Apical routing of MRP2

Role of the N-terminal transmembrane region of the multidrug resistance protein MRP2

in routing to the apical membrane in MDCKII cells

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Summary

In polarized cells, the multidrug resistance protein MRP2 is localized in the apical plasma membrane, whereas MRP1, another MRP family member, is localized in the basolateral membrane. MRP1 and MRP2 are thought to contain an N-terminal region of five transmembrane segments (TMD0) coupled to two times six transmembrane segments via an intracellular loop (L0). We previously demonstrated for MRP1 that a mutant lacking TMD0 but still containing L0, called L0ΔMRP1, was functional and routed to the lateral plasma membrane. To investigate the role of the TMD0L0 region of MRP2 in routing to the apical membrane, we generated mutants similar to those made for MRP1. In contrast to L0-MRP1, L0ΔMRP2 was associated with an intracellular compartment, most likely endosomes. Co-expression with TMD0, however, resulted in apical localization of L0ΔMRP2 and transport activity. Uptake experiments with vesicles containing L0-MRP2 demonstrated that the molecule is able to transport LTC4. A MRP2 mutant without TMD0L0, ΔMRP2, was only core glycosylated and localized intracellularly. Co-expression of ΔMRP2 with TMD0L0 resulted in an increased protein level of ΔMRP2, full glycosylation of the protