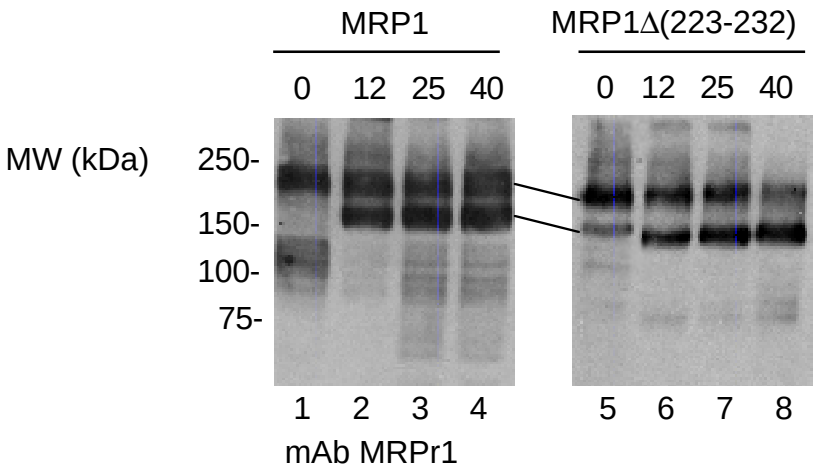


Fig. 4.4. Deglycosylation of MRP1 and ΔMRP1.

Western blot with lysates from MDCKII-MRP1 and MDCKII-MRP1Δ(223-232) cells, treated with tunicamycin (3 µg/ml) for 0, 12, 25 and 40 hours.

4.2.3 Subcellular localization of MRP1Δ(223-232) in MDCKII monolayers

By confocal laser scanning microscopy (CLSM), it was investigated if the deletion of the α-helix was affecting basolateral routing of the protein. Fig. 4.5 shows the immunolocalization by CLSM of the mutant MRP1Δ223-323 in MDCKII-derived monolayers. The upper part shows a top view of the monolayer (X/Y section), whereas the lower part shows a vertical (X/Z) laser scan perpendicular to the monolayer. Like wild-type MRP1, MRP1Δ223-323 is routed to the lateral plasma membrane. The 10 amino acid deletion in this mutant did not affect the sorting of this mutant indicating that these residues are not required for the lateral routing of MRP1.

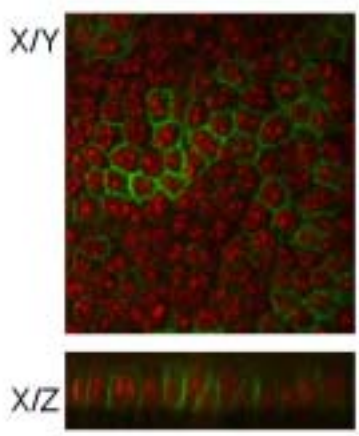


Fig. 4.5. Immunolocalization of MRP1Δ(223-232) in MDCKII monolayers.

MRP1Δ223-323 was detected by indirect immunofluorescence with mAb MRPr1 (green signal). Nucleic acids were counterstained with propidium iodide (red signal).

4.2.4 Characterization of the transport activity of MRP1Δ(223-232) in MDCKII cells

4.2.4.1 [^{14}C]DNP–GS transport in MDCKII monolayers: experimental procedure

It has been shown before that dinitro-phenyl-glutathione (DNP-GS) is transported into inside-out membrane vesicles isolated from MRP1 overexpressing cells (Jedlitschky et al., 1994; Müller et al., 1994). Subsequently, it was demonstrated that DNP-GS is a substrate for MRP1 in intact cells. This finding was useful for the development of a sensitive assay to measure the rate of MRP1-mediated transport from polarized cells overproducing MRP1 in the basolateral membrane (Evers et al., 1996). The general principle of this system is shown in fig. 4.6: Madin-Darby Canine Kidney (MDCKII) cells grow in so called double-well plates on a porous membrane where they form a tight monolayer. The porous membrane separates the culture plate into two compartments, an apical and a basolateral compartment. The cells form the physical barrier between both compartments (panel A). Typically non-metabolized drugs are added to either the apical or basolateral compartment and the amount of drug accumulating in the opposite compartment is measured. For transport experiments with chloro-dinitrobenzene (CDNB), the substrate is added to both compartments.

To measure transport of DNP-GS, the culture medium is replaced by a buffer containing radioactive [^{14}C]CDNB at t = 0. This hydrophobic compound rapidly enters the cell by diffusion. In the cytoplasm, it is metabolized to [^{14}C]dinitro-phenyl-glutathione (DNP-GS) by glutathione S-transferases (GST) (panel B). [^{14}C]DNP-GS a hydrophilic compound, which can only leave the cell by the mediation of an active transporter (panel C). Therefore, the amount of [^{14}C]DNP-GS detected in the buffer has been actively transported out of the cell. As [^{14}C]CDNB is also present in the medium both [^{14}C]DNP-GS and [^{14}C]CDNB would contribute to the total radioactivity measured. To measure only [^{14}C]DNP-GS, the two substances are separated by extraction with ethyl acetate. [^{14}C]DNP-GS remains in the aqueous phase, while [^{14}C]CDNB partitions into the hydrophobic phase. Samples of medium are taken from the apical and the

basolateral compartment at fixed time-points. The time-dependent accumulation of [^{14}C]DNP-GS in both compartments is determined.

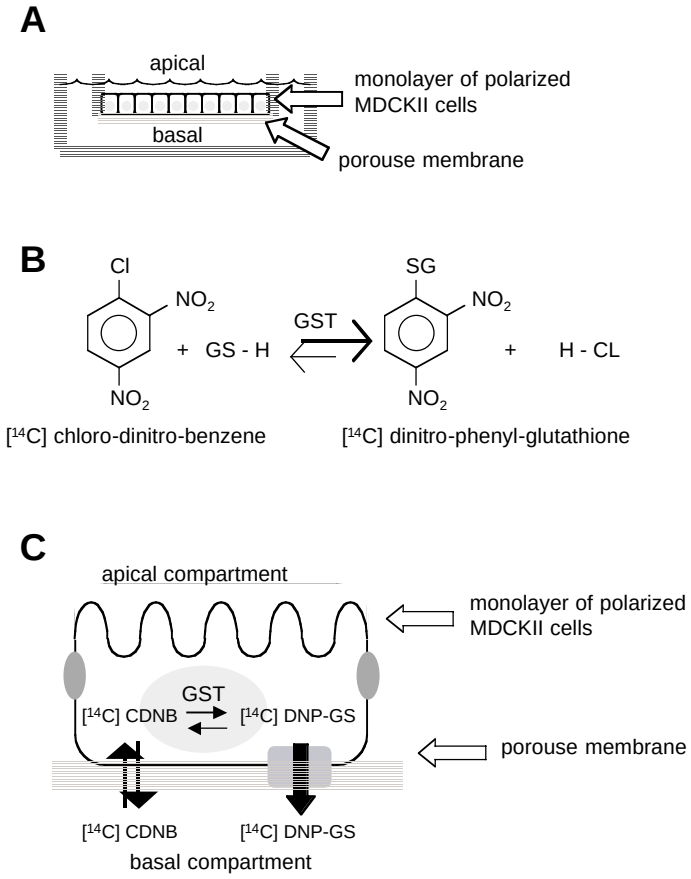


Fig. 4.6. Transport of DNP-GS in MDCKII monolayers.

A. MDCKII monolayers are grown on a double-well plate. Support is a porous membrane.
B. Radioactive [^{14}C] CDNB is added instead of the culture medium. It is metabolized to DNP-GS by cellular glutathione S-transferases (GST).
C. The exit of radioactive [^{14}C] DNP-GS is mediated by an active transporter.

4.2.4.2 Transport of [^{14}C]DNP–GS in MDCKII-MRP1Δ(223-232) monolayers

Fig. 4.7 shows the transport activity of MRP1(Δ223-323) in MDCKII monolayers. [^{14}C]DNP-GS transport was performed as described above (fig. 4.6) and the activities of MRP1 and MRP1(Δ223-323) were compared. Wild-type MDCKII cells were used as a negative control. Samples were taken at t = 0, 1, 3, 6, 12 and 20 minutes

and extracted with ethyl acetate. The experiments were performed twice in duplicate. The results clearly indicate that the deletion of amino acids 223-232 completely abolished transport activity.

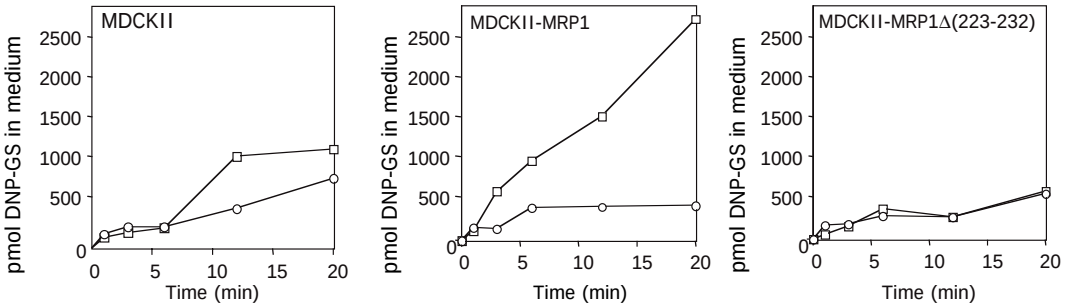


Fig. 4.7. Transport of DNP-GS by MDCKII derived monolayers.

Cells were incubated in the presence of [^{14}C]CDNB at room temperature and the amount of DNP-GS exported into the medium was determined. Squares indicate transport into the basolateral compartment, circles the transport to the apical compartment. Samples were taken at 0, 1, 3, 6 and 20 min.

4.3 Co-expression of the cytoplasmic loop L_0 with ΔMRP1 in MDCKII cells

To investigate the role of the cytoplasmic loop L_0 in more detail, L_0 was expressed together with the inactive C-terminal core of MRP1, ΔMRP1 (amino acids 280-1531). These two truncated mutants of MRP1 are shown schematically in fig. 4.8. The construct L_0 was cloned into the pBabe-puro-CMV vector, and introduced into MDCKII cells by retroviral transduction as described in 4.3.1.

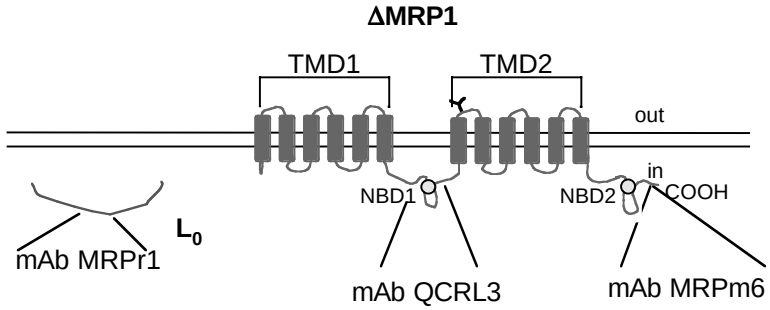


Fig. 4.8. Expression of L_0 and DMRP1 in MDCKII cells.

Schematic representation of the truncated mutants of MRP1. The intracellular loop L_0, containing the epitope for mAb MRPr1, and ΔMRP1, containing the epitopes for the monoclonal antibodies QCRL3 and MRPm6.

4.3.1 Generation of stable MDCKII cell lines expressing L_0 and ΔMRP1

Retroviruses are an efficient method to stably deliver DNA to a wide range of mammalian cell types. They integrate with high efficiency mediating stable expression. The retroviral vectors are transiently transfected into a helper packaging cell line (Phoenix cells) that produces all viral proteins required for the packaging of the retroviral DNA except the packaging signal, which is contained in the retroviral vector.

Currently available retroviral vectors for dividing mammalian cells are based on the Moloney murine leukemia virus (MMULV). Packaging cell lines are either ecotropic (capable of delivering genes to dividing murine or rat cells) or amphotropic (capable of delivering genes to dividing cells of most mammalian species, including human). The Phoenix cell lines are based on the HEK293T cell line (a human embryonic kidney cell line), which can be efficiently transfected by either calcium phosphate-mediated transfection or liposome-based transfection protocols. Fifty percentage or higher of cells can be transiently transfected. Virus producing cells continuously generate retroviral vector particles, which are released into the growth medium.

To isolate MDCKII cells stably expressing the L_0 construct I first transfected Phoenix packaging cells using the calcium phosphate technique with the pBabe-puro-CMV-L_0 vector. The supernatant from the virus producing Phoenix cells was used to retrovirally-transduce MDCKII-ΔMRP1 cells. To generate a cell line derived from one single positive cell, the density of the transduced cellular pool was reduced by serial dilution. Cells were selected with puromycin to isolate clones as the L_0 pBabe-puro-CMV vector contains the puromycin resistance gene. Isolated single resistant clones were cultured and analyzed by Western blot analysis.

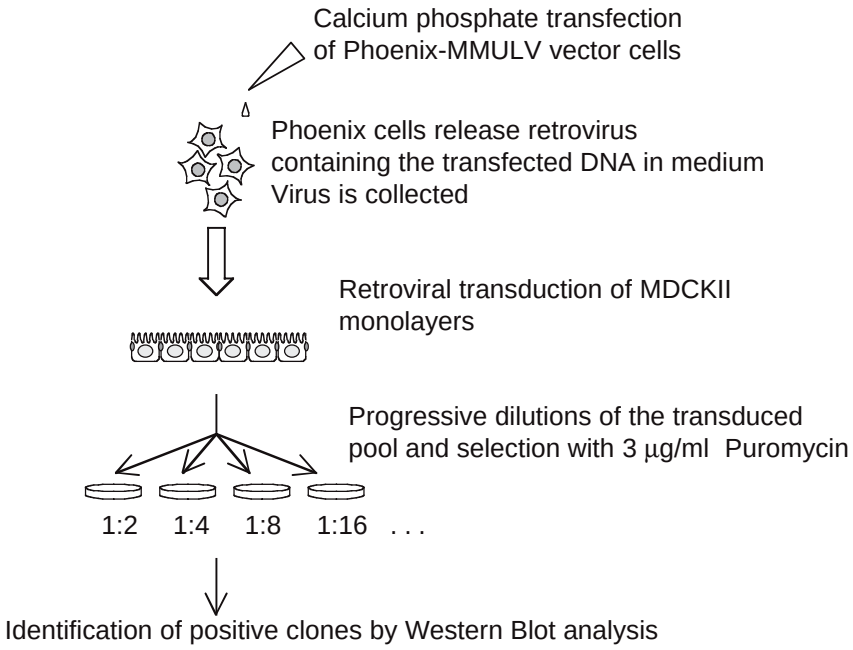


Fig. 4.9. Generation of stable MDCKII cell lines expressing TMD (MRP1)L_0(MRP2) by retroviral transduction.

Transfection of Phoenix packaging cells, collection of the retroviral supernatant, transduction of MDCKII and MDCKII-ΔMRP1 and selection with puromycin of positive clones.

4.3.2 Immunolocalization of L_0 in MDCKII-L_0+ΔMRP1 monolayers

It was found by Bàkos et al. (1998) that the presence of L_0 is necessary to route the mutant L_0ΔMRP1 to the basolateral plasma membrane. I therefore wanted to investigate whether the peptide L_0 is able to reach the plasma membrane if co-produced in MDCKII-ΔMRP1 cells.

Figure 4.10 shows the localization of the loop L_0 co-expressed with ΔMRP1 in MDCKII monolayers by CLSM. The isolated clone of MDCKII-ΔMRP1 cells expressing the loop L_0 was part of a pool of puromycin resistant cells. Clear basolateral localization of L_0 was detected in small groups of cells. Since only a small percentage of cells of the pool showed staining with mAb MRPr1 it was impossible to isolate single clones. Consequentially I could not perform Western blot analysis or transport experiments. L_0 was not detectable in wild-type MDCKII cells retrovirally-transduced with the L_0 construct. Probably the peptide is not stable and rapidly degraded.

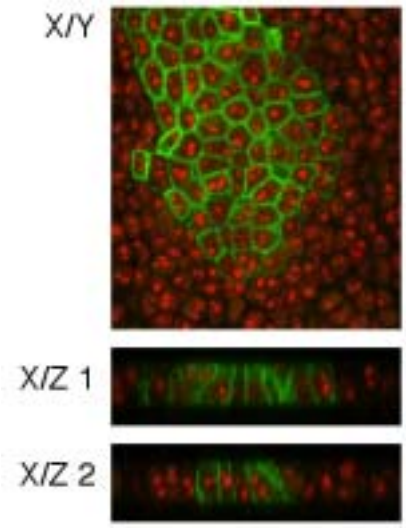


Fig. 4.10. Immunolocalization of L_0 in MDCKII monolayers by confocal laser scanning microscopy.

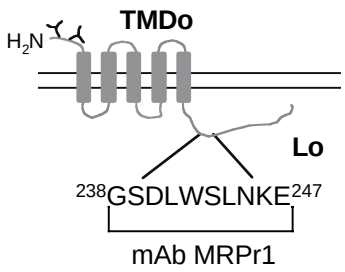
The upper picture represents a top view of the monolayer (X/Y), the lower pictures are vertical sections of the monolayers (X/Z). Nuclei were counterstained with propidium iodide (red signal). L_0 was detected with mAb MRPr1 (green signal).

4.4 Characterization of the N-terminal region of MRP1 (TMD_0L_0) in MDCKII polarized cells

4.4.1 Isolation of stable MDCKII clones expressing TMD_0L_0

As it was not possible to isolate a cell line stably expressing the loop L_0 alone, I selected MDCKII cells producing the TMD_0L_0 (amino acids 1-281) polypeptide. TMD_0L_0 consists of the first transmembrane domain of MRP1, TMD_0, extended with the intracellular loop L_0. Cell lysates of single clones were analyzed by Western blotting (fig. 4.11, Panel B). TMD_0L_0 appeared as a smear of bands with a molecular weight ranging from 40-70 kDa. This might be due to glycosylation as the protein contains two of the three glycosylation sites of MRP1 (Asn 19 and Asn 23, and see fig. 4.1). To better estimate the amounts of TMD_0L_0 produced by the different clones, 4 and 20 µg of protein were size fractionated. For further characterization of the protein in MDCKII cells, I chose the clone showing the highest expression level: clone #25 was selected for further experiments.

A



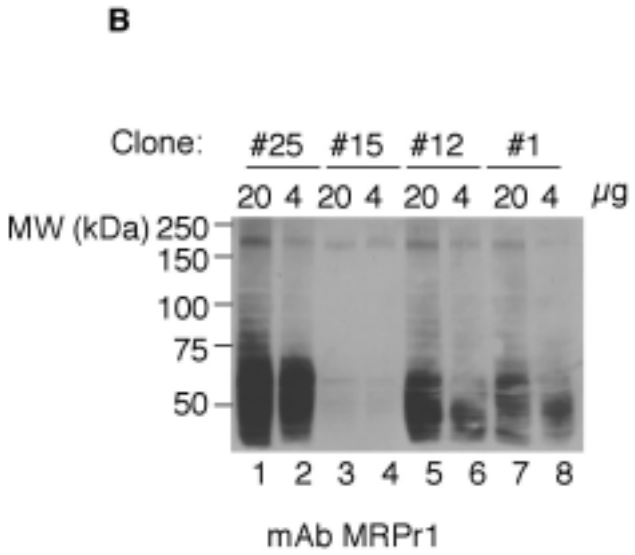


Fig. 4.11 Expression of TMD_0L_0 in MDCKII cells.

A. Schematic representation of TMD_0L_0. Glycosylation sites and the epitope of mAb MRPr1 are shown. B. Detection of TMD_0L_0 by Western blotting. Lysates from independent clones of MDCKII-TMD_0L_0 cells were analyzed. Proteins were size-fractionated in a 7,5% acrylamyde gel. To compare the relative expression levels, 4 and 20 µg of protein were loaded. The blot was incubated with mAb MRPr1.

4.4.2 Immunolocalization of TMD_0L_0 in MDCKII monolayers

Figure 4.12 shows the detection with mAb MRPr1 of TMD_0L_0 in MDCKII monolayers by CLSM. A high amount of intracellular staining was observed, but in several vertical sections some apparent lateral localization was detected. In the X/Y section a clear dark rim is visible around the nucleus in some cells, suggesting that in these cells TMD_0L_0 is localized in the plasma membrane. The vertical sections (X/Z) confirm this: besides intracellular staining in the majority of the cells, staining of the lateral membrane was observed in some cells (*e.g.* X/Z section 1).

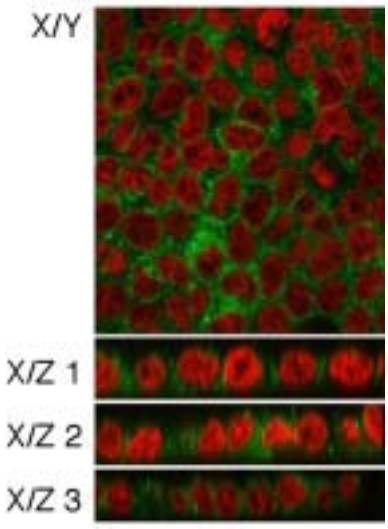


Fig. 4.12. Immunolocalization of TMD_0L_0 in MDCKII monolayers by confocal laser scanning microscopy.

The upper picture represents a top view of the monolayer (X/Y), the lower pictures are vertical sections of the monolayers (X/Z 1-3). Nuclei were counterstained with propidium iodide (red signal), TMD_0L_0 was detected with mAb MRPr1 (green signal).

4.4.3 Cell surface biotinylation of MDCKII-TMD_0L_0 monolayers

4.4.3.1 The principle of cell surface biotinylation

Biotin is a vitamin with a molecular weight of 244 Dalton present in blood and in tissue. Gitlin et al. found in 1987 that biotin binds with very high affinity to avidin, a basic glycoprotein found in the egg white and tissue of birds, reptiles and amphibia. This protein is a tetramer with a total molecular weight of 67000 Dalton. The avidin-biotin interaction is the strongest non-covalent biological interaction known between protein and ligand, and it is only disrupted under drastic conditions.

I used biotin hydrazide for incorporation of biotin into the carbohydrate groups of glycoproteins proteins after their oxidation with sodium periodate. Oxidation generates aldehyde groups that can react spontaneously with the hydrazide as shown in

fig. 4.13. This way several biotin molecules can be coupled to a protein, enabling the biotinylated protein to bind more than one molecule of avidin.

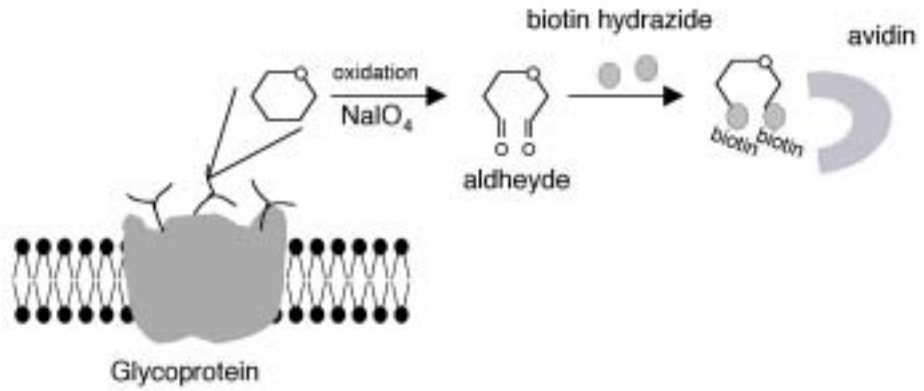


Fig. 4.13. Biotinylation of glycoproteins.

Biotin hydrazide binds covalently to the carbohydrates of glycoproteins after the oxidation to aldehydes. Avidin reacts selectively with biotinylated proteins: a protein can be conjugated with several molecules of biotin.

As the plasma membrane is not permeable to biotin, glycoproteins localized in the apical or basolateral plasma membrane can be biotinylated selectively in cells grown in monolayer by adding biotin hydrazide to either the apical or the basolateral compartment (fig. 4.14).

The biotinylated proteins could be detected by confocal laser scanning microscopy after binding to fluorescently labeled avidin (fig. 4.14, panel A). Alternatively, biotinylated proteins can be precipitated by incubation of cell lysates with agarose-bound avidin and detected by Western blot analysis (fig. 4.14, panel B).

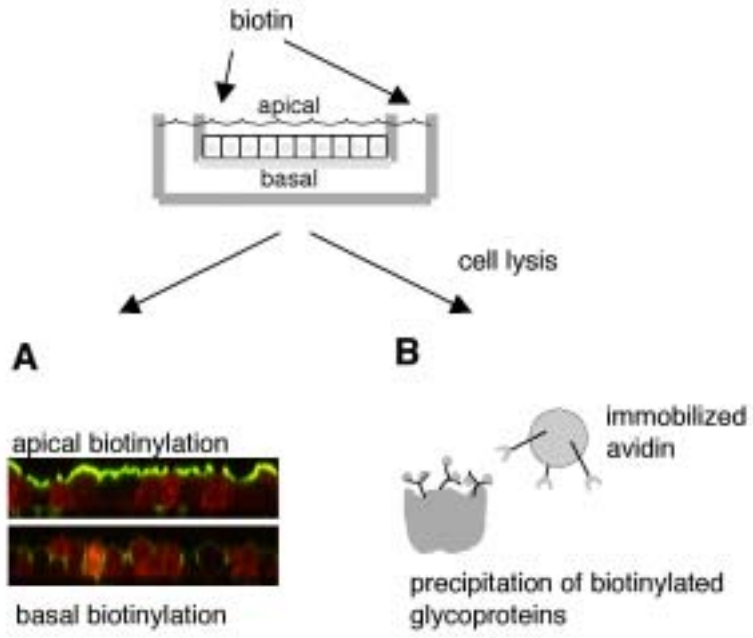


Fig. 4.14. Selective cell surface biotinylation of a monolayer of polarized cells.

A. Biotinylated surface proteins can be detected by reaction with fluorescent avidin. The upper picture shows selective biotinylation of apical proteins in a MDCKII monolayer, the lower shows basolateral biotinylation.
B. Biotinylated proteins can be isolated from a whole lysate by precipitation if avidin is fixed to an inert solid support.

4.4.3.2 Precipitation of biotinylated proteins and identification of TMD_0L_0 by Western blot analysis

To investigate further whether part of TMD_0L_0 was localized in the lateral membrane (fig. 4.12), I carried out a cell surface biotinylation with MDCKII-TMD_0L_0 cells. MDCKII-MRP1 cells were used as a positive control in these experiments. Fig. 4.15, lane 5 shows the precipitated amount of MRP1 protein. The signal in lane 4 (containing the non-precipitated fraction) was weak, indicating that only a small amount of MRP1 was non-biotinylated. Precipitated protein from MDCKII-TMD_0L_0 lysates was size fractionated in lane 2. After biotinylation, a significant amount of TMD_0L_0 was binding to avidin, indicating that part of the molecules is located in the plasma

membrane. The relative high amount of unprecipitated TMD_0L_0 (Lane 1) reflects the high quantity of cytoplasmic TMD_0L_0 also seen by immunolocalization.

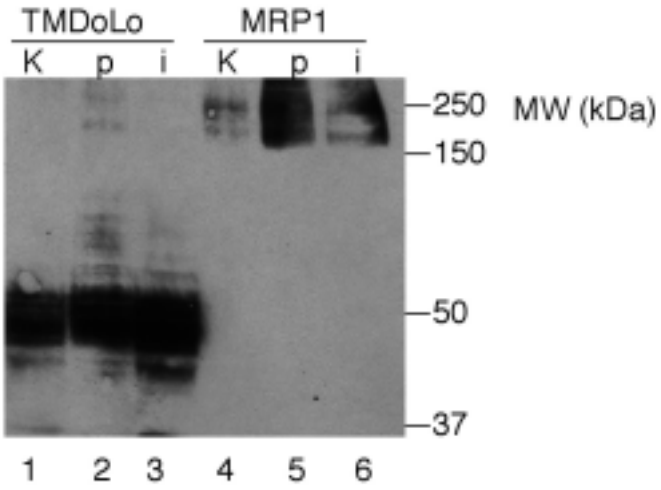


Fig. 4.15. Cell surface biotinylation of MDCKII- TMD_0L_0 and MDCKII-MRP1 monolayers.

The surface of the monolayer was biotinylated by treatment with biotin hydrazide after oxidation with sodium periodate. After biotinylation the monolayers were lysed and the biotinylated proteins were precipitated with immobilized avidin beads. The precipitated proteins were eluted with D-biotin and then separated by size fractionation in a 7,5% polyacrylamide gel under denaturing conditions. MRP1 and TMD_0L_0 were detected with mAb MRPr1.
Lanes marked with p represent the precipitated fraction; i indicates the input (10% of the lysate before precipitation). As control, after precipitation 10% of the supernatant, containing non-precipitated, cytoplasmic protein was size fractionated (lanes marked with K).

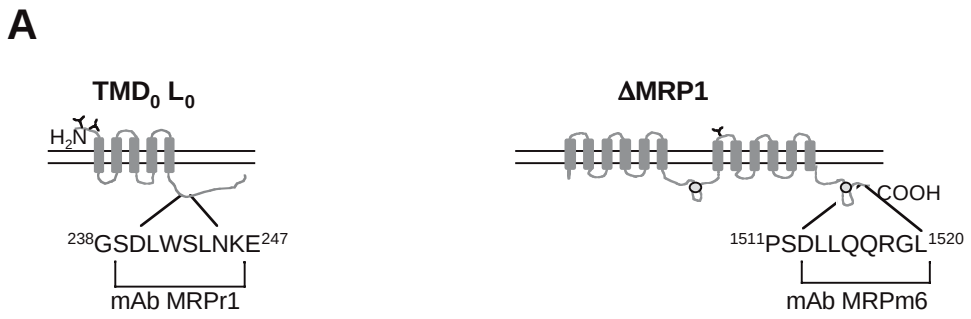
4.5 Characterization of the N-terminal region of MRP1 (TMD_0L_0) in MDCKII-ΔMRP1 polarized cells

4.5.1 Generation of an MDCKII cell line stably expressing TMD_0L_0 and ΔMRP1

It was tried to investigate if the L_0 peptide was able to rescue ΔMRP1 to the plasma membrane(Bàkos et al. 2000), but technically it was not possible to isolate a MDCKII-ΔMRP1 clone stably expressing L_0. As an alternative approach, I therefore

transduced MDCKII-ΔMRP1 cells with the retroviral vector pCMV-neo-TMD_0L_0. After selection with G-418, several clones were isolated stably co-expressing TMD_0L_0 and ΔMRP1. A schematic representation of the constructs is shown in figure 4.16 (panel A). Together the two mutant peptides make-up a full-length MRP1 molecule.

Panels B and C show the Western blot analysis of lysates from MDCKII-TMD_0L_0+ΔMRP1 cells. On the blots, the MRP1 fragments expressed in one cell (MDCKII-ΔMRP1+TMD_0L_0 cells) are compared to cells expressing ΔMRP1 or TMD_0L_0 alone (MDCKII-ΔMRP1 and MDCKII-TMD_0L_0 cells, respectively). TMD_0L_0 detected with mAb MRPr1 in lysates from MDCKII-ΔMRP1+ TMD_0L_0 cells (panel B, lane 1) looks similar to that in lysates from MDCKII-TMD_0L_0 cells (lane 3). As a negative control lysates from MDCKII-ΔMRP1 cells were size fractionated (Lane 2). No signal was detected in these cells with mAb MRPr1. As a positive control, lysates from MDCKII-MRP1 monolayers were analyzed (lane 4). In panel C, proteins were detected using mAb MRPm6. In lane 1 the signal of ΔMRP1 in MDCKII-ΔMRP1+TMD_0L_0 cells is shown, while ΔMRP1 in MDCKII-ΔMRP1 lysates is shown in lane 2. There is a clear difference between the signals detected in the two cell lines: in the MDCKII-ΔMRP1+TMD_0L_0 cells an additional band running at a somewhat higher apparent molecular weight is detectable that is absent in MDCKII-ΔMRP1 cells. As a negative control lysates from MDCKII-TMD_0L_0 cells were size fractionated in lane 3; as a positive control, lysates from MDCKII-MRP1 monolayers were analyzed in lane 5.



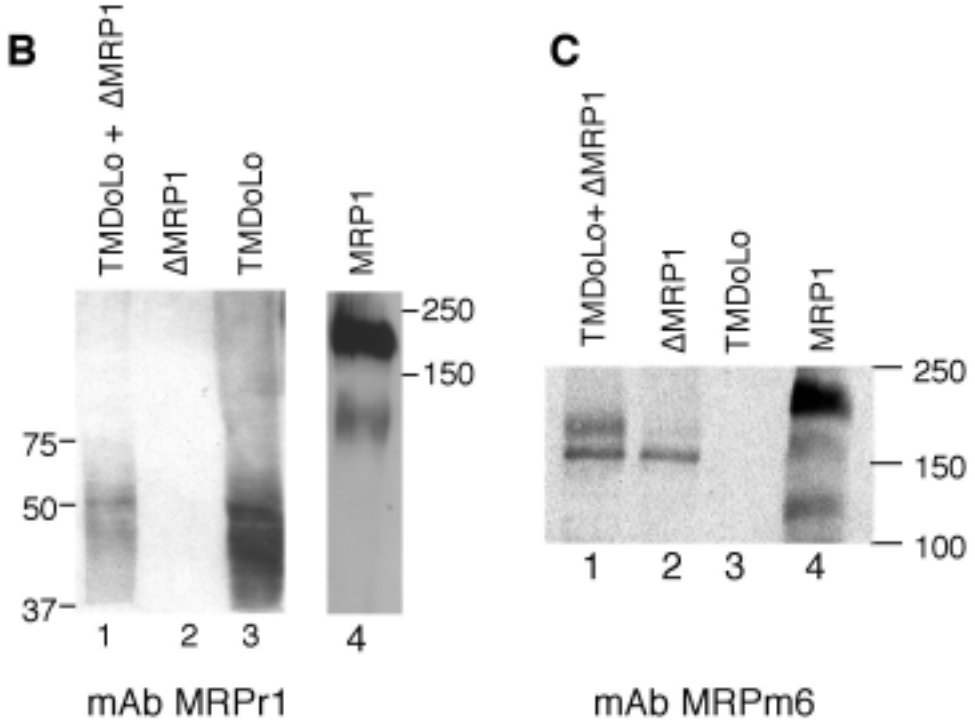


Fig. 4.16. Co-expression of TMD_0L_0 and ΔMRP1 in MDCKII cells.

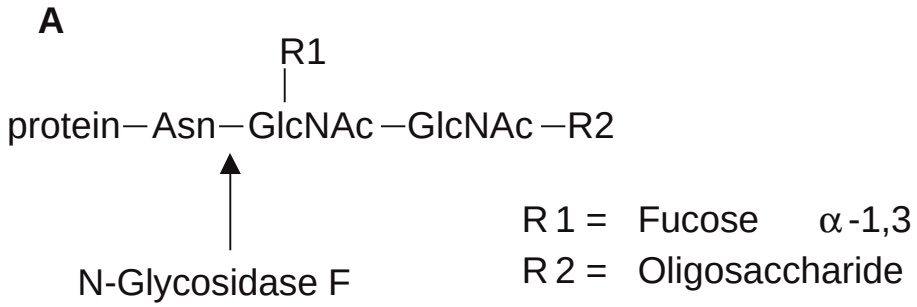
A. Schematic representation of the truncated mutants of MRP1, TMD_0L_0 and ΔMRP1. TMD_0L_0 contains the epitope for mAb MRPr1, and ΔMRP1 contains the epitopes for mAbs MRPm6 and QCRL3.
B, C. Western blot analysis of lysates from MDCKII-TMD_0L_0+ΔMRP1, MDCKII-ΔMRP1, MDCKII-TMD_0L_0 and MDCKII-MRP1 cells. Twenty µg of protein were size fractionated in a 7,5% polyacrylamide gel under denaturing conditions. After electroblotting one blot was incubated with mAb MRPr1 (panel B); a parallel blot was incubated with mAb MRPm6 (panel C).

4.5.2 Glycosylation state of TMD_0L_0 and ΔMRP1 in MDCKII-TMD_0L_0+ΔMRP1 monolayers

In order to investigate the glycosylation state of the truncation mutants of MRP1 in MDCKII-TMD_0L_0+ΔMRP1 cells, membranes were isolated from these cells and incubated with N-glycosidase F. N-glycosidase F digests virtually any N-linked sugars from glycoproteins (Tarentino and Plummer, 1994). Sugars are removed by enzymatic cleavage of the bond between the first N-acetylglucosamine and the asparagine residue of the protein (Figure 4.17, panel A).

Panels B and C in figure 4.17 show a Western blot of membrane fractions of MDCKII-TMD_0L_0+ΔMRP1 cells. Like in fig. 4.16 (panel C), the signal for ΔMRP1 in MDCKII-TMD_0L_0+ΔMRP1 lysates consisted of two bands. The proteins had an apparent molecular weight of about 150 kDa and 170 kDa, respectively. In contrast, the signal for ΔMRP1 in MDCKII-ΔMRP1 lysates consisted of only one band with an apparent molecular weight of 150 kDa. In order to investigate whether the additional 170 kDa band detected in MDCKII-TMD_0L_0+ΔMRP1 lysates is due to glycosylation, membrane extracts from these cells were treated with N Glycosidase F. As positive control I used membranes isolated from MDCKII-MRP1 cells. In figure 4.17 (Panel B) lysates from MDCKII-MRP1 and MDCKII-ΔMRP1+ TMD_0L_0 cells treated (lanes 2 and 4) and untreated (lanes 1 and 3) with N-glycosidase F were size fractionated. The disappearance of the signal at 170 kDa in lane 4, indicates that part of the ΔMRP1 molecules is glycosylated in the presence of TMD_0L_0.

In panel C deglycosylation of proteins in membrane extracts from MDCKII-TMD_0L_0 and MDCKII-TMD_0L_0+ΔMRP1 cells is shown. TMD_0L_0 was detected with mAb MRPr1. The results for the two cell lines are similar: after deglycosylation only one polypeptide with an apparent molecular weight of about 22 kDa was detected.



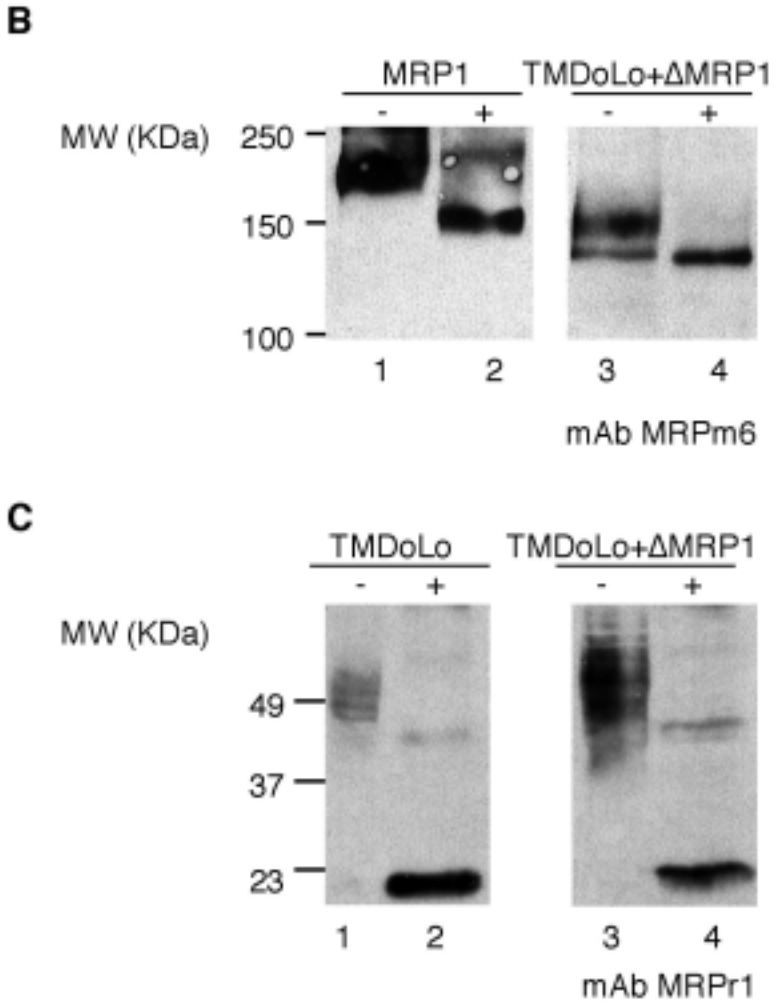


Fig. 4.17. Deglycosylation of TMD_0L_0 and ΔMRP1 in MDCKII- TMD_0L_0+ΔMRP1 monolayers.

A. Mechanism of action of N-glycosidase F. N-glycans are digested from asparagine residues.
B, C. Detection by Western blotting of MRP1, TMD_0L_0+ΔMRP1 and TMD_0L_0 after treatment with N-glycosidase. MDCKII-MRP1, MDCKII- TMD_0L_0 and MDCKII-TMD_0L_0 +ΔMRP1 membranes were incubated with N-glycosidase F for 1h at 37°C (lanes marked with "+"). As a negative control membranes were incubated under the same conditions without enzyme (lanes marked with "-").

Panel B. MRP1 and TMD_0L_0+ΔMRP1 were size-fractionated in a denaturing 7,5% polyacrylamide gel after incubation with (+) or without (-) N-glycosidase F. Western blots were incubated with mAb MRPm6. Panel C. Membrane fractions from MDCKII- TMD_0L_0 and MDCKII- TMD_0L_0+ΔMRP1 were size-fractionated in a denaturing 11% polyacrylamide gel after treatment with (lanes 2 and 4) or without (lanes 1 and 3) N-glycosidase F. The Western blots were incubated with mAb MRPr1.

4.5.3 Immunolocalization of TMD_0L_0 and of ΔMRP1 in MDCKII-TMD_0L_0+ΔMRP1 and MDCKII-ΔMRP1 monolayers

To verify if TMD_0L_0 affects the subcellular localization of ΔMRP1 in MDCKII cells co-expressing these two fragments, I analyzed MDCKII- TMD_0L_0+ΔMRP1 monolayers by confocal laser scanning microscopy.

Panel A in fig. 4.18 shows the immunolocalization of ΔMRP1 in MDCKII cells when TMD_0L_0 is absent. The picture shows that ΔMRP1 is not localized in the plasma membrane and that its detection is restricted to the cytoplasm, in accord with what has been shown before by Bàkos et al. (1998).

Panel B shows the immunolocalization of TMD_0L_0 and ΔMRP1 in MDCKII-TMD_0L_0+ΔMRP1 cells. The results for TMD_0L_0 confirm what was shown in figures 4.12 and 4.15 for MDCKII-TMD_0L_0 cells; the mutant is partially localized in the plasma membrane. TMD_0L_0 was detected by indirect immunofluorescence using mAb MRPr1.

In contrast to what is shown in panel A, in MDCKII-TMD_0L_0+ΔMRP1 cells ΔMRP1 is routed to the plasma membrane due to the presence of TMD_0L_0. Like in panel A, ΔMRP1 was detected with the mAb QCRL3.

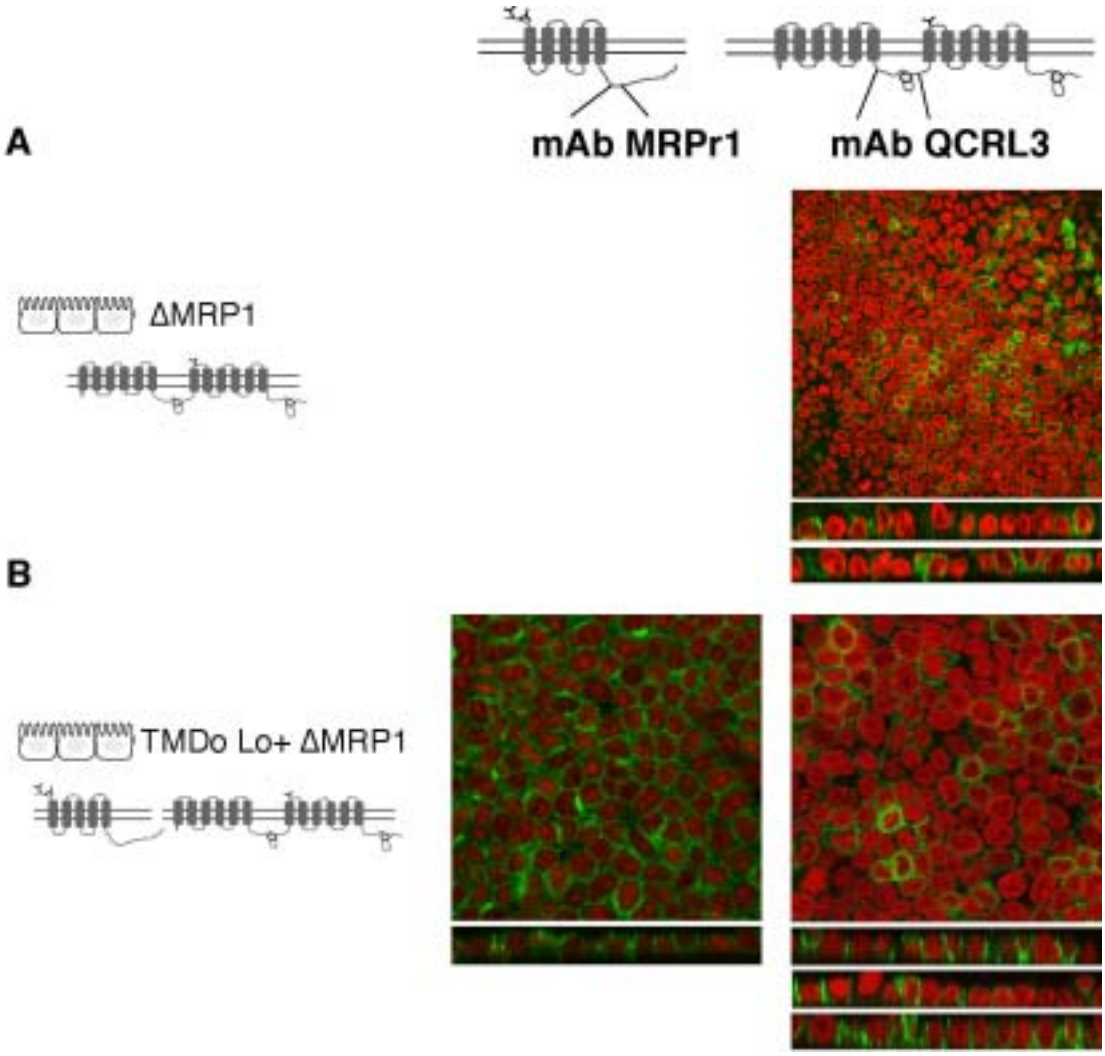


Fig. 4.18. Immunolocalization of TMD_0L_0 and ΔMRP1 in MDCKII- TMD_0L_0+ΔMRP1 monolayers.

A. Immunolocalization by confocal laser scanning microscopy of ΔMRP1 in MDCKII monolayers. ΔMRP1 was detected by indirect immunofluorescence with mAb QCRL3.
B. Immunolocalization of TMD_0L_0 and of ΔMRP1 in MDCKII monolayers co-expressing both proteins. Proteins were detected by indirect immunofluorescence with mAb MRPr1, (left side of panel B) which epitope is in the region L_0 between aa 238-247, and with mAb QCRL3 (right site panel B).
In both panels antibody signals are represented in green and nucleic acids are counterstained with propidium iodide, giving a red signal In each picture a top view (X/Y) of the monolayers and vertical X/Z sections are shown.

4.5.4 Transport properties of TMD_0L_0+ΔMRP1 in MDCKII monolayers.

Since part of ΔMRP1 was demonstrated to be routed to the lateral plasma membrane in MDCKII-TMD_0L_0+ΔMRP1 monolayers, I investigated whether co-expression of TMD_0L_0 with ΔMRP1 resulted in reconstitution of transport activity. I compared the transport of the typical MRP1 substrates DNP-GS and daunorubicin by MDCKII-TMD_0L_0+ΔMRP1 monolayers to transport observed in MDCKII-ΔMRP1 and MDCKII-TMD_0L_0 monolayers. As positive control the transport by MDCKII-MRP1 monolayers was determined, as negative control transport by wild-type MDCKII monolayers.

In fig. 4.19 (panel A), I measured the amount of [^{14}C]DNP-GS transported to the apical and to basolateral side in the various cell lines. Neither in MDCKII-TMD_0L_0 nor in MDCKII-ΔMRP1 monolayers transport activity was detectable. In contrast, basolateral transport of DNP-GS by MDCKII-TMD_0L_0+ΔMRP1 (TL19 in the figure) monolayers was comparable to the amount transported by MDCKII-MRP1 cells.

Panel B shows the amount of [^{3}H]-Daunorubicin transported to the apical and to the basolateral side by the same cell lines as in panel A. While MDCKII-TMD_0L_0 and MDCKII-ΔMRP1 cells did not show transport, MDCKII-TMD_0L_0+ΔMRP1 monolayers demonstrated a significantly increased basolateral transport of daunorubicin.

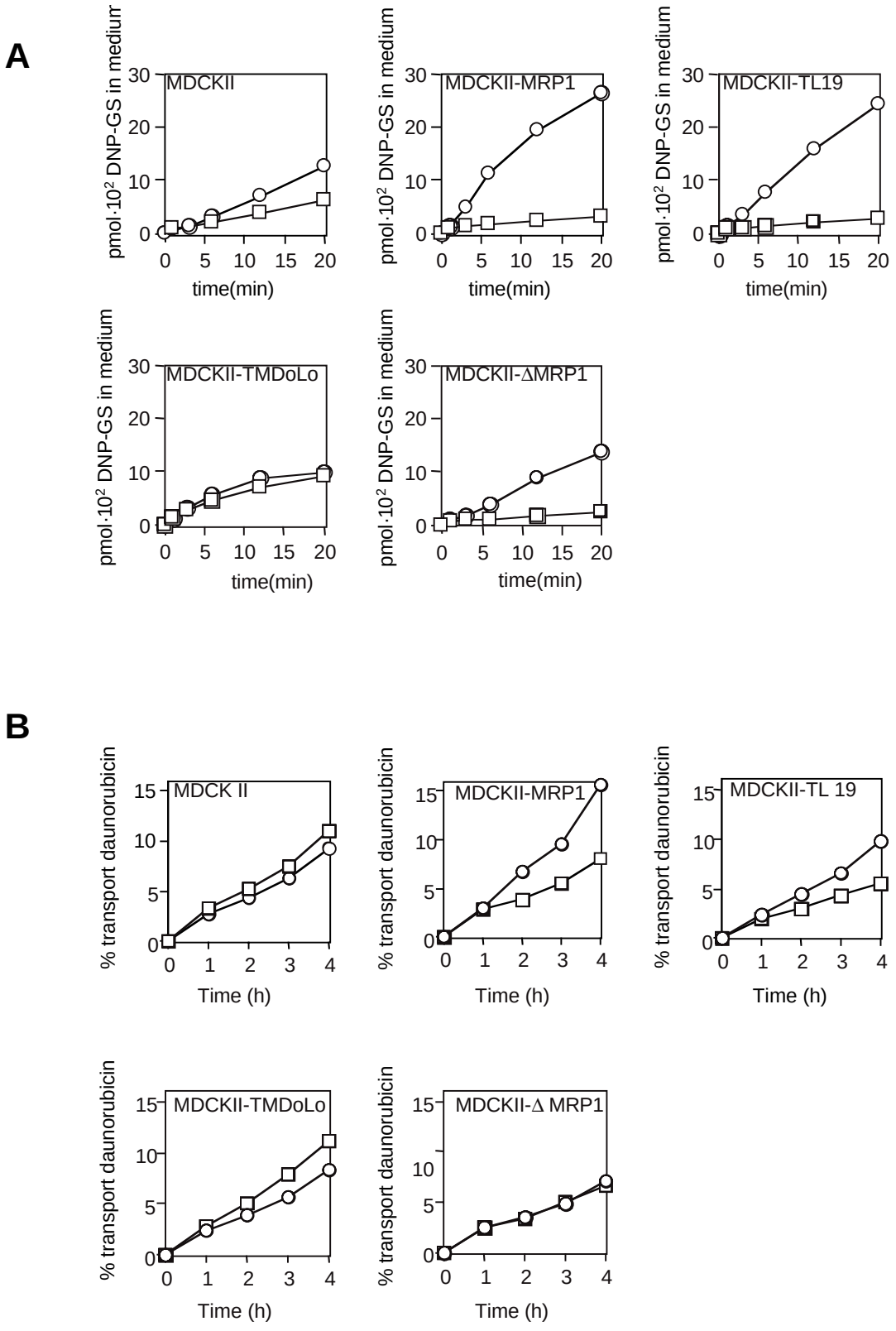


Fig. 4.19. Transport properties of TMD_0L_0+ΔMRP1 in MDCKII monolayers.

A. Transport of DNP-GS by MDCKII, MDCKII-MRP1, MDCKII-TMD_0L_0 and MDCKII-TMD_0L_0+ΔMRP1 (TL19) cells. Cells grown on filters for three days were incubated with 2 µM [^{14}C]CDNB in the apical and in the basal compartment. This compound enters the cells by diffusion; intracellularly it is conjugated to glutathione by the mediation of glutathione-S-transferases. MRP1 is able to transport DNP-GS out of the cells. Samples were taken from both compartments at t = 0, 1, 3, 6, 12 and 20 minutes. Squares, transport to the basal compartment; circles, transport to the apical compartment. The experiments were performed in duplicate and repeated twice.
B. Transport of [^{3}H]-daunorubicin by MDCKII, MDCKII-MRP1 and TMD_0L_0+MDCKII-ΔMRP1 cells. Transport is expressed as the percentage of total radioactivity added at the beginning of the experiment at t = 0. Samples from both compartments were taken at t= 0, 1, 2, 3, 4h. PSC 833 (0,1µM) was present in both compartments to inhibit endogenous MDR1 Pgp (Bàkos et al., 1998).

4.5.5 Demonstration of the interaction between TMD_0L_0 and ΔMRP1

4.5.5.1 Immunoprecipitation and co-immunoprecipitation

The principle of immunoprecipitation exploits the possibility to use an antibody to separate an antigen recognized by the antibody from a mixture of proteins in solution, for example a cell lysate. In general the antibody-antigen complex itself is soluble, so that a direct separation after complex formation is not possible. To separate the complex by precipitation, the antibody is fixed to a solid support (*e.g.* beads coated with protein A or G), containing immunoglobulin-binding proteins with high affinity to the antibody.

After coupling of the monoclonal antibody to the beads, they are incubated with the cell lysate in the presence of detergent. An incubation period follows, to allow the recognition of the antigen by the antibody resulting in the formation of an antigen-antibody complex. The resulting complex may also include proteins interacting with the antigen recognized by the antibody.

The beads I used for our experiments were paramagnetic, so that the separation of the bound proteins from the non-bound fraction could be done by magnetic separation. After separation from the solution, the precipitated protein complexes are disrupted under denaturing conditions and analyzed by SDS-Page.

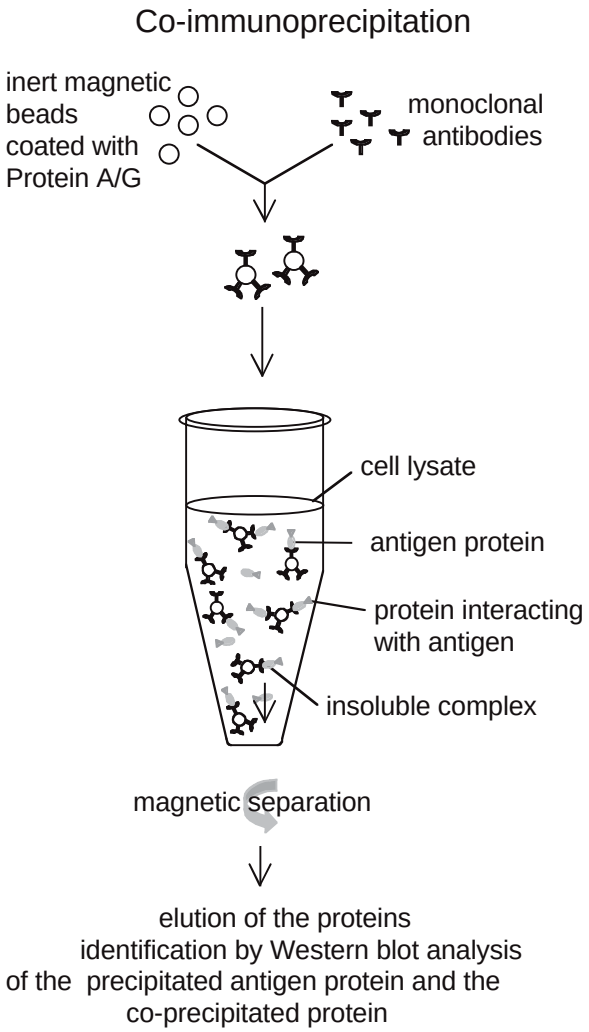


Fig. 4.20. Scheme representing the mechanism of immunoprecipitation.

A monoclonal antibody is fixed to a solid support through protein A or G. The antibody-coated beads are incubated with the solution containing the antigen (ovals in figure). The antibody recognizes the antigen and an insoluble complex is formed. The complex includes also an antigen-binding protein (triangles in figure). After separation from the solution, the proteins present in the complex can be identified by SDS-page.

4.5.5.2 Co-immunoprecipitation of TMD_0L_0 and ΔMRP1 in MDCKII-TMD_0L_0+ΔMRP1 cells

The data demonstrating the glycosylation of ΔMRP1 in MDCKII-TMD_0L_0+ΔMRP1 cells (fig. 4.17), the MRP1-like transport activity (fig. 4.19, panel B) and the partial basolateral localization of ΔMRP1 (fig. 4.18, panel A) in these cells suggest that the TMD_0L_0 and ΔMRP1 molecules interact with each other. In order to verify this hypothesis, I performed an immunoprecipitation. Lysates of MDCKII-TMD_0L_0+ΔMRP1 cells were incubated with mAb QCRL3 coupled to paramagnetic beads. The precipitated proteins were identified by Western blot analysis as shown in figure 4.21. As control I used MDCKII-TMD_0L_0 cells. Eluted proteins were size fractionated in lanes 1 and 3 (lanes marked with IP). Ten percent of the incubation mixture was removed from the cell lysate before precipitation, and size fractionated as an input control (lanes 2 and 4 (input)). After Western blotting, TMD_0L_0 was detected with mAb MRPr1. TMD_0L_0 was co-immunoprecipitated from lysates made from cells co-expressing ΔMRP1 and TMD_0L_0 (lane 1). That this interaction was specific was suggested by the finding that no TMD_0L_0 protein was immunoprecipitated from cells expressing TMD_0L_0 alone (lane 3).

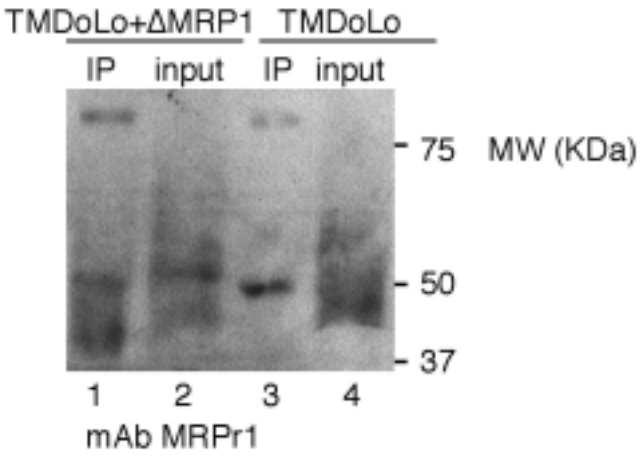


Fig. 4.21. Co-immunoprecipitation of TMD_0L_0 with ΔMRP1 from MDCKII-TMD_0L_0+ΔMRP1 cell lysates.

For precipitation mAb QCRL3 was used. Detection by Western Blot analysis.

Precipitated proteins were eluted from Dynal beads with 2x SDS sample buffer and size fractionated in a 7,5% denaturing polyacrylamide gel (lanes labeled with IP). The Western blot was incubated with mAb MRPr1. As control, 10% of the input lysates was taken before precipitation and size fractionated. (Lanes signed with input).

4.6 Characterization of the chimeric construct $TMD_0(MRP1)L_0(MRP2)$ in MDCKII cells

4.6.1 Generation of $TMD_0(MRP1)L_0(MRP2)$ by overlap PCR

A possible explanation for the results described above is that the TMD_0L_0 fragment of MRP1 contains a basolateral routing signal To investigate whether the routing signal was contained in L_0, I generated a chimeric cDNA consisting of TMD_0 of MRP1 and L_0 of MRP2. The chimeric cDNA encoding $TMD_0(MRP1)L_o(MRP2)$ was cloned using overlap PCR.

For the PCR overlap technique complementary oligodeoxyribonucleotide (oligo) primers and the polymerase chain reaction are used to generate two DNA fragments with overlapping ends at their 3' and 5' ends, respectively. These fragments are combined in a subsequent PCR reaction in which the overlapping ends of the two fragments anneal to each other, allowing the 3' overlap of each strand to serve as a primer for the 3' extension of the complementary strand. The resulting fusion product is amplified further by PCR using the 5' primer from the original upstream fragment (Oligo 1 in fig. 4.22, panel A) and the 3' primer from the original downstream fragment 2 (Oligo 4 in fig. 4.22, panel A). Specific mutations in the nucleotide sequence can be introduced by incorporating nucleotide changes into the overlapping oligo primers.

For the PCR amplification of the cDNA fragment coding for T1 MRP1 cDNA was used as template. Primer 1, flanking the 5' -end contained a *Bam HI* restriction site. The primer flanking the 3' end of T1 (primer 2) included in its sequence the initial bases of the sequence of L_0 (MRP2), followed by TMD_0 sequence of MRP1. The upstream primer for the generation of fragment L2 contained at its 5' end nucleotides encoding

TMD_0 of MRP1 followed by sequence encoding L_0 of the MRP2 cDNA. The 3' Primer 4 contained a *Bam HI* restriction site. MRP2 cDNA was used as the template for the amplification of L2.

After the amplification of fragments L1 and L2, a second PCR reaction was performed. T1 and L2 were used as template DNA, and primer 1 and primer 4 were used for the PCR amplification. As T1 and L2 had complementary sequences at their 3' and 5' end, respectively, a cDNA encoding TMD_0(MRP1)L_0(MRP2) was obtained. The fragment contained *Bam HI* restriction sites both ends, which were used for cloning into the pGEM-7Zf plasmid vector. Sequence analysis showed that the amplified fragment did not contain mutations.

The T1L2 fragment was isolated from the amplified pGEM-7Zf expression vector after digestion with *Bam HI*, and cloned into the *Bam HI* digested retroviral pBabe-puro-CMV vector containing the strong viral CMV promoter upstream of the cloned cDNA and a puromycin resistance gene. MDCKII-T1L2 and MDCKII-T1L2+ΔMRP1 stable cell lines were generated by retroviral transduction as described in 4.3.1. For selection of clones expressing the transduced constructs, cells were grown in medium containing puromycin and positive clones were isolated (fig. 4.22, panel B).

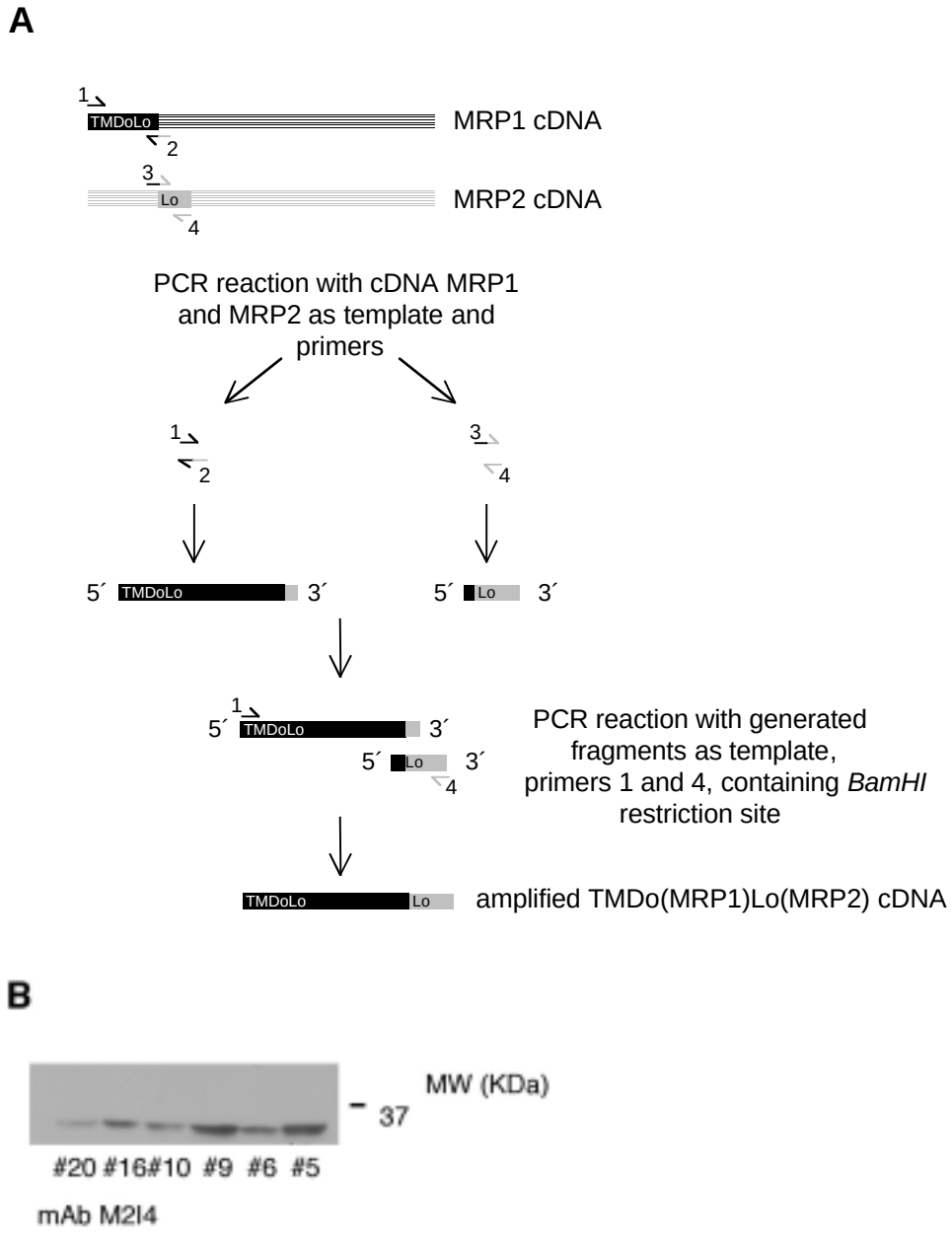


Fig. 4.22. Generation of the chimeric $TMD_0(MRP1)L_0(MRP2)$ cDNA by overlap PCR.

A. Primer 1 initiates at bp 169 of MRP1, primer 2 at bp 702 (MRP2), primer 3 at bp 769 (MRP1) and primer 4 at bp 900 (MRP2). Primer 1 and primer 4 contain the *Bam HI* restriction site.
B. Example of Western Blot analysis of $TMD_0(MRP1)L_0(MRP2)$ clones isolated after selection with Puromycin. The blot was incubated with anti-MRP2 mAb M2I4.

4.6.2 Western blot analysis of MDCKII-TMD$_0$(MRP1)L$_o$(MRP2) and of MDCKII-TMD$_0$(MRP1)L$_0$ (MRP2)+ΔMRP1 lysates

The selected clones of MDCKII-TMD$_0$(MRP1)L$_0$(MRP2) and MDCKII-TMD$_0$(MRP1)L$_0$(MRP2)+ΔMRP1 cells were analyzed by Western Blot analysis.

The Western blot in fig. 4.23 (panel A) was incubated with the anti MRP2 mAb M$_2$I4, recognizing an epitope within the cytoplasmic loop of MRP2 between aa 215 and 310. Proteins from lysates of both cell lines were size fractionated in an 11% polyacrylamide denaturing gel. The amount of chimeric protein produced by the two cell lines is similar (panel A, lanes 1 and 2).

In panel B the same lysates as in panel A were size fractionated. The blot was incubated with mAb MRPm6 to verify the presence of ΔMRP1 in MDCKII-TMD$_0$(MRP1)L$_0$(MRP2)+ΔMRP1 cells. ΔMRP1 in these cells was detectable as it can be seen in panel B, lane 2. As expected no ΔMRP1 protein was detectable in the wild type MDCKII cells expressing T1L2.

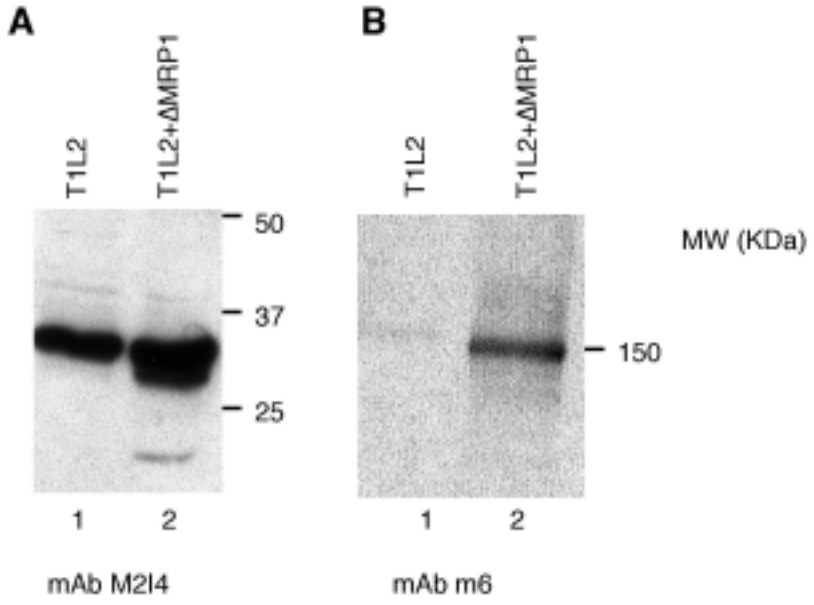


Fig. 4.23. Western blot analysis of MDCKII-TMD$_0$(MRP1)L$_0$(MRP2) and of MDCKII-TMD$_0$(MRP1)L$_0$ (MRP2)+ΔMRP1 lysates.

A. Detection of $TMD_0(MRP1)L_0(MRP2)$ in parental cells and in ΔMRP1 expressing MDCKII cells by western blot analysis. Twenty µg of protein were size-fractionated on an 11% polyacrylamide gel. After electroblotting the protein was revealed using anti-hMRP2 mAb M_2I4.
B. Detection of ΔMRP1 in $TMD_0(MRP1)L_0(MRP2)$ expressing MDCKII-ΔMRP1 cells. Twenty µg of protein were size-fractionated on a 7,5% polyacrylamide gel. After electroblotting ΔMRP1 was detected using mAb MRPm6.

4.6.3 Glycosylation state of $TMD_0(MRP1)L_0(MRP2)$ in MDCKII and in MDCKII-ΔMRP1 cells.

In order to verify the glycosylation state of $TMD_0(MRP1)L_0(MRP2)$ I treated cell lysates containing the chimeric protein with N-glycosidase F and with Endoglycosidase-H. Endoglycosidase-H only recognizes high-mannose and hybrid-type N-glycans and cleaves between the two N-acetylglucosamine residues in the core of the oligosaccharide, generating a truncated sugar molecule with one N-acetylglucosamine residue remaining on the asparagine. In contrast, as shown above (fig. 4.17, panel A), N-glycosidase F removes complex oligosaccharide (fig. 4.24, panel A).

After the deglycosylation reaction, proteins were analyzed by Western blot analysis. Fig. 4.24 (panel B), shows the deglycosylation of $TMD_0(MRP1)L_o(MRP2)$ expressed in MDCKII-ΔMRP1 (lanes 1 and 2) and in MDCKII cells (lanes 3 and 4). Membrane extracts were treated with N-glycosidase F as described. $TMD_0(MRP1)L_o(MRP2)$ was glycosylated in both cell lines as demonstrated by the detection of a low molecular weight band after glycosidase treatment. After treatment, the signal revealed for the protein by Western blot analysis changed to an apparent molecular weight of approximately 28 kDa.

Treatment of MDCKII and MDCKII-ΔMRP1 lysates with Endoglycosidase-H (fig. 4.26, panel C) gave similar results as with N-glycosidase F. In lanes 1 and 2 the Western blot of Endoglycosidase H treated lysates from MDCKII-$TMD_0(MRP1)L_o(MRP2)$+ΔMRP1 cells is shown, and in lanes 3 and 4, lysates from MDCKII cells expressing the chimeric protein. Corresponding to the results of deglycosylation with N-glycosidase F, after treatment with Endoglycosidase-H the apparent molecular weight of the mutant protein decreased to about 28 kDa. As a control I incubated cell lysates from MDCKII-TMD_0L_0 cells with Endoglycosidase-H.

After incubation, the proteins were size fractionated in a 7,5% polyacrylamide gel (panel D, lanes 1 and 2). TMD_0L_0 was not affected by this enzyme, confirming that TMD_0L_0 is not localized in the endoplasmic reticulum.

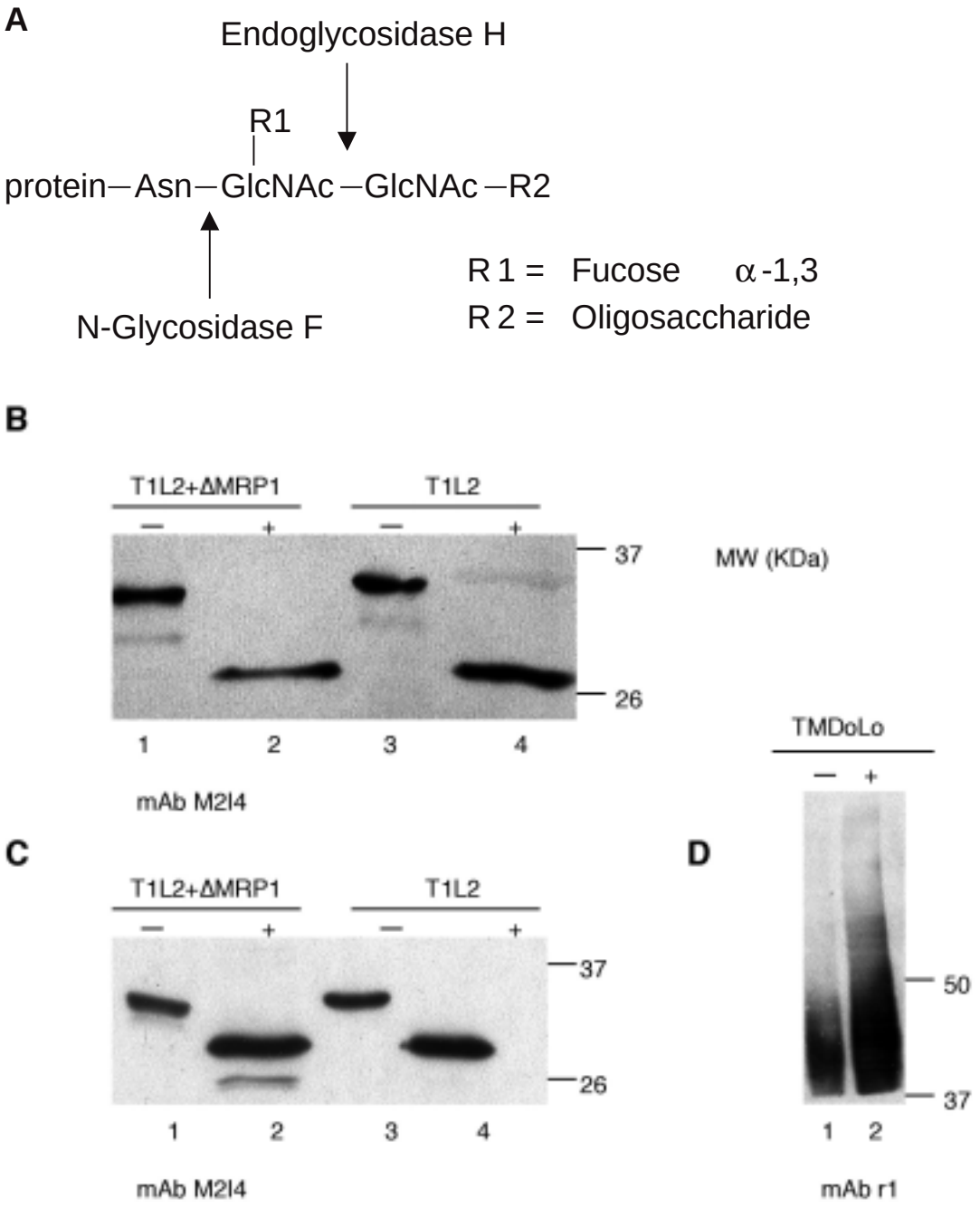


Fig. 4.24. Glycosylation of TMD_0(MRP1)L_0(MRP2) in parental MDCKII and in MDCKII-ΔMRP1 cells.

A. Enzymatic cleavage by N-glycosidase F and Endoglycosidase H.
B. Western blot analysis of membrane extracts of $TMD_0(MRP1)L_0(MRP2)$+MDCKII-ΔMRP1 cells and $TMD_0(MRP1)L_0(MRP2)$-MDCKII cells, treated (lanes signed with +) with N-glycosidase F. Membranes were incubated for 1h with N-glycosidase F at 37°C. As control, untreated membranes (lanes signed with -) are shown. Proteins were size-fractionated in an 11% polyacrylamide denaturing gel. After electroblotting proteins were detected with M_2I4 antibody.
C. Treatment with Endoglycosidase-H of lysates from $TMD_0(MRP1)L_0(MRP2)$+MDCKII-ΔMRP1 cells and $TMD_0(MRP1)L_0(MRP2)$-MDCKII cells. Membranes were incubated for 1h with Endoglycosidase-H at 37°C. Lanes signed with - indicate untreated controls. As in (A), proteins were size-fractionated in an 11% polyacrylamide denaturing gel, and after electroblotting they were revealed with mAb M_2I4.
D. Incubation of MDCKII-TMD_0L_0 cell lysates with Endoglycosidase-H, at same conditions as in (B). The lane signed with - indicates untreated lysates. Proteins were size-fractionated in a 7,5% polyacrylamide denaturing gel, and after electroblotting they were revealed with mAb MRPr1.

4.6.4 Immunolocalization of $TMD_0(MRP1)L_0(MRP2)$ in MDCKII and in MDCKII-ΔMRP1 monolayers.

By indirect immunocytochemistry I analyzed if the substitution of L_0 of MRP1 for L_0 of MRP2 in TMD_0L_0 affected its subcellular localization. $TMD_0(MRP1)L_0(MRP2)$was detected with the anti MRP2 mAb M_2I4 in MDCKII and MDCKII-ΔMRP1 monolayers.

Panel A of fig. 4.25 shows that $TMD_0(MRP1)L_0(MRP2)$ is not routed to the plasma membrane in MDCKII cells.

In panel B the localization of the chimeric protein in MDCKII-ΔMRP1 monolayers is demonstrated. Like the result shown in panel A, the protein was localized intracellularly. The picture on the right side shows the immunolocalization of ΔMRP1 in the same monolayers. In contrast to cells co-expressing TMD_0L_0 and ΔMRP1, the subcellular localization of ΔMRP1 was not affected in cells co-expressing ΔMRP1 and $TMD_0(MRP1)L_0(MRP2)$. Immunolocalization of ΔMRP1 was performed using the antiMRP1 mAb QCRL3.

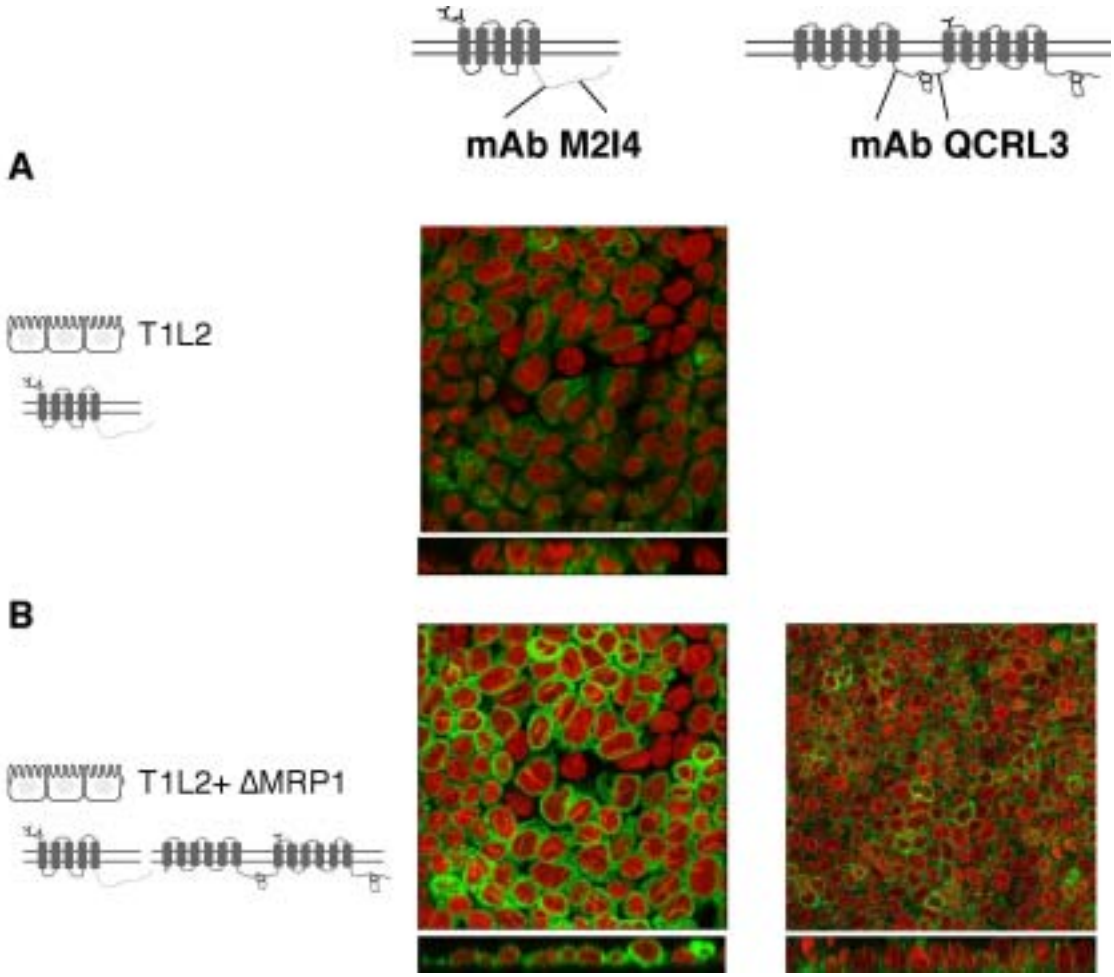


Fig. 4.25. Immunolocalization of $TMD_0(MRP1)L_o(MRP2)$in MDCKII and in MDCKII-ΔMRP1 monolayers.

A. Immunolocalization of $TMD_0(MRP1)L_0(MRP2)$in MDCKII monolayers.
B. Immunolocalization of $TMD_0(MRP1)L_0(MRP2)$ in MDCKII-ΔMRP1 monolayers. $TMD_0(MRP1)L_0(MRP2)$ was detected with anti MRP2 mAb M_2I4, ΔMRP1 was detected with mAb QCRL3 (green signals). Nucleic acids were counterstained with propidium iodide. In the schematic representation of the constructs, parts in dark gray indicate MRP1, parts in light gray indicate MRP2. The $TMD_0(MRP1)L_0(MRP2)$ molecule is abbreviated as T1L2.

5 DISCUSSION

The N-terminal domain TMD_0L_0 (amino acids 1-282) of MRP1 consists of the first transmembrane domain TMD_0 and the cytoplasmic linker L_0. Previously, Bákos et al. (1998, 2000) examined the function of the TMD_0L_0 domain and found that the linker peptide L_0 is required for transport and might contain a signal required for routing to the lateral plasma membrane of the protein. The focus of this thesis was to investigate the role of the N-terminal domain L_0 of MRP1 in transport and routing to the lateral plasma membrane in polarized MDCKII cells. As MRP1 may cause clinical chemotherapy resistance in human tumors (Hipfner et al., 1999; Borst and Oude Elferink, 2002), information regarding its key functional domains and the identification of small specific peptide regions will help to design efficient inhibitors for this drug pump. The elucidation of the mechanisms of action of MRP1 might contribute to the understanding of the multidrug resistance phenotype of cancer cells, potentially allowing the design of mechanism-based drugs.

I searched for regions that are important for routing of MRP1 to the basolateral plasma membrane by expression and co-expression of various MRP1 mutants in polarized MDCKII cells. An evolutionary conserved α-helix within the linker loop L_0 was identified that is important for the transport function of MRP1, since a mutant lacking this helix did not mediate efflux of DNP-GS and daunomycin. Routing of this protein was not affected by this deletion (fig. 4.6), indicating that the function of MRP1 as transporter is not necessarily coupled to its ability to route to the lateral membrane. If the intracellularly localized core of MRP1, ΔMRP1, was co-expressed with the isolated L_0 peptide in MDKCII cells, I found that L_0 was routed to the lateral membrane (fig. 4.10). L_0 in these cells was probably rapidly degraded, as I was not able to isolate clones stably expressing L_0. I therefore established MDCKII cell lines expressing the TMD_0L_0 region alone or together with ΔMRP1 (figures 4.12 and 4.18). I found that in cells strongly overproducing TMD_0L_0 part of the molecules were localized laterally. In MDCKII-TMD_0L_0+ΔMRP1 cells both deletion fragments were routed to the lateral plasma membrane, supporting the hypothesis that the L_0 region contains information required for lateral routing. The co-expression of TMD_0L_0 with ΔMRP1 also resulted in

increased transport of the anthracycline daunorubicin and the organic anion DNP-GS. Transport was similar to that observed in MDCKII-MRP1 cells (figure 4.19), suggesting that TMD_0L_0 and ΔMRP1 dimerize with each other and do not have to be covalently bound in order to form a functional transporter. Further support for dimerization of the two co-expressed mutant fragments was obtained in co-immunoprecipitation experiments that demonstrated that TMD_0L_0 was binding to ΔMRP1 (fig. 4.21). To investigate whether L_0 from MRP2 could substitute for L_0 of MRP1, a chimeric molecule was constructed consisting of TMD_0 of MRP1 and L_0 of MRP2. In MDCKII cells the resulting protein was localized in an intracellular compartment, probably the endoplasmic reticulum (figures 4.24 and 4.25).

5.1 The N-terminal region of MRP 1

The N-terminal transmembrane domain TMD_0 is found in several MRP family members and although this region shows a low overall amino acid identity among these proteins, it is structurally conserved in evolution (Tusnady et al., 1997). MRP1 was the first ABC transporter in which topology predictions suggested the presence of this domain and it was speculated that it might be essential for transport activity. It is evident from our experiments that L_0 is essential for the function of MRP1, but the role of TMD_0 is not clear. The possibility that TMD_0 has no function is improbable, since this domain is evolutionary conserved. TMD_0 could have a regulatory role by interacting with other membrane proteins, be required for the stabilization of the protein, be important for the efficient transport of some substrates, or contribute to proper protein routing in other cells than canine kidney derived MDCKII cells. That TMD_0 may have a stabilizing role is suggested by the fact that despite various attempts, establishing cell lines stably expressing L_0 failed. I was able to establish, however, MDCKII cell lines stably expressing TMD_0L_0. The hypothesis that TMD_0 plays a role in routing in some MRP orthologs was supported by recent findings of Mason and Michaelis (2002). These authors showed that the first transmembrane domain plays a critical role in trafficking to the vacuolar membrane of Ycf1p, a yeast homolog of MRP1 localized in the vacuolar membrane. Fragments of Ycf1p were expressed

separately or together, following a similar approach as used to characterize the various MRP1 domains (Bàkos et al., 1998, 2000; Gao et al., 1998; this thesis). In contrast to what I found for MRP1, the first transmembrane domain was essential for routing of Ycf1p to the vacuolar membrane. Similarly Mateus et al. (2002) demonstrated recently that TMD_0 of MRP2 is required to remain stably localized in the apical membrane or for the routing of MRP2 to the apical plasma membrane in MDCKII cells. These data together indicate that at least in some MRP family members, the first transmembrane domain is required for proper the routing of the protein.

Yang et al. (2002) constructed mutant forms of MRP1 in which N-terminal cysteine residues at position 7 or 32 were mutated to an alanine. Mutation of Cys7 resulted in a strong decrease in the Vmax for transport of LTC_4 whereas the Km was hardly affected. Mutation of Cys7 also resulted in loss of resistance against doxorubicin, vincristine, VP-16 and colchicine. It was shown that mutation of Cys7 resulted in a change in the N-terminal conformation of MRP1. A role of TMD_0 in transport activity was also suggested by experiments by Gao et al. (1998). They found a loss of transport activity as a result of the deletion of the first transmembrane α-helix of MRP1. These data suggest that the N-terminus of MRP1 is important in maintaining the proper structure of MRP1 and that mutations disturb the proper folding of the whole molecule. Probably removal of the whole TMD_0 domain restores transport activity (Bàkos et al., 1998), as the mutated region no longer interferes with the L_0ΔMRP1 region.

5.2 Identification of an amphipathic α-helix within L_0

The presence of the L_0 domain is characteristic for all known MRP family members including the sulfonyl urea receptor (SUR1/2, ABCC8 and -9) and CFTR (ABCC7). Our aim was to examine the apparent important role of this region in plasma membrane targeting and transport of MRP1.

Co-expression in Sf9 cells of the non-functional core of MRP1, ΔMRP1, with the L_0 fragment resulted in an active transporter with similar transport characteristics as wild type MRP1 (Bàkos et al., 2000). This indicates that a covalent linkage between

ΔMRP1 and the L_0 region is not required. It was found that the isolated L_0 peptide was attached to membranes and could only be solubilized by treatment with urea or detergents. Detergents typically solubilize proteins with hydrophobic regions interacting with the phospholipid bilayer. The computer-based secondary structure predictions shown in fig. 1.6 suggests that the L_0 region includes two alpha-helical regions, one of which, between amino acids 221-233, is an amphipathic helix conserved in all members of the MRP family. This amphipathic helix may produce hydrophobic interactions with the lipid bilayer of the cell membrane or with hydrophobic regions of other membrane proteins. The identification of an α-helix may explain the binding of the L_0 peptide to the membrane. In amphipathic helices with a structure corresponding to the predictions shown in figure 1.6, the hydrophobic amino acids of the helix are exposed to the lipid inner core of the membrane. At the bilayer interface rings of tryptophan and tyrosine residues are present and flanking these residues hydrophilic amino acids are localized. This might be the molecular basis for the interaction between the amphipathic helix in L_0 and the plasma membrane.

A mutant MRP1 molecule containing a ten amino acid deletion (Δ223-232) was constructed in order to analyze the role of the amphipathic alpha-helical region in plasma membrane routing or function of MRP1. Tunicamycin treatment demonstrated that MRP1Δ(223-232) is N-glycosylated in MDCKII cells (fig. 4.4). MRP1Δ(223-232) appears to be glycosylated like the wild-type protein, suggesting that the two proteins might follow the same post-translational pathway. That this may indeed be the case is indicated by the lateral localization of MRP1Δ(223-232) in MDCKII monolayers as detected by CLSM (fig. 4.5).

In figure 4.7 it is demonstrated that the α-helix is required for the transport function of MRP1. Similarly, it was demonstrated (Mason and Michaelis, 2002) that the homologous amphipathic helical region in L_0 of the yeast protein Ycf1p is important for transport activity. Taken together with the results shown in figure 4.5, this indicates that basolateral routing and transport activity of MRP1 are not coupled. This is probably also true for MRP2, as Mateus et al. (2002) found that an MRP2 mutant lacking the TMD_0 domain was not routed to the apical plasma membrane in MDCKII cells, but still able to transport LTC_4.

Similar to Bàkos et al. (1998) who constructed an MRP1 fragment extending from amino acid 204 to the C-terminus, Gao et al. (1998) constructed an MRP1 mutant also lacking the first transmembrane domain TMD_0 but starting at amino acid 229. In contrast to the mutant starting at amino acid 204, the mutant of Gao et al. did not show any transport activity. This is most likely explained by the fact that in this mutant the α-helical region is disrupted. Critical amino acids for the function of MRP1 were identified by others in transmembrane helices in the C-terminal transmembrane region (Haimeur et al., 2002; Conrad et al., 2002; Zhang et al., 2001; Ito et al., 2001). In order to explain why the absence of the helix in L_0 compromises the function of the protein it is possible to speculate that it interferes with conformational changes occurring in the protein during the transport cycle.

5.3 Routing to the plasma membrane in polarized cells

Polarity in epithelial cells results from domain-specific sorting of proteins. Most newly synthesized proteins are believed to be sorted at the level of the Golgi complex to either the apical or the basolateral membrane after recognition of signals located within the protein itself (Caplan, 1997). Such specific signals are required for inclusion of proteins into vesicles destined for the apical or basolateral membrane. In many cases, once delivered to the appropriate domain, proteins are anchored to or in the membrane by interactions with the cytoskeleton or with other membrane proteins (Yeaman et al., 1999).

A family of adaptor protein complexes (AP) can mediate incorporation into transport vesicles by interacting with the sorting signals presented in cytosolic domains of the transmembrane proteins, once they are matured in the Trans Golgi Network (TGN; Boman et al., 2001). Four adaptor protein complexes, AP1-4 have been characterized (Bonifacino and Dell'Angelica, 1999; Robinson and Bonifacino, 2001). Recently is was demonstrated (Simmen et al., 2002) that AP4 can bind different types of cytosolic signals known to mediate basolateral transport in epithelial cells. Under the apical sorting mechanisms, clustering into cholesterol and sphingolipid-rich, detergent

insoluble microdomains (rafts) has been speculated to be important for the formation of vesicles from the TGN for some membrane proteins (Simons and Ikonen, 1997). Nevertheless the raft hypothesis is still very controversial It has been demonstrated that the apical targeting of mutant forms of hemagglutinin (Lin et al., 1998) and of serpins (Larsen et al., 2002) in MDCKII cells takes place independently from their association with rafts.

Several different basolateral sorting signals have been identified. In many cases they have been located in the cytoplasmic tail of the protein. The determinant for basolateral sorting may coincide with tyrosine-based or dileucine-based endocytosis signals, which are well known as clathrin-coated pit internalization signals (Matter and Mellman, 1994). It has been reported that the basolateral sorting of the transferrin receptor TfR is mainly determined by the GDNS sequence downstream of the YTRF endocytosis/coated pit localization signal (Dargemont et al., 1993; Odorizzi and Trowbridge, 1997a). Most of the tyrosine-based sorting signals fit to the consensus sequence YXXØ, where Y is a tyrosine, X any amino acid and Ø a hydrophobic amino acid (Ohno et al., 1995, 1999; Boll et al., 1996; Dell'Angelica et al., 1997; Aguilar et al., 2001). A recent example is the localization of the basolateral routing signal of the chicken AE1-4 anion exchanger in MDCK cells, which demonstrated to be dependent upon a tyrosine and a leucine residue that resides within the sequence YVEL, matching the YXXØ motif (Adair-Kirk et al., 2003). As adaptor protein (AP) complexes of clathrin coats interact, at least in vitro, directly with sorting signals that contain critical tyrosine residues (in the context of the consensus sequence YXXØ) it had been hypothesized that an AP- or AP-like complex may play a similar role in basolateral sorting in epithelial cells (Hunziker et al., 1991).

Alternative to tyrosine dependent signals, a dileucine motif has been identified for the first time in the cytoplasmic tail of the mouse macrophage Fc receptor isoform II-B2 (FcRII-B2). Another signal, identified in the IgG Fc receptor and the human invariant chain, only requires a central di-hydrophobic doublet (LL and ML, respectively) (Hunziker and Fumey, 1994; Odorizzi and Trowbridge, 1997b). The LL signal is required both for basolateral sorting and endocytosis (Matter et al., 1994; Hunziker and Fumey, 1994). Another type of dileucine motif identified in the

endoprotease furin, requires an additional upstream acidic amino acid (Simmen et al., 1999). A highly conserved dileucine motif is responsible for the basolateral targeting of E-cadherin, a major adherens junction protein of epithelial cells (Miranda, 2001). Interestingly the cytoplasmic routing signal of the water channel Aquaporin 4 (AQP4) is composed of two independent basolateral sorting signals. One signal consists of a dileucine-based motif preceded by a cluster of acidic amino acids (ETEDLIL), while the other involves the tyrosine-based motif (YMEV) (Madrid et al., 2001). Likewise two discontinuous motifs responsible for basolateral sorting of the human Interleukine-6 (IL-6) receptor were identified: a tyrosine-based motif (YSLG) and a dileucine-type motif (LI) (Martens et al., 2000).

Alternatively, basolateral routing motifs can be unrelated to coated-pit internalization signals, dependent or independent of critical tyrosine residues (Aroeti et al., 1998). Also other basolateral sorting signals, different form the classical tyrosine and dileucine motifs have been identified (Le Gall et al., 1997; Distel et al., 1998). The presence of a bipartite basolateral sorting motif composed by the amino acids QETE and (EP)LT has been recognized in the cytoplasmic juxtamembrane region of the receptor tyrosine kinase ERBB2 (Dillon et al., 2002).

Sorting and/or retention of proteins in their membrane domains can also be mediated by PDZ domain containing proteins. PDZ domains are conserved 70-90 amino acid domains with a defined secondary structure (Doyle et al., 1996), that bind to short peptide sequences at the C-terminus of other proteins, which are called PDZ-interacting domains. This interaction requires the sequence (T/S)XV, where at the –2 position serine or threonine residues are permitted, at position –1 any amino acid and at position 0, the C-terminus, a valine or another amino acid with a hydrophobic side-chain. PDZ proteins are cytosolic, generally restricted to specific subcellular domains as synapses, tight junctions, or the apical or the basolateral cell surface. Their function is to localize their ligands to the appropriate subcellular domain, *e.g.* anchoring and clustering transmembrane proteins or recruiting cytosolic proteins to membrane complexes (Fanning and Anderson, 1999).

A PDZ interaction is required for the polarization of CFTR to the apical plasma membrane (Moyer et al., 2000). Recently it was demonstrated that in this case the PDZ

interacting domain of CFTR does not mediate apical sorting but retention of the protein in the plasma membrane. Two PDZ proteins belonging to the same gene set, LIN-10 and LIN-7 are responsive for the basolateral sorting of the EGF receptor (Whitfield et al., 1999) and of the potassium channel Kir 2.3, respectively (Olsen et al., 2002). A PDZ protein was found to interact with the C-terminus of MRP2 (Kocher et al., 1999), but mutation analyses by Nies et al. (2002) and Mateus et al., (2002) found that the apical routing of MRP2 is independent of a PDZ interaction. Recently, it was shown that in knock-out mice lacking the PDZK1 protein, the routing of Mrp2 was not impaired (Kocher et al., 2003).

5.4 Role of the cytoplasmic linker L_0

By co-expression of L_0 with ΔMRP1 in MDCKII cells, it was found that L_0 was routed to the lateral plasma membrane (fig. 4.10), most likely together with ΔMRP1. It was shown (Bàkos et al., 2000) that when the two peptides were co-expressed in Sf9 cells a functional MRP1 protein was obtained. These results strongly suggest that the isolated L_0 peptide is able to associate with ΔMRP1 and that, consequently, the protein regains its function and is routed to the lateral membrane. Transport by the co-produced proteins could not be measured in MDCKII cells, as for technical reasons it was not possible to isolate single clones. To obviate the difficulty of generating an MDCKII-L_0 cell line, I cloned the complete N-terminal fragment TMD_0L_0 (amino acids 1-281) and expressed it in MDCKII cells. Bàkos et al. (1998) detected by indirect immunofluorescence the presence of TMD_0L_0 in large intracellular vesicles. In this thesis, however, partial lateral localization of the polypeptide was observed by CLSM (fig. 4.12), a finding, which was confirmed by the cell surface biotinylation experiments (fig.4.15). An explanation for this incongruity is that the MDCKII- TMD_0L_0 cell line I analyzed produced relatively high amounts of the TMD_0L_0 peptide (fig. 4.11, panel B). The protein is probably rapidly turned over, as it is only a relatively small non-functional fragment. The lateral staining is possibly the result of a equilibrium between endocytosis and expression rate. I therefore can not rule out that the partial lateral localization of the TMD_0L_0 polypeptide is an artifact of the relatively high expression in

the clone analyzed. A more attractive hypothesis, however, is that a basolateral routing signal is present in TMD_0L_0. Detailed mutagenesis studies are required to discriminate between these two possibilities.

5.5 Co-expression of TMD_0L_0 and ΔMRP1 in MDCKII cells

A stable MDCKII cell line co-expressing TMD_0L_0 and ΔMRP1 was selected. The first indication that TMD_0L_0 had an effect on ΔMRP1 was the presence of an additional band running at a somewhat higher apparent molecular weight in Western blots with MDCKII-TMD_0L_0+ΔMRP1 lysates (fig. 4.16). Enzymatic digestion with N-glycosidase F showed that this additional band was due to glycosylation of ΔMRP1 (fig. 4.17). Peptide-N-glycosidase F from *Flavobacterium meningosepticum* cleaves almost all N-glycans from glycoproteins (Tarentino and Plummer, 1994). It is interesting to note that while TMD_0L_0 is sensitive to the treatment with N-glycosidase F (fig. 4.17, panel C), it is not affected by the activity of Endoglycosidase H (fig. 4.24, panel D). Endoglycosidase H (Endo H) from *Streptomyces plicatus* only releases high-mannose and hybrid type glycans, non-mature intermediates formed during the processing of complex oligosaccharides on glycoproteins in the Golgi. That Endoglycosidase H fails to remove sugar residues from TMD_0L_0 provides an additional indication that this protein moves through the secretory pathway to the plasma membrane.

ΔMRP1 is routed to the lateral plasma membrane in the presence of TMD_0L_0 (fig. 4.18 panel B) and the co-expression of the two truncated mutants results in a functional DNP-GS and daunomycin transporter (fig. 4.19). This is in accordance with data published by Bàkos et al. in 1998 showing that the co-expression of these truncated forms of MRP1 in Sf9-derived vesicles results in full MRP1-like ATP dependent transport activity. Gao et al. (1998) also found independently that when the two half-molecules were co-expressed transport activity was restored. In an analogous way, it was found that the co-expression of a truncated mutant extending from the N-terminus to the first ABC cassette with the complementary half protein resulted in an active transporter in insect cells (Gao et al., 1996). A similar situation was found in yeast,

where co-expression of parts of Ycf1p requires results in an active protein (Mason and Michaelis, 2002). Some ABC transporters are half-molecules, containing only one membrane spanning domain and one nuclear binding domain. These half-transporters function either as homo- or heterodimers. Examples are the peptide transporter TAP1 and TAP2, which acts a heterodimer (Trowsdale et al., 1990; Shepherd et al., 1993) and BCRP, which acts as a homodimer (Kage et al., 2002). The separation or fusion of functional domains may not be of importance for the activity of ABC transporters. The data reported in figures 4.17, 4.18 and 4.19 suggest that the two proteins recognize each other as portions of wild-type MRP1 either in the plasma membrane or in the secretory pathway en route to the plasma membrane. The latter hypothesis is the most likely one as ΔMRP1 is only glycosylated if co-expressed with TMD_0L_0. That TMD_0L_0 and ΔMRP1 physically interact is shown by the result shown in fig. 4.21; TMD_0L_0 is co-immunoprecipitated with ΔMRP1 in lysates from MDCKII-TMD_0L_0+ΔMRP1 lysates using mAb QCRL3, an antibody recognizing an epitope at the C-terminus of MRP1. Results obtained by Loo and Clarke (1995) by co-immunoprecipitations indicate as well that there are specific noncovalent associations between the various domains of P-glycoprotein. Not only an association between the two transmembrane domains was detected, but unexpectedly also between the nucleotide-binding domains and between the nucleotide-binding domains and the transmembrane domains of the same half-protein. These results suggest that structure and function of P-glycoprotein are influenced by noncovalent interactions between the domains of the protein. The authors propose that noncovalent interaction between the domains of P-glycoprotein may contribute to structure and function of P-glycoprotein. Gu et al. (2001a) analyzed the trafficking and activity of Adenylyl cyclases. These proteins possess similar structures as ABC transporters. They consist of two tandem six-transmembrane segments containing domains, each followed by a highly homologous ATP-binding cytosolic loop. It was demonstrated by co-immunoprecipitation of various truncated and mutated molecules, and functional assays that cooperation between the two intact transmembrane domains was essential and sufficient to target the enzyme to the plasma membrane of the cell.

5.6 A comparison of MRP1 with the homologous protein MRP2

MRP1 and MRP2 transport a more or the less overlapping range of chemotherapeutic drugs (Borst et al., 2000). While MRP1 is routed to the lateral membrane in polarized cells, MRP2 is routed to the apical membrane. Mateus et al. (2002) constructed similar MRP2 mutants as those described in this thesis. Neither the N-terminal TMD_0L_0 nor ΔMRP2, the C-terminal core, are routed to the plasma membrane. It is interesting to note that the construct L_0ΔMRP2 was not sorted to the apical membrane, but found in endosomes (Mateus et al., 2002). In vitro uptake experiments, however, with vesicles made from baculovirus infected Sf9 cells producing L_0ΔMRP2 showed that the protein was able to transport LTC_4. This indicates that like in the case of MRP1 routing and transport activity are not linked. In contrast to what was found for MRP1, L_0 was not sufficient for routing of MRP2 to the plasma membrane. The TMD_0 domain of MRP2 is clearly important to for routing to the apical plasma membrane. Considering that all TMD_0L_0 of MRP2 was detected intracellularly if expressed alone, it is possible that MRP2 contains multiple signals required for routing. I can not exclude that this is also the case for MRP1 as only a small fraction of the TMD_0L_0 fragment of MRP1 was routed basolaterally.

5.7 Construction of a chimeric MRP1/MPR2 peptide

The norepinephrine transporter (NET) is localized predominantly in the basolateral plasma membrane, while the dopamine receptor (DAT) is localized in the apical membrane (Gu et al., 1996). Both proteins share 67% overall amino acid sequence identity. Recently, Gu et al. (2001b) characterized by immunocytochemistry and transport experiments chimeric proteins consisting of fragments of NET and DAT. These studies resulted in the identification of a dileucine basolateral sorting signal in the N-terminal cytoplasmic tail of NET. Using a similar approach Dunbar et al. (2000) described the routing behavior of chimeric proteins containing part of the Na/K ATPase α subunit (basolateral localization) and of the H,K-ATPase α subunit (apical

localization). The two loops flanking the fourth transmembrane helix of the H,K-ATPase but also the fourth helix itself were able to redirect the Na/K ATPase to the apical membrane. These experiments revealed the complexity that routing signals can have in polytopic transporters. Following a similar approach I constructed an MRP1/MRP2 chimeric mutant analog to TMD_0L_0 (figures 4.22-25), consisting of the N-terminal transmembrane domain TMD_0 of MRP1 and the cytosolic loop L_0 of MRP2. In lysates made from MDCKII cells producing this peptide the chimeric polypeptide was deglycosylated by N-glycosidase F and Endoglycosidase H (fig. 4.24). This indicates that this mutant probably does not pass the quality control in the endoplasmic reticulum. This finding was confirmed by the immunolocalization of the mutant protein, indicating that staining was restricted to the cytoplasm (fig. 4.25).

The intracellular localization of TMD_0 (MRP1) L_0 (MRP2) could be due to misfolding of the peptide. The deglycosylation of the protein by Endoglycosidase H showed that the maturation process of the carbohydrate chains was not complete. For certain proteins, accurate N-glycosylation is required for proper folding and misfolded polypeptides are retained in the ER (reviewed in Ma and Hendershot, 2001), where they are degraded by proteasomes. It has been demonstrated by Simons and Wardinger-Ness (1990) that in polarized cells apical and lateral transmembrane proteins are contained in the same rough ER and Golgi vesicles along the secretory pathway. The sorting to specific transport vesicles of apical and basolateral directed proteins occurs within the *trans* Golgi network. Therefore, an alternative explanation for the intracellular localization of TMD_0 (MRP1) L_0 (MRP2) is that the transit of the protein through the secretory pathway is blocked, as it does neither contain basolateral nor apical dominant sorting signals. To identify the signals responsible for the basolateral routing of MRP1, more detailed mutational analyses are required.

6 ABBREVIATIONS

APS	ammonium persulfate
AZT-MP	azidothymidine monophosphate
BBB	blood-brain barrier
BSA	bovine serum albumine
BSO	buthionine sulfoximine
CDNB	1-chloro-2,4-dinitro-benzene
CLSM	confocal laser scanning microscopy
CMV	cytomegalovirus
DMEM	Dulbecco’ s modified
DMSO	dimethil sulfoxyde
DNP-GS	dinitro-phenyl-glutathione
DTT	Dithiothreitol
EA	ethacrynic acid
ECL	enhanced chemiluminescence
EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid
FITC	Fluorescein isothiocyanate
HA	Hemagglutinin
HBSS	Hanks buffered saline solution
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
Kb	kilo Bases
kDa	kilo Dlaton
LB medium	Luria-Bertani medium
Luminol	5-Amino-2,3-dihydro-1,4-phthalazinedione
mAb	monoclonal antibody
MDCKII	Madin Darby canine kidney type II
MMULV	Moloney murine leukemia virus

MTX	methotrexate
NEM-GS	N-ethylmaleimide glutathione
OD	optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
SDS	Sodium Dodecyl Sulfate-Lauryl
Sf9	Spodoptera frugiperda insect cells
TEMED	N,N N',N'-Tetramethylethylenediamine

7 REFERENCES

Bàkos E, Hegedus T, Hollo Z, Welker E, Tusnady GE, Zaman GJ, Flens MJ, Varadi A, Sarkadi B.1996. Membrane topology and glycosylation of the human multidrug resistance-associated protein. *J Biol Chem*. **271:** 12322-6

Adachi M, Sampath J, Lan LB, Sun D, Hargrove P, Flatley R, Tatum A, Edwards MZ, Wezeman M, Matherly L, Drake R, Schuetz J. 2002. Expression of MRP4 confers resistance to ganciclovir and compromises bystander cell killing. *J Biol Chem.* **277:** 38998-39004

Adair-Kirk TL, Dorsey FC, Cox JV. 2003. Multiple cytoplasmic signals direct the intracellular trafficking of chicken kidney AE1 anion exchangers in MDCK cells. *J Cell Sci.* **116:** 655-663

Aguilar RC, Boehm M, Gorshkova I, Crouch RJ, Tomita K, Saito T, Ohno H, and Bonifacino JS. 2001. Signal-binding specificity of the {micro}4 subunit of the adaptor protein complex AP-4. *J. Biol. Chem*. **276***:* 13145-13152

Allen JD, Brinkhuis RF, van Deemter L, Wijnholds J, Schinkel AH. 2000. Extensive contribution of the multidrug transporters P-glycoprotein and Mrp1 to basal drug resistance. *Cancer Res.* **60:** 5761-5766

Almquist KC, Loe DW, Hipfner DR, Mackie JE, Cole SPC, Deeley RG. 1995. Characterization of the M(r) 190,000 multidrug resistance protein (MRP) in drug-selected and transfected human tumor cell.*Cancer Res.* **55:** 102-110

Aroeti B, Okhrimenko H, Reich V, Orzech E. Polarized trafficking of plasma membrane proteins: emerging roles of SNAREs, GTPases, and their link to the cytoskeleton. 1998. *Biochim et Biophys Acta*. **1376:** 57-90

Baekelandt MM, Holm R, Nesland JM, Troupe CG, Kristensen GB. 2000. P-glycoprotein expression is a marker for chemotherapy resistance and prognosis in advanced ovarian cancer. *Anticancer Res.* **20 :** 1061-1067

Bàkos E, Evers R, Calenda G, Tusnady GE, Szakacs G, Varadi A, Sarkadi B.2000.Characterization of the amino-terminal regions in the human multidrug resistance protein (MRP1). *J Cell Sci.* **24:** 4451-4461

Bàkos É, Evers R, Szakács G, Tusnády GE, Welker E, Szab K, de Haas M, van Deemter L, Borst P, Váradi A, et al. 1998. Functional multidrug resistance protein (MRP1) lacking the N-terminal transmembrane domain. *J Biol Chem*. **273:** 32167-32175

Bechinger B. 2000. Understanding peptide interactions with the lipid bilayer: a giude to membrane protein engineering. *Curr. Opin. Chem*. Biol. **4:** 639-644

Belinsky MG, Bain LJ, Balsara BB, Testa JR, Kruh GD. 1998. Characterization of MOAT-C and MOAT-D, new members of the MRP/cMOAT subfamily of transporter proteins.*J Natl Cancer Inst.* **90:** 1735–1741

Belinsky MG, Chen ZS, Shchaveleva I, Zeng H, Kruh GD. 2002. Characterization of the drug resistance and transport properties of multidrug resistance protein 6 (MRP6, ABCC6). *Cancer Res*. **62:** 6172-6177

Belinsky MG, Kruh GD. 1999. MOAT-E (ARA) is a full-length MRP/cMOAT subfamily transporter expressed in kidney liver.*Br. J.Cancer.* **80:** 1342– 1349

Belinsky MG, Zeng H, Tosolini A, Testa JR, Kruh GD. 2001. Analysis of the structure and expression pattern of MRP7 (ABCC10), a new member of the mrp subfamily. *Cancer Lett*. **162:** 181–191

Bera TK, Iavarone C, Kumar V, Lee S, Lee B, Pastan I. 2002. MRP9, an unusual truncated member of the ABC transporter superfamily, is highly expressed in breast cancer. *Proc Natl Acad Sci USA*. **99:** 6997-7002

Berger W, Hauptmann E, Elbling L, Vetterlein M, Kokoschka EM, Micksche M. 1997. Possible role of the multidrug resistance-associated protein (MRP) in chemoresistance of human melanoma cells. *Int J Cancer*. **71:** 108-115

Bolhuis H, van Veen HW, Molenaar D, Poolman B, Driessen AJ, Konings WN. 1996. Multidrug resistance in Lactococcus lactis: evidence for ATP-dependent drug extrusion from the inner leaflet of the cytoplasmic membrane. *EMBO J*.**15:** 4239-4245

Boll W, Ohno H, Songyang Z, Rapoport I, Cantley LC, Bonifacino JS, and Kirchhausen T. 1996. Sequence requirements for the recognition of tyrosine-based endocytic signals by clathrin AP-2 complexes. *EMBO J*. **15:** 5789-5795

Boman AL. GGA proetins: new players in the sorting game. 2001. *J. Cell Sci.* **144:** 3413-3418

Bonifacino JS and Dell´Angelica EC. 1999. Molecular bases for the recognition of tyrosine-based sorting signals. *J. Cell Biol*. **145:** 923-926

Borst P, Evers R, Kool M, Wijnholds J. 2000. A family of drug transporters: the multidrug resistance-associated proteins.*J Natl Cancer Inst.* **92:** 1295-1302

Borst P, Oude Elferink R. 2002. Mammalian ABC transporters in health and desease. *Annu Rev Biochem*. **71:** 537–592

Buchler M, König J, Brom M, Kartenbeck J, Spring H, Horie T, Keppler D. 1996. cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. *J Biol Chem*. **271:** 15091–15098

Burg D, Wielinga P, Zelcer N, Saeki T, Mulder GJ, Borst P. 2002. Inhibition of the multidrug resistance protein 1 (MRP1) by peptidomimetic glutathione-conjugate analogs. *Mol Pharmacol.* **62:** 1160-1166

Caplan MJ. 1997. Membrane polarity in epithelial cells: protein sorting and establishment of polarized domains. *Am. J. Physiol Renal Physiol.* **272:** F425-F429

Caplan, MJ. 1997. Ion pumps in epithelial cells: sorting, stablilization and polarity. *Am J Physiol*. **272:** G1304-G1313

Chang G, Roth CB. 2001. Structure of MsbA from E. coli: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. *Science.* **293:** 1793-1800

Chen ZS, Lee K, Kruh GD. 2001. Transport of cylic nucleotides and estradion 17β-D-glucuronide by multridrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine. *J Biol Chem*. **276:** 33747–33754

Chen ZS, Lee K, Kruh GD. 2001.Transport of cyclic nucleotides and estradiol-17β-D-glucuronide by multidrug resistance protein 4. *J Biol Chem.* **276:** 33747-33754

Childs S, Yeh RL, Hui D, Ling V. Taxol resistance mediated by transfection of the liver-specific sister gene of P-glycoprotein. 1998. *Cancer Res.* **58:** 4160-4167

Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart Kurz EU, Duncan AM, Deeley RG.1992. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science*. **258:** 1650-1654

Cole SPC, Deeley RG. 1998. Multidrug resistance mediated by the ATP-binding cassette transporter protein MRP. *Bioessays.* **20:** 681-688

Cole SPC. 1992. The 1991 Merck Frosst Award. Multidrug resistance in small cell lung cancer.*Can J Physiol Pharmacol*. **70:** 313-329

Conrad S, Kauffmann HM, Ito K, Leslie EM, Deeley RG, Schrenk D, Cole SP. 2002. A naturally occurring mutation in MRP1 results in a selective decrease in organic anion transport and in increased doxorubicin resistance. *Pharmacogenetics* **12:** 321-330

Cui Y, König J, Buchholz U, Spring H, Leier I, Keppler D. 1999. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells.*Mol Pharmacol*. **55:** 929-937

Dargemont, C., Le Bivic, A., Rothenberger, S., Iacopetta, B., and Kuhn, L.C. 1993. The internalization signal and the phosphorylation site of transferrin receptor are distinct from the main basolateral sorting information. *EMBO J*. **12***:* 1713-1721

Dean M, Allikmets R.1995. Evolution of ATP-binding cassette transporter genes. *Curr Opin Genet Dev*. **5:** 779-785

Dell'Angelica, E.C., Ohno, H., Ooi, C.E., Rabinovich, E., Roche, K.W., and Bonifacino, J.S. 1997. AP-3: an adaptor-like protein complex with ubiquitous expression. *EMBO J.* **16:** 917-928

Dillon C, Creer A, Kerr K, Kumin A, Dickson C. 2002. Basolateral targeting of ERBB2 is dependent on a novel bipartite juxtamembrane sorting signal but independent of the C-terminal ERBIN-binding domain. *Mol Cell Biol.* **22:** 6553-6563

Distel B, Bauer U, Le Borgne R, Hoflack B. 1998. Basolateral sorting of the cation-dependent mannose 6-phosphate receptor in Madin Darby canine kindey cells. Identifiaction of a basolateral determinant unrelated to clathrin-coated pit localization signals. *J Biol Chem* **273:** 186-193

Doerrler WT, Reedy MC, Raetz CR. 2001. An Escherichia coli mutant defective in lipid export. *J Biol Chem.* **276:** 11461-11464

Doyle DA, Lee A, Lewis J, Kim E, Sheng M, MacKinnon R. 1996. Crystal structure of a complexed and peptm ide free membrane preotein-binding domain: molecular basis for peptide recognition by PDZ. *Cell.* **85:** 1067-1076

Draper MP, Martell RL, Levy SB. 1997. Active efflux of the free acid form of the fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein in multidrug-resistance-protein-overexpressing murine and human leukemia cells. *Eur J Biochem.* **243:** 219-224

Draper MP, Martell RL, Levy SB. 1997. Indomethacin-mediated reversal of multidrug resistance and drug efflux in human and murine cell lines overexpressing MRP, but not P-glycoprotein.*Br J Cancer* **75:** 810–815

Duffy CP, Elliott CJ, O'Connor RA, Heenan MM, Coyle S, Cleary IM, Kavanagh K, Verhaegen S, O'Loughlin CM, NicAmhlaoibh R, Clynes M.1998. Enhancement of chemotherapeutic drug toxicity to human tumour cells in vitro by a subset ofnon-steroidal anti-inflammatory drugs (NSAIDs).*Eur J Cancer* **34:** 1250 –1259

Dunbar LA, Aronson P, Caplan MJ. (2000). A transmembrane segment determines the steady-state localization of an ion-transporting adenosine triphosphatase. *J. Cell Biol.* **148:** 769-778

Dunbar LA and Caplan MJ. 2000. The cell biology of ion pumps: sorting and regulation. *Eur J Cell Biol.* **79:** 557-563

Esterbauer H, Schaur RJ, Zollner, H. 1991. Chemistry and biochemistry of 4-hydroxinonenal, malondialdehyde and relates aldehydes. *Free radical biology & medicine*. **11:** 81-128

Evers R, Cnubben NHP, Wijnholds J, van Demeeeter L, van Bladeren PJ, Borst P. 1997.Transport of glutathione prostaglandin A conjugates by the multidrug resistance protein I. *FEBS Lett*. **419:** 112-116

Evers R, de Haas M, Sparidans R, Beijnen J, Wielinga PR, Lankelma J, Borst P, 2000. Vinblastine and sulfinpyrazone export by the multidrug resistance protein MRP2 is associated with glutathione export. *Br J Cancer*. **83:** 375–383

Evers R, Kool M, van Deemter L, Janssen H, Calafat J, Oomen LC, Paulusma CC, Oude Elferink RP, Baas F, Schinkel AH, Borst P. 1998. Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. *J Clin Invest.* **101:** 1310-1319

Evers R, Zaman GJR, van Deemter L, Jansen H, Calafat J, oomen LCJM, Oude Elfernik RPJ, Borst P, Schinkel AH. 1996. Basolateral localization and export activity of the human multidrug resistance-asssociated protein in polarized pig kidney cells. *J Clin Invest*. **97:** 1211-1218

Fanning AS and Anderson JM. PDZ domains: fundamental building blocks in the organization of protein complexes at the plasma membrane. 1999. *J Clin Invest*. **103:** 767-772

Ferry DR, Traunecker H, Kerr DJ. 1996. Clinical trials of P-glycoprotein reversal in solid tumors. *Eur J Cancer*. **32A:** 1070-1081

Flens MJ, Izquierdo MA, Scheffer GL, Fritz JM, Meijer CJ, Scheper RJ, Zaman GJ.1994. Immunochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. *Cancer Res*. **54:** 4557-4563

Flens MJ, Zaman GJ, van der Valk P, Izquierdo MA, Schroeijers AB, Scheffer GL, van der Groep P, de Haas M, Meijer CJ, Scheper RJ. 1996. Tissue distribution of the multidrug resistance protein. *Am J Pathol.* **148:** 1237-47

Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesman MM, Pastan I. 1987. Expression of a mutidrug resistance gene in human tumors and tissues. *Proc Natl Acad Sci USA.* **84:** 265-269

Furuta K, Tomokiyo K, Tien Kuo M, Ishikawa T, Suzuki M.1999. Molecular design of glutathione-derived biochemical probes targeting the GS-X pump. *Tetrahedron*. **55:** 7529–7540

Gao M, Loe DW, Grant CE, Cole SP, Deeley RG. 1996. Reconstitution of ATP-dependent leukotriene C4 transport by Co-expression of both half-molecules of human multidrug resistance protein in insect cells. *J Biol Chem.* **271:** 27782-27787

Gao M, Yamazaki M, Loe DW, Westlake CJ, Grant CE, Cole SP, Deeley RG.1998. Multidrug resistance protein. Identification of regions required for active transport of leukotrieneC4. *J Biol Chem*. **273:** 10733-40

Gekeler V, Ise W, Sanders KH, Ulrich WR, Beck J.1995. The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. *Biochem Biophys Res Commun.* **208:** 345-352

Gitlin G, Bayer EA, Wilchek M.1987. Studies on the biotin-binding site of avidin. Lysine residues involved in the active site. *Biochem J*. **242:** 923-926

Glatt H. 2000. Sulfotransferases in the bioactivation of xeno-biotics. *Chem Biol Interact*. **129:** 141–170

Goldstein LJ, Galski H, Fojo A, Willingham M, Lai SL, Gazdar A, Pirker R, Green A, Crist W, Brodeur GM.1989. Expression of a mutidrug resistance gene in human cancers. *J Natl Cancer Inst*. **81:** 116-124

Gottesman MM, Foji T, Bates SE. 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nature Rev Cancer.* **2:** 48-58

Grant CE, Kurz EU, Cole SP, Deeley RG.1997. Analysis of the intronexon organization of the human multidrug-resistance protein gene (MRP) and alternative splicing of its mRNA**.** *Genomics.* **45:** 368-78

Greiner B, Eichelbaum M, Fritz P, Kreichgauer HP, von Richter O, Zundler J, Kroemer HK.1999. The role of intestinal P-glycoprotein in the interaction iof digoxin and rifampin. *J Clin Invest*. **104:** 147-153

Gu C, Sorkin A, Cooper DM. 2001a. Persistent interactions between the two transmembrane clusters dictate the targeting and functional assembly of adenylyl cyclase. *Curr Biol* **11:**185-190

Gu HH, Wu X, Giros B, Caron MG, Caplan MJ, Rudnick G. 2001b. The NH_2-terminus of norepinephrine transporter contains a baslolateral localization signal for epithelial cells. *Mol Biol Cell*. **12:** 3797-3807

Gu HH, Ahn J, Caplan MJ, Blakely RD, Levey AI, Rudnick G.1996. Cell-specific sorting of biogenic amine transporters expressed in epithelial cells. *J. Biol. Chem.* **271:** 18100-18106

Haimeur A, Deeley RG, Cole SP. 2002. Charged amino acids in the sixth transmembrane helix of multidrug resistance protein 1 (MRP1) are critical determinants of transport activity. *J Biol Chem* **277:** 41326-41333

Heijn M Hooijberg JH, Scheffer GGl, Szabo G, Westerhoff HV, Lankelma J. 1997. Anthracyclines modulate multidrug resistance protein (MRP) mediated organic anion transport. *Biochim Biophys Acta*. **1326:** 12-22

Higgins C, Linton KJ. 2001. Structural biology. The xyz of ABC transporters. *Science.* **293:** 1782-1784

Higgins FC. ABC transporters: from microorganisms to man. 1992. *Annu Rev. Cell. Biol*. **8:** 67-113

Hipfner DR, Almquist KC, Leslie EM, Gerlach JH, Grant CE, Deeley RG, Cole SP. 1997. Membrane topology of the multidrug resistance protein (MRP). A study of

glycosylation-site mutants reveals an extracytosolic NH2 terminus. *J Biol Chem*. **272:** 23623-23630

Hipfner DR, Deeley RG and Cole SP. 1999. Structural, mechanicistic and clinical aspects of MRP1. *Biochim. Biophys. Acta*. **1461:** 359-376

Hipfner DR, Gao M, Scheffer G, Scheper RJ, Deeley RG, Cole SP.1998. Epitope mapping of monoclonal antibodies specific for the 190-kDa multidrug resistance protein (MRP). *Br J Cancer*. **78:** 1134-1140

Hipfner DR, Gauldie SD, Deeley RG, Cole SP. 1994. Detection of the M(r) 190,000 multidrug resistance protein, MRP, with monoclonal antibodies. *Cancer Res.* **54:** 5788-5792

Hipfner DR, Mao Q, Qiu W, Leslie EM, Deeley RG, Cole SPC. 1999. Monoclonal antibodies that inhibit the transport function of the 190-kDa multidrug resistance protein, MRP. Localization of their epitopes to the nucleotide-binding domains of the protein. *J Biol Chem.* **274:** 15420-15426

Hirohashi T, Suzuki H, Chu XY, Tamai I, Tsuji A, Sugiyama Y. Function and expression of multidrug-resistance-associated protein family in human colon adenocarcinoma cells (Caco-2). 2000. *J Oharmacol Exp Ther.* **292:** 265-270

Hirohashi T, Suzuki H, Ito K, Ogawa K, Kume K, Shimizu T, Sugiyama Y. 1998. Hepatic expression of multidrug resistance-associated protein-like proteins maintained in eisai hyperbilirubinemic rats. *Mol Pharmacol.* **53:** 1068-1075

Hirrlinger J, König J, Keppler D, Lindenau J, Schulz JB, Dringen R. 2001. The multidrug resistance protein MRP1 mediates the release of glutathione disulfide from rat astrocytes during oxidative stress. *J neurochem.* **76:** 627-636

Hitzl M, Klein K, Zanger UM, Fritz P, Nussler AK, Neuhaus P, Fromm MF. 2003. Influence of omeprazole on multidrug resistance protein 3 expression in human liver. J *Pharmacol Exp Ther*. **304:** 524-530

Holland IB, Blight MA. 1999. ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. *J Mol Biol*. **293:** 381-399

Hollo ZS, Homoloya L, Hegedüs T, Sarkadi B. 1996. Transport properties of the multidrug resistance-associated protein (MRP) in human tumor cells. *FEBS Lett*. **383:** 99-104

Hooijberg JH, Broxterman HJ, Kool M, Assaraf YG, Peters GJ, Noordhuis P, Scheper RJ, Borst P, Pinedo HM, Jansen G. 1999.Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. *Cancer Res.* **59:** 2532-2535

Hopfner KP, Karcher A, Shin DS, Craig L, Arthur LM, Carney JP, Tainer JA. 2000. Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. *Cell*. **101:** 789-800

Hopper E, Belinsky MG, Zeng H, Tosolini A, Testa JR, Kruh GD. 2001. Analysis of the structure and expression pattern of MRP7 (ABCC10), a new member of the MRP subfamily. *Cancer Lett.* **162:** 181–191

Hung LW, Wang IX, Nikaido K, Liu PQ, Ames GF, Kim SH. 1998. Crystal structure of the ATP-binding subunit of an ABC transporter. *Nature*. **396:** 703-707

Hunziker W and Fumey C.1994. A dileucine motif mediates endocytosis and basolateral sorting of macrophage IgG Fc receptors in MDCK cells. *EMBO J.* **13:** 2963-2967

Hunziker W, Harter C, Matter K, and Mellman I. 1991. Basolateral sorting in MDCK cells requires a distinct cytoplasmic domain determinant. *Cell.* **66:** 907-920

Ilias A, Urban Z, Seidl TL, Le Saux O, Sinko E, Boyd CD, Sarkadi B, Varadi A.2002. Loss of ATP-dependent transport activity in pseudoxanthoma elasticum-associated mutants of human ABCC6 (MRP6). *J Biol Chem*. **277:** 16860-16867

Ishikawa T, Müller M, Klunemann C, Schaub T, Keppler D. 1990. ATP-dependent primary active transport of cysteinyl leukotrienes across liver membrane. Role of the ATP-dependent transport system for glutathione S-conjugates. *J Biol Chem.* **265:** 19279-19286

Ishikawa T. 1992.The ATP-dependent glutathione S-conjugates export pump. *Trends Biochem Sci*. **17:** 463-468

Ito K, Olsen SL, Qiu W, Deeley RG, Cole SP. 2001.Mutation of a single conserved tryptophan in multidrug resistance protein 1 (MRP1/ABCC1) results in loss of drug resistance and selective loss of organic anion transport. *J Biol Chem*. **276:** 15616-24

Jedlitkschky G, Leier I, Buchholz U, Barnouin K, Kurz G, Keppler D. 1996. Transport of glutathione, glucuronate and sulfate conjugates by the MRP gene-encoded conjugate exort pump. *Cancer Res*. **56:** 988-994

Jedlitschky G, Burchell B, Keppler D. 2000. The multidrug resistance protein 5 function as an ATP-dependent export pump for cyclic nucleotides. *J Biol Chem.* **275:** 30069-30074

Jedlitschky G, Leier I, Buchholz U, Center M, Keppler D. 1994. ATP-dependent transport of glutathione S-conjugates by the multidrug resistance-associate protein. *Cancer Res*. **54:** 4833-4836

Jedlitschky G, Leier I, Buchholz U, Hummel-Eisenbeiss J, Burchell B, Keppler D. 1997. ATP dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2.*Biochemistry J.* **327:** 305-310

Kage K, Tsukahara S, Sugiyama T, Asada S, Ishikawa E, Tsuruo T, Sugimoto Y. 2002. Dominant-negative inhibition of breast cancer resistance protein as drug efflux pump through the inhibition of S-S dependent homodimerization. *Int J Cancer*. **97:** 626-630

Karow M, Georgopoulos C. 1993. The essential Escherichia coli msbA gene, a multicopy suppressor of null mutations in the htrB gene, is related to the universally conserved family of ATP-dependent translocators. *Mol Microbiol*. **7:** 69-79

Kartenbeck J, Leuschner U, Mayer R, Keppler D. 1996. Absence of the canalicular isoform of the MRP gene-en-coded conjugate export pump from the hepatocytes in Dubin–Johnson syndrome. *Hepatology*. **23:** 1061–1066

Kast C, Gros P. 1997. Topology mapping of the amino-terminal half of multidrug resistance-associated protein by epitope insertion and immunofluorescence.*J Biol Chem.* **272:** 26479-26487

Kast C, Gros P. 1998. Epitope insertion favors a six transmembrane domain model for the carboxy-terminal portion of the multidrug resistance-associated protein.*Biochemistry.* **37:** 2305-2313

Keppler D, Jedlitschky G, Leier I. 1998. Transport function and substrate specificity of multidrug resistance protein. *Methods Enzymol.* **292:** 607-616

Keppler D, Leier I, Jedlitkschky G. 1997. Transport of glutathione conjugates and glucuronides by the multidrug resistance proteins MRP1and MRP2. *J Biol Chem*. **378:** 787-791

Kessel D, Hall TC, Roberts D.1968. Modes of uptake of methotrexate by normal and leukemic human leukocytes in vitro and their relation to drug response. *Cancer Res.* **28:** 564-570

Kiuchi Y, Suzuki H, Hirohashi T, Tyson CA, Sugiyama Y. 1998. cDNA cloning and inducible expression of human multidrug resistance associated protein 3 (MRP3). *FEBS Lett*. **433:** 149–152

Kocher O, Comella N, Gilchrist A, Rinku P, Tognazzi K, Brown LF, Knoll JHM. PDZK1, a novel PDZ domain-containing protein up regulated incarcinomas and mapped to chromosome 1q21, interacts with cMOAT (MRP2), the multidrug resistance associated protein. 1999. *Lab Invest*. **79:** 1161-1170

Kocher O, Pal R, Roberts M, Cirovic C, Gilchrist A. 2003. Targeted Disruption of the PDZK1 Gene by Homologous Recombination. *Mol Cell Biol.* **23:** 1175-1180

Koike K, Kawabe T, Tanaka T, Toh S, Uchiumi T, Wada M, Akiyama S, Ono Kuwano M.1997.A canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells. *Cancer Res.* **57:** 5475-5479

Koike K, Kawabe T, Tanaka T, Toh S, Uchiumi T, Wada M, Akiyama S, Ono M, Kuwano M. 1997. A canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells. *Cancer Res.* **57:** 5475–5479

König J, Nies AT, Cui Y, Leier I, Keppler D. 1999a. Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. *Biochim Biophys Acta*. **1461:** 377-394

König J, Rost D, Cui Y, Keppler D. 1999b. Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. *Hepatology*. **29:** 1156 –1163

König P, Giraldo R, Chapman L, Rhodes D. 1996. The crystal structure of the DNA-binding domain of yeast RAP1 in complex with telomeric DNA.*Cell.* **85:** 125–36

Kool M, De Haas M, Scheffer GL, Scheper RJ, van Eijk MJT, Juijn JA, Baas F, Borst P. 1997. Analysis of expression of cMOAT *(MRP2), MRP3, MRP4,* and *MRP5,* homologues of the multidrug resistance associated protein gene (*MRP1*), in human cancer cell lines. *Cancer Res*. **57:** 3537– 47

Kool M, van der Linden M, de Haas M, Baas F, Borst P. 1999a. Expression of human MRP6, a homologue of the multidrug resistance protein gene *MRP1*, in tissues and cancer cells. *Cancer Res.* **59:** 175-182

Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, Elferink RP, Baas F, Borst P.1999b. MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc Natl Acad Sci USA.* **96:** 6914-6919

Kuss BJ, O'Neill GM, Eyre H, Doggett NA, Callen DF, Davey RA. 1998 ARA, a novel ABC transporter, is located at 16p13.1, is deleted in inv(16) leukemias, and is shown to be expressed in primitive hematopoietic precursors.*Genomics.* **51:** 455–458

Lai L, Tan TM. 2002. Role of glutathione in the multidrug resistance protein 4 (MRP4/ABCC4)-mediated efflux of cAMP and resistance to purine analogues. *Biochem J.* **361:** 497-503

Laing NM, Belinsky MG, Kruh GD, Bell DW, Boyd JT, Barone L, Testa JR, Tew KD.1998. Amplification of the ABC-binding cassette 2 transporter gene is functionally linked with enhanced efflux of estramustine in ovarian carcinoma cells. *Cancer Res.* **58:** 1332-1337

Larsen JE, Sjostrom H, Noren O, Vogel LK. 2002. Serpins are apically secreted from MDCK cells independently of their raft association. *Biochem Biophys Res Commun.* **299:** 35-41

Le Gall AH, Powell SK, Yeaman CA, Rodriguez-Boulan E. 1997. The neural cell adhesion molecule expresses a tyrosine-indipendent basolaterl sorting signal *J Biol Chem*.**272:** 4559-4567

Lee K, Klein-Szanto AJ, Kruh GD. Analysis of the MRP4 drug resistance profile in transfected NIH3T3 cells. 2000. *J Natl Cancer Inst.* **92:** 1934-1940

Leier I, Jedlitschky G, Buchholz U, Center M, Cole SP, Deeley RG, Keppler D. 1996. ATP-dependent glutathione sulfide transport mediated by the MRP gene-encoded conjugate export pump (GS-X pump). *Biochem J*. **314:** 433-437

Leier I, Jedlitschky G, Buchholz U, Cole SPC, Deeley RG, Keppler D. 1994. The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates.*J Biol Chem.* **269:** 27807-27810

Leslie EM, Ito KI, Upadhyaya P, Hecht SS, Deeley RG, Cole SPC. 2001. Transport of the β-O-Glucuronide conjugate of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by the Multidrug Resistance Protein 1 (MRP1). Requirement for glutathione or a non-sulfur-containing analog. *J Biol Chem* **276:** 27846–27854

Leslie EM, Mao Q, Oleschuk CJ, Deeley RG, Cole SPC. 2001. Modulation of multidrug resistance protein 1 (MRP1/ABCC1) transport and ATPase activities by interaction with dietary flavonoids. *Mol. Pharmacol*. **59:** 1171–1180

Lin S, Naim HY, Rodriguez AC, Roth MG. 1998. Mutations in the middle of the transmembrane domain reverse the polarity of transport of the influenza virus hemagglutinin in MDCK epithelial cells. *J Cell Biol.* **142:** 51-57

Loe DW, Almquist KC, Cole SPC, Deeley RG. 1996. ATP-dependent 17 beta-estradiol 17-(beta-D-glucuronide) transport by multidrug resistance protein (MRP). Inhibition by cholestatic steroids. *J Biol Chem.* **271:** 9683-9689

Loe DW, Almquist KC, Deeley RG, Cole SPC. 1996.Multidrug resistance protein (MRP)-mediated transport of leukotriene C_4 and chemotherapuetic agents in membrane vesicles: demonstration of glutathione-dependent vincristine transport. *J Biol Chem.* **271:** 9675-9682

Loe DW, Deeley RG, Cole SPC. 1998. Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione. *Cancer Res.* **58:** 5130-5136

Loe DW, Steward RK, Massey TE, Deeley RG, Cole SPC. 1997. ATP-dependent transport of aflatoxin B1 and its glutathione conjugates by the product of the multidrug resistance protein (MRP) gene. *Mol Pharmacol*. **51:** 1034-1041

Longhurst TJ, O'Neill GM, Harvie RM, Davey RA. 1996. The anthracycline resitance-associated (*ara*) gene, a novel gene associated with multidrug resistance in a human leukaemia. *Br J Cancer*. **74:** 1331–1335

Loo TW, Clarke DM.1995. P-glycoprotein. Associations between domains and between domains and molecular chaperones. *J Biol Chem*. **270:** 21839-21844

Lorico A, Rappa G, Finch RA, Yang D, Flavell RA, Sartorelli AC. 1997. Disruption of the murine MRP (multidrug resistance protein) gene leads to increased sensitivity to etoposide (VP-16) and increased levels of glutathione.*Cancer Res.* **57:** 5238-5242

Lu SC, Cai J, Kuhlenkamp J, Sun WM, Takikawa H, Takenaka O, Horie T, Yi J, Kaplowitz N. 1996. Alterations in glutathione homeostasis in mutant Eisai hyperbilirubinemic rats. *Hepatology.* **24:** 253–258

Ma Y, Hendershot LM. 2001.The unfolding tale of the unfolded protein response. *Cell*. **107:** 827-830

Madon J, Hagenbuch B, Landmann L, Meier PJ, Stieger B. 2000. Transport function and hepatocellular localization of mrp6 in rat liver. *Mol. Pharmacol*. **57:** 634–641

Madrid R, Le Maout S, Barrault MB, Janvier K, Benichou S, Merot J. 2001. Polarized trafficking and surface expression of the AQP4 water channel are coordinated by serial and regulated interactions with different clathrin-adaptor complexes. *EMBO J.* **20:** 7008-7021

Maliepaard M, Scheffer GL, Faneyte IF, van Gastelen MA, Pijnenborg AC, Schinkel AH, van De Vijver MJ, Scheper RJ, Schellens JH.2001. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer Res.* **61:** 3458-3464

Mao Q, Qiu W, Weigl KE, Lander PA, Tabas LB, Shepard RL, Dantzig AH, Deeley RG, Cole SP. 2002. GSH-dependent photolabeling of multidrug resistance protein MRP1 (ABCC1) by [125I]LY475776. Evidence of a major binding site in the COOH-proximal membrane spanning domain. *J Biol Chem.* **277:** 28690-28699

Martens AS, Bode JG, Heinrich PC, Graeve L. 2000. The cytoplasmic domain of the interleukin-6 receptor gp80 mediates its basolateral sorting in polarized madin-darby canine kidney cells. *J Cell Sci*. **113:** 3593-602

Mason DL, Michaelis S. 2002. Requirement of the N-Terminal Extension for Vacuolar Trafficking and Transport Activity ofYeast Ycf1p, an ATP-binding Cassette Transporter. *Mol Biol Cell*. **13:** 4443-55

Mateus Fernández SB, Hollo Z, Kern A, Bàkos E, Fischer PA, Borst P, Evers R. Role of the N-terminal transmembrane region of te multidrug resistance protein MRP2 in routing to the apical membrane in MDCKII cells. 2002. *J. Biol. Chem.* **277:** 31048-31055

Matter K, and Mellman, I. 1994. Mechanisms of cell polarity: sorting and transport in epithelial cells. *Curr. Opin. Cell Biol.* **6:** 545-554

Matter K, Yamamoto EM, Mellman I. 1994. Structural requirements and sequence motifs for polarized sorting and endocytosis of LDL and Fc receptors in MDCK cells. *J Cell Biol*. **126:** 991-1004

McAleer MA, Breen MA, White NL, Matthews N. 1999. pABC11 (also known as MOAT-C and MRP5), a member of the ABC family of proteins, anion transporter activity but does not confer multidrug resistance when overexpressed in human embryonic kidney 293 cells. *J Biol Chem*. **274:** 23541–23548

Melaine N, Lienard MO, Dorval I, Le Goascogne C, Lejeune H, Jegou B. 2002. Multidrug resistance genes and p-glycoprotein in the testis of the rat, mouse, Guinea pig, and human. *Biol Reprod*. **67:** 1699-1707

Miranda KC, Khromykh T, Christy P, Le TL, Gottardi CJ, Yap AS, Stow JL, Teasdale RD. 2001. A dileucine motif targets E-cadherin to the basolateral cell surface in Madin-Darby canine kidney and LLC-PK1 epithelial cells.*J Biol Chem* **276:**22565-2257

Mirski SEL, Gerlach JH, Cole SPC.1987. Multidrug resistance in a human small cell lung cancer cell line selected in adriamycin. *Cancer Res*. **47:** 2594-2598

Miyake K, Mickley L, Litman T, Zhan Z, Robey R, Cristensen B, Brangi M, Greenberger L, Dean M, Fojo T, Bates SE.1999. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transporters. *Cancer Res.* **59:** 8-13

Momburg F, Roelse J, Howard JC, Butcher GW, Hammerling GJ, Neefjes JJ. 1994. Selectivity of MHC-encoded peptide transporters from human, mouse and rat. *Nature*. **367:** 648-651

Mottino AD, Hoffman T, Jennes L, Vore M. 2000. Expression and localization of multidrug resistant protein mrp2 in rat small intestine. *J Pharmacol Exp Ther*. **293:** 717–723

Moyer BD, Duhaime M, Shaw C, Denton J, Reynolds D, Karlson KH, Pfeiffer J, Wang S, Mickle JE, Milewski M, Cutting GR, Guggino WB, Li M, Stanton BA. 2000.The PDZ-interacting domain of cystic fibrosis transmembrane conductance regulator is required for functional expression in the apical plasma membrane. *J Biol Chem* **275:** 27069-27074

Müller M. http://nutrigene.4t.com/humanabc.htm

Müller M, Meijer C, Zaman GJR, Borst P, Scheper RJ, Mulder NH, de Vries EGE, Jansen PLM. 1994. Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport.*Proc Natl Acad Sci USA*. **91:** 13033-13037

Müller M, Roelofsen H, Jansen PLM. 1996. Secretion of organic anions by hepatocytes: involvement of homologues of the multidrug resistance protein. *Seminars in liver desease.* **16:** 211-220

Nies AT, König J, Cui Y, Brom M, Spring H, Keppler D. Structural requirement for the apical sorting of human multidrug resistance protein 2 (ABCC2). *Eur J Biochem*. **269:** 1866-1876

Nies AT, König J, Pfannschmidt M, Klar E, Hofmann WJ, Keppler D. 2001. Expression of the multidrug resistance proteins MRP2 and MRP3 in human hepatocellular carcinoma. *Int J Cancer.* **94:** 492-499

Nooter K, Westerman AM, Flens MJ, Zaman GJ, Scheper RJ, van Wingerden, Burger H, Oostrum R, Boersma T, Sonneveld P, et al. 1995. Expression of the multidrug resistance associated protein (MRP) gene in human cancers. *Clin Cancer Res.* **1:** 1301-1310

Norman BH, Gruber JM, Hollinshead SP, Wilson JW, Starling JJ, Law KL, Self TD, Tabas LB, Williams DC, Paul DC, Wagner MM, Dantzig AH. 2002. Tricyclic isoxazoles are novel inhibitors of the multidrug resistance protein (MRP1*). Bioorg Med Chem Lett.* **12:** 883-886

Odorizzi G, and Trowbridge IS. 1997a. Structural requirements for basolateral sorting of the human transferrin receptor in the biosynthetic and endocytic pathways of Madin-Darby canine kidney cells. *J. Cell Biol.* **137:** 1255-1264

Odorizzi G and Trowbridge IS. 1997b. Structural requirements for major histocompatibility complex class II invariant chain trafficking in polarized Madin-Darby canine kidney cells. *J. Biol. Chem.* **272:** 11757–11762

O'Neill GM, Peters GB, Harvie RM, MacKenzie HB, Henness S, Davey RA. Amplification and expression of the ABC transporters ARA and MRP in a series ofmultidrug-resistant leukaemia cell sublines.1998. *Br J Cancer* **77:** 2076 –2080

Ogawa K, Suzuki H, Hirohashi T, Ishikawa T, Meier PJ, Hirose K, Akizawa T, Yoshioka M, Sugiyama Y. Characterization of inducible nature of MRP3 in rat liver. 2000. *Am J Physiol Gastrointest Liver Physiol*. **278:** G438–G446

Ohno H, Stewart J, Fournier MC, Bosshart H, Rhee I, Miyatake S, Saito T, Gallusser A, Kirchhausen T, and Bonifacino JS. (995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science.* **269:** 1872-1875

Ohno, H., Tomemori, T., Nakatsu, F., Okazaki, Y., Aguilar, R.C., Foelsch, H., Mellman, I., Saito, T., Shirasawa, T., and Bonifacino, J.S. 1999. Mu1B, a novel adaptor medium chain expressed in polarized epithelial cells. *FEBS.* Lett. **449:** 215-220

Oka M, Fukuda M, Sakamoto A, Takatani H, Fukuda M, Soda H, Kohno S.1997. The clinical role of MDR1 gene expression in human lung cancer. *Anticancer Res.* **17:** 721-724

Olsen O, Liu H, Wade JB, Merot J, Welling PA. 2002. Basolateral membrane expression of the Kir 2.3 channel is coordinated by PDZ interaction with Lin-7/CASK complex. *Am J Physiol Cell Physiol.* **282:**C183-95

Ortiz DF, Li S, Iyer R, Zhang X, Novikoff P, Arias IM. 1999. MRP3, a new ATP-binding cassette protein localized to the canalicular domain of the hepatocyte. *Am J Physiol.* **276:** G1493-1500

Oude Elferink RP, Bakker CT, Roelofsen H, Middelkoop E, Ottenhoff R, Heijn M, Jansen PL. 1993. Accumulation of organic anion in intracellular vesicles of cultured rat

hepatocytes is mediated by the canalicular multispecific organic anion transporter. *Hepatology*. **17:** 434-444

Pascolo L, Fernetti C, Pirulli D, Bogoni S, Garcia-Mediavilla MV, Spano A, Puzzer D, Tiribelli C, Amoroso A, Crovella S. 2000. Detection of MRP1 mRNA in human tumors and tumor cell lines by in situ RT-PCR. *Biochem Biophys Res Commun.* **275:** 466-471

Paulusma CC, Bosma PJ, Zaman GJR, Bakker CTM, Otter M, Scheffer GL, Scheper RJ, Borst P, Oude Elfernik RPJ. 1996. Congenital jaundice in rats with a mutation in a multidrug resistance associated protein gene. *Science.* **271:** 1126 –28

Paulusma CC, Kool M, Bosma PJ, Scheffer GL, ter Borg F, Scheper RJ, Tytgat GN, Borst P, Baas F, Oude Elferink RP.1997. A mutation in the human canalicular multispecific oragnic anion transporter gene causes the Dubin-Jonson syndrome. *Hepatology.* **25:** 1539-1542

Paulusma CC, Oude Elferink RPJ. 1997. The canalicular multispecific organic anion transporter and conjugated hy-perbilirubinemia in rat and man. *J Mol Med*. **s** 420–428

Paulusma CC, van Geer MA, Evers R, Heijn M, Ottenhoff R, Borst P, Oude Elferink RPJ. 1999. Canalicular multispecific organic anion transporter/ multidrug resistance protein 2 mediates low-affinity transport of re-duced glutathione. *Biochem J*. **338:** 393–401

Paumi CM, Ledford BG, Smitherman PK, Townsend AJ, Morrow CS. 2001. Role of the multidrug resistance protein 1 (MRP1) and glutathione S-transferase A1-1 in alkylating agent resistance: kinetics of gluathione conjugate formation and efflux govern differential cellular sensitivitiy to chlorambucil versus melphalan toxicity. *J Biol Chem*. **276:** 7952–7956

Qian YM, Grant CE, Westlake CJ, Zhang DW, Lander PA, Shepard RL, Dantzig AH, Cole SP, Deeley RG. 2002. Photolabeling of human and murine multidrug resistance protein 1 with the high affinity inhibitor [125I]LY475776 and azidophenacyl-[35S]glutathione. *J Biol Chem.* **277:** 35225-35231

Qian YM, Qiu W, Gao M, Westlake CJ, Cole SP, Deeley RG. 2001 Characterization of binding of leukotriene C4 by human multidrug resistance protein 1: evidence of differential interactions with NH2- and COOH-proximal halves of the protein. *J Biol Chem.* **276:** 38636-386344

Qian YM, Song, WC, Cui HR, Cole SPC, Deeley RG. 2001. Glutathione stimulate sulfated estrogen trans-port by multidrug resistance protein 1. *J Biol Chem.* **276:** 6404–6411

Rao VV, Dahlheimer JL, Bardgett ME, Snyder AZ, Finch RA, Sartorelli AC, Piwnica-Worms D. 1999. Choroid plexus epithelial expression of *MDR1* P-glycoprotein and multidrug resistance-associated protein contribute tho the blood-cerebrospinal-fluid drug-permeability barrier. *Proc Natl Acad Sci USA.* **96:** 3900-3905

Ren XQ, Furukawa T, Aoki S, Sumizawa T, Haraguchi M, Nakajima Y, Ikeda R, Kobayashi M, Akiyama S. 2002. A positively charged amino acid proximal to the C-terminus of TM17 of MRP1 is indispensable for GSH-dependent binding of substrates and for transport of LTC4. *Biochemistry.* **41:** 14132-14140

Renes J, de Vries EE, Hooiveld GJ, Krikken I, Jansen PL, Müller M.. 2000. The multidrug resistance protein MRP1 protects against the toxicity of the major lipid peroxidation product 4—hydroxinonenal *Biochem J*. **350:** 555-561

Renes J, de Vries EGE, Nienhuis EF, Jansen PLM, Müller M. 1999. ATP- and glutathione-dependent transport of chemotherapeutic drugs by the multidrug resistance protein MRP1. *Br J Pharmacol.* **126:** 681-688

Ringpfeil F, Lebwohl MG, Christiano AM, Uitto J. 2000. Pseudoxanthoma elasticum: mutations in the MRP6 gene encoding a transmembrane ATP binding cassette (ABC) trnaporter.*Proc Natl Acad Sci USA* . **97:** 6001–6006

Ritter JK. 2000. Roles of glucuronidation and UDP-glu-curonosyltransferases in xenobiotic bioactivation reactions. *Chem Biol Interact*. **129:** 171–193

Robbiani DF, Finch DA, Jager D, Müller WA, Sartorelli AC, Randolph GJ. 2000. The leukotriene C(4) transporter MRP1 regulates CCL19 (MIP-3beta, ELC)-dependent mobilization of dendritic cells to lymph nodes.*Cell.* **103:** 757-768

Robinson MS and Bonifacino JS, 2001. Adaptor-related proteins. *Curr Opin Cell Biol*. **13:** 444-453

Rosenberg MF, Callaghan R, Ford RC, Higgins CF. 1997. Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by microscopy and image analysis. *J Biol Chem*. **272:** 10685-10694

Rosenberg MF, Mao Q, Holzenburg A, Ford RC, Deeley RG, Cole SPC. 2001a. The structure of the multidrug resistance protein 1 (MRP1/ABCC1). Cristallization and single-particle analysis. *J Biol Chem.* **276:** 16076-16082

Rosenberg MF, Velarde G, Ford RC, Martin C, Berridge G, Kerr ID, Callaghan R, Schmidlin A, Wooding C, Linton KJ, Higgins CF. 2001b. Repacking of the transmembrane domains of P-glycoprotein during the transport ATPase cycle. *EMBO J***. 20:** 5615-5625

Ross DD, Yang W, Abruzzo LV, Dalton WS, Schneider E, Lage H, Dietel M, Greenberger L, Cole SP, Doyle LA. 1999. Atypical multidrug resistance: breast cancer resistance protein messenger RNA expression in mitoxantrone-selected cell lines. *J Natl Cancer Inst.* **91:** 429-433

Ruetz S, Gros P. 1994. Phosphatidiylcholine translocase: a physiologiacal role for the *Mdr2* gene. *Cell.* **77:** 1071-1081

Sakamoto H, Hara H, Hirano K, Adachi T. 1999. En-hancement of glucuronosyl etoposide transport by glu-tathione in multidrug resistance-associated protein-overexpressing cells. *Cancer Lett* **135:** 113–119

Salerno M, Garnier-Suillerot A. 2001. Kinetics of glutathione and daunorubicin efflux from multidrug resistance protein overexpressing small-cell lung cancer cells. *Eur J Pharmacol*. **42:** 1-9

Schaub T, Ishikawa T, Keppler D. 1991. ATP-dependent leukotriene export from mastocytoma cells. *FEBS Lett*. **279:** 83-86

Schaub TP, Kartenbeck J, König J, Spring H, Dorsam J, Staehler G, Storkel S, Thon WF, Keppler D. 1999. Expression of the MRP2 gene-encoded conjugate export pump in human kidney proximal tubules and in renal cell carcinoma. *J Am Soc Nephrol.* **10:** 1159–1169

Scheffer GL, Kool M, de Haas M, de Vree JM, Pijnenborg AC, Bosman DK, Elferink RP, van der Valk P, Borst P, Scheper RJ. 2002. Tissue distribution and induction of human multidrug resistant protein 3. *Lab Invest*. **82:** 193-201

Schinkel AH, Mayer U, Wagenaar E, Mol CA, van Deemter L, Smit JJ, van der Valk, MA, Voordouw AC, Spits H, van Tellingen O, Zijlmans JM, Fibbe WE, Borst P1997. Normal viability and altered pharmacokinetics in mice lacking Mdr1-type (drug transporting) P-glycoproteins. *Proc Natl Acad Sci USA*. **94:** 4028-4033

Schinkel AH, Wagenaar E, Mol CA, van Deemter L. 1996. P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *J Clin Invest*. **97:** 2517-2524

Schuetz JD, Connelly MC, Sun D, Paibir SG, Flynn PM, Srinivas RV, Kunar A, Fridland A. 1999. A previously unidentified factor in resistance to nucleoside-based antiviral drugs. *Nat Med.* **5:** 1048-1051

Seelig A, Blatter XL, Wohnsland F. 2000. Substrate recognition by P-glycoprotein and the multidrug resistance-associated protein MRP1: a comparison. *Int J Clin Pharmacol Ther.* **38:** 111–121

Shen D, Pastan I, Gottesman MM. 1998. Cross-resistance to methotrexate and metals in human cisplatin-resistant cell lines results from a pleiotropic defect in accumulation of these compounds associated with reduced plasma membrane binding proteins.1998. *Cancer Res*. **58:** 268-275

Shen DW, Goldenberg S, Pastan I, Gottesman MM.2000. Decreased accumulation of [14C]carboplatin in human cisplatin-resistant cells results from reduced energy-dependent uptake.*J. Cell Physiol*. **183:** 108-116

Shen ZX, Chen GQ, Ni JH, Li XS, Xiong SM, Qiu QY, Zhu J, Tang W, Sun GL, YanKQ, Chen Y, Zhou L, Fang ZW, Wang YT, Ma J, Zhang P, Zhang TD, Chen SJ Chen Z, Wang ZY. 1997. Use of arsenic trioxide (As2O3) in the treatment of acute

promyelocytic leukemia (APL):Clinical efficacy and pharmacokinetics in relapsed patients. *Blood.* ***8*9:** 3354-3360

Shepherd JC, Schumacher TN, Ashton-Rickardt PG, Imaeda S, Ploegh HL, Janeway CA Jr, Tonegawa S. 1993. TAP1-dependent peptide translocation in vitro is ATP dependent and peptide selective. *Cell.* **74:** 577-584

Simmen T, Nobile M, Bonifacino JS and Hunziker W. 1999. Basolateral sorting of furin in MDCK cells requires a phenylalanine–isoleucine motif together with an acidic amino acid cluster. *Mol. Cell. Biol.* **19:** 3136–3144

Simons K and Ikonen E. 1997. Functional rafts in cell membranes. *Nature*. **387:** 569-572

Simons K, Wandinger-Ness. (1990). Polarized sorting in epithelia. *Cell* **62**: 207-210

Slovak ML, Ho JP, Bhardwaj G, Kurz EU, Deeley RG, Cole SPC. Localization of a novel multidrug resistance-associated gene in the HT1080/DR4 and H69ARhuman tumor cell lines. *Cancer Res*. **53:** 3221-3225

St.Pierre MV, Serrano ME, Macias RIR, Dubs U, Hoechli M, Lauper U, Meier PJ, Marin JJG. 2000. Expression of members of the multidrug resistance protein family in human term placenta. *Am J Physiol*. **279:** R1495–R1503

Stockel B, König J, Nies AT, Cui Y, Brom M, Keppler D. 2000. Characterization of the 5' flanking region of the human multidrug resistance protein 2 (MRP2) gene and its regulation in comparison with the multidrug resisatance protein 3 (MRP3) gene. *Eur J Biochem.* **267:** 1347-1358

St-Pierre MV, Serrano MA, Macias RI, Dubs U, Hoechli M, Lauper U, Meier Marin JJ.2000. Expression of members of the multidrug resistance protein family in human term placenta. *Am J Physiol Regul Integr Comp Physiol*. **279:** R1495-503.

Strautnieks SS, Bull LN, Knisely AS, Kocoshis SA, Dahl N, Arnell H, Sokal E, Dahan K, Childs S, Ling V, Tanner MS, Kagalwalla AF, Nemeth A, Pawlowska J, Baker A, Mieli-Vergani G, Freimer NB, Gardiner RM, Thompson RJ. 1998. A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nature Genetic*. **20:** 233-238

Suzuki H, Sugiyama Y. 1998. Excretion of GSSG and glutathione conjugates mediated by MRP1 and cMOAT/MRP2. *Semin Liver Dis.* **18:** 359-376

Tammur J, Prades C, Arnould I, Rzhetsky A, Hutchinson A, Adachi M, Schuetz J, Swoboda KJ, Ptacek LJ, Rosier M, Dean M, Allikmets R.2001.Two new genes from the human ATP-binding cassette transporter superfamily, ABCC11 and ABCC12, tandemly duplicated on chromosome 16q12. *Gene.* **273:** 89-96

Tarentino AL, Plummer TH. 1994. Enzymatic deglycosylation of asparagine-linked glycans: purification, properties, and specificity of oligosaccharide-cleaving enzymes from Flavobacterium meningosepticum. *Methods Enzymol*. **230:** 44-57

Toh S, Wada M, Uchiumi T, Inokuchi A, Makino Y, Horie Y, Adachi Y, Sakisaka S, Kuwano M. 1999. Genomic structure of the canalicular multispecific organic anion-transporter gene (MRP2/cMOAT) and mutations in the ATP-binding-cassette region in Dubin-Johnson syndrome. *Am J Hum Genet*. **64:** 739-746

Tomii K, Kanehisa M. 1998. A comparative analysis of ABC transporters in complete microbial genomes.*Genome Res*. **8:** 1048-1059

Trauner M, Arrese M, Soroka CJ, Ananthanarayanan M, Koeppel TA, Schlosser SF, Suchy FJ, Keppler D, Boyer JL. 1997. The rat canalicular conjugate export pump (Mrp2) is down-regulated in intrahepatic and obstructive cholestasis. *Gastroenterology*. **113:** 255-264

Trock BJ, Leonessa F, Clarke R. 1997.Multidrug resistance in breast cancer: a meta analysis od MDR/GP170 expression and its possible funcional significance. *J Natl Cancer Inst*. **89:** 917-931

Trowsdale J, Hanson I, Mockridge I, Beck S, Townsend A, Kelly A.1 990. Sequences encoded in the class II region of the MHC related to the 'ABC' superfamily of transporters. *Nature*. **348:** 741-744

Tusnady GE, Bàkos E, Varadi A, Sarkadi B. 1997. Membrane topology distinguishes a subfamily of the ATP-binding cassette (ABC) transporters. *FEBS Lett.* **402:** 1-3

Van Aubel RAMH, Koenderink JB, Peters JGP, van Os CH, Russel FGM. 1999. Mechanisms and interac-tion of vinblastine and reduced glutathione transport in membrane vesicles by the rabbit multidrug resistanceprotein Mrp2 expressed in insect cells. *Mol Pharmacol***. 56:** 714–719

Van Aubel RAMH, Smeets PHE, Peters JGP, Bindls RJM, Russel FGM. 2002. The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proxilam tubules: putative efflux pump for urinary cAMP and cGMP. *J Am Soc Nephrol*. **13:** 595-693

Van Bladeren PG. 2000. Glutathione conjugation as a bioac-tivation reaction. *Chem Biol Interact*. **129:** 61–76

Vanhoefer U, Yin MB, Harstrick A, Seeber S, Rustum YM.1997. Carbamoylation of glutathione reductase by N,N-bis(2-chloroethyl)-N- nitrosourea associated with inhibition of multidrug resistance protein (MRP) function. *Biochem Pharmacol*. **53:** 801-809

van Veen HW, Higgins CF, Konings WN.2001. Molecular basis of multidrug transport by ATP-binding cassette transporters: a proposed two-cylinder engine model. *J Mol Microbiol Biotechnol.* **3:** 185-192

Versantvoort CH, Broxterman HJ, Bagrij T, Scheper RJ, Twentyman PR. 1995 Regulation by glutathione of drug transport in multidrug-resistant human lung tumour

cell lines overexpressing multidrug resistance-associated protein. *Br J Cancer*. **72:** 82-89

Walker JE, Saraste M, Runswick MJ, Gay NJ. 1982. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J*. **1:** 945-951

Wang W, Ballatori N. 1998. Endogenous glutathione conjugates: occurence and biological functions. *Pharmacol Rev*. **50:** 335-356

Whitfield CW, Benard C, Barnes T, Hekimi S, Kim SK. Basolateral localization of the Caenorbitis elegans epidermal growth factor receptor in epitheliac cells by the PDZ protein LIN-10. 1999. *Mol Biol Cell*. **10:** 2087-2100

Wielinga PR, Reid G, Challa EE, Van Der Heijden I, Van Deemter L, De Haas M, Mol C, Kuil AJ, Groeneveld E, Schuetz JD, Brouwer C, De Abreu RA, Wijnholds J, Beijnen JH, Borst P. 2002. Thiopurine Metabolism and Identification of the Thiopurine Metabolites Transported by MRP4 and MRP5 Overexpressed in Human Embryonic Kidney Cells. *Mol Pharmacol*. **62:** 1321-1331

Wijnholds J, De Lange ECM, Scheffer GL, Van den Berg DJ, Mol CAAM, van der Valk M, Schinkel AH, Scheper RJ, Breimer DD, Borst P. 2000a. Mutlidrug resistance protein I protects the choroid plexis epithelium and contributes to the blood-cerebrospinal fluid barrier. *J Clin Invest.* **105:** 279-285

Wijnholds J, Evers R, van Leusden MR, Mol CA, Zaman GJ, Mayer U, Beijnen JH, van der Valk M, Krimpenfort P, Borst P. 1997. Increased sensitivity to anticancer drugs and decreased inflammatory response in mice lacking the multidrug resistance-associated protein. *Nat Med* **11:** 1275–79

Wijnholds J, Mol CAAM, Van Deemter L, De Haas M, Scheffer GL, Baas F, Beijnen JH, Scheper RJ, Hatse, De Clercq E, Balzarini J, Borst P. 2000b. Multidrug resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs. *Proc Natl Acad Sci USA.* **97:** 7476–7481

Wijnholds J, Scheffer GL, van der Valk M, van der Valk P, Beijnen JH, Scheper RJ, Borst P. 1998. Mutlidrug resistance protein I portects the oropharingeal mucosal layer and the testicular tubules against drug-induced damage. *J Exp Med.* **188:** 797-808

Xie R, Hammarlund-Udenaes M, de Boer AG, de Lange EC. 1999. the role of P-glycoprotein in blood-brain barrier transport of morphine: transcortical microdialysis studies in Mdr1 $a^{(-/-)}$ and Mdr1 $a^{(+/+)}$ mice. *Br J Pharmacol*. **128:** 563-568

Yang Y, Chen Q, Zhang JT. 2002. Structural and functional consequences of mutating cysteine residues in the amino terminus of human multidrug resistance-associated protein 1 (MRP1). *J Biol Chem*. **277:** 44268-44277

Yeaman C, Grindstaff KK, Nelson WJ. New perspectives on mechanisms involved in generating epithelial cell polarity. 1999. *Physiol Rev*. **79:** 73-98

Young LC, Campling BG, Cole SP, Deeley RG, Gerlach JH. 2001. Multidrug resistance proteins MRP3, MRP1, and MRP2 in lung cancer: correlation of protein levels with drug response and messenger RNA levels. *Clin Cancer Res*. **7:** 1798-1804

Young LC, Campling BG, Voskoglou-Nomikos T, Cole SP, Deeley RG, Gerlach JH. 1999. Expression of multidrug resistance protein-related genes in lung cancer: correlation with drug response. *Clin Cancer Res*. **5:** 673-680

Yuan YR, Blecker S, Martsinkevich O, Millen L, Thomas PJ, Hunt JF. 2001. The crystal structure of the MJ0796 ATP-binding cassette. Implications for the structural consequences of ATP hydrolysis in the active site of an ABC transporter. *J Biol Chem.* **276:** 32313-32321

Zaman GJ, Cnubben NH, van Bladeren PJ, Evers R, Borst P. 1996. Transport of the glutathione conjugate of ethacrynic acid by the human multidrug resistance protein MRP. *FEBS Lett*. **391:** 126–130

Zaman GJ, Lankelma J, van Tellingen O, Beijnen J, Dekker H, Paulusma C, Oude Elferink RP, Baas F, Borst P. 1995. Role of glutathione in the export of compuonds from cells by the multidrug resitance-associates protein. *Proc Natl Acad Sci USA.* **92:** 7690-7694

Zelcer N, Reid G, Wielinga P, Kuil A, Van Der Heijden I, Schuetz JD, Borst P. 2003a. Steroid- and bile acid-conjugates are substrates of human MRP4 (ABCC4). *Biochem J*. [epub ahead of print]

Zelcer N, Saeki T, Reid G, Beijnen JH, Borst P. 2001. Characterization of drug transport by the human multidrug resistance protein 3 (ABCC3). *J. Biol. Chem.* **276:** 400–407

Zelcer N, Saeki T, Bot I, Kuil A, Borst P. 2003b. Transport of bile acids in multidrug-resistance-protein 3-overexpressing cells co-transfected with the ileal Na+-dependent bile-acid transporter. *Biochem J.* **369:** 23-30

Zeng H, Bain LJ, Belinsky MG, Kruh GD. 1999. Expression of multidrug resistance protein-3 (multispecific organic anion transporter-D) in human embryonic kidney 293 cells confers resistance to anticancer agents. *Cancer Res*. **59:** 5964–5967

Zhang DW, Cole SP, Deeley RG. 2001. Identification of a nonconserved amino acid residue in multidrug resistance protein 1 important for determining substrate specificity: evidence for functional interaction between transmembrane helices 14 and 17. *J Biol Chem.* **276:** 34966-34974

Zhang Y, Han HY Elmquist WF, Miller DW. 2000. Expression of various multidrug resistance-associated protein (MRP) homologues in brain microvessel endothelial cells. *Brain Res.* **876:** 148–153

Zhou Y, Gottesman MM, Pastan I. Studies of human MDR1-MDR2 chimeras demonstrate the functional exchangeability of a major transmenbrane segment of the

multidrug transporter and phosphatidylcholine flippase. 1999. *Mol Cell Biol.* **19:** 1450-1459

Zhou Z, White KA, Polissi A, Georgopoulos C, Raetz CR. 1998. Function of Escherichia coli MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis. *J Biol Chem.* **273:** 2466-2475

8 APPENDIX

CURRICULUM VITAE

Name:	Giulia Calenda
Geschlecht:	weiblich
Geburtsdatum:	19.08.1973
Geburtsort:	Salzburg, Österreich
Familienstand:	verheiratet
Staatsangehörigkeit:	italienisch

1992/07	Diploma di Maturitá Scientifica (Abitur) an der Schule Liceo Scientifico Giuseppe Berto
1992/10-1999/12	Studium an der Universitá degli Studi di Padova
1999/12	Laurea (Diplom) in dem Fach “Pharmazeutische Chemie und Technologie” mit der Arbeit: “Vergleichsstudie über die Transfektionseffizienz von Mäuse-Muskelzellen mit nicht-viralen Vektoren”
1999/02-1999/04	Teilnahme an an das Europeische Austauschsprogramm „Leonardo“ am Universitätsklinikum Frankfurt/Main, AG Pr. Dr. K. Strebhart
1999/05-2003	Doktorandin am chemotherapeutischem Forschungsinstitut Georg-Speyer-Haus in Frankfurt/Main, AG Dr Raymond Evers. Titel der Arbeit: “Role in routing to the plasma membraneof the L_0 domain of the multidrug resistance protein MRP1”

Publikationen:

Bàkos E*, Evers R*, **Calenda G**, Tusnady GE, Szakacs G, Varadi A, Sarkadi B. Characterization of the amino-terminal regions in the human multidrug resistance protein (MRP1). J Cell Sci. 2000 Dec;113 Pt 24:4451-61.

*These authors contributed equally to this work

Abstracts:

Evers R, **Calenda G** (2000). Routing of the multidrug resistance protein MRP1 in MDCKII cells. ELSO-meeting, Genf, Schweiz, September 2-6, 2000.

Calenda G, Evers R (2001). Characterization of the multidrug resistance protein MRP1 in polarized MDCKII cells. PharmaConference 2001, Interlaken, Schweiz, August 5-10, 2001.

Sprachkenntnisse:

Italienisch – Muttersprache

Deutsch – Muttersprache

Englisch – fließend in Schrift und Sprache

Spanisch – Grundwissen

Computerkenntnisse:

Windows und Macintosh: Office, Photoshop, Netscape Anwendungen

Thanks...

To Dr. Raymond Evers, who gave me the opportunity to remarkably improve my experience and my knowledge. I also thank him for his constant and intense attention in the lab and from far and for the inspiring phone calls that encouraged me when I was losing motivation.

To Dr. Martin Zörnig and his group who welcomed me so kindly, for their precious practical support, and their friendship. I had a good time with you all!

To my friends, Claudia Bürger, who lightened me in my fight “against” the scientific German language and Stefan Theis, who was always willing to help me.

To all the friends, that made the time in Frankfurt such a special time, I miss you so much!

To my husband Ringo, for being always on my side, for his great encouragement, the technical help and his astonishing talent to stand me in every time…

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Frankfurt am Main, den 21. März 2003

Giulia Calenda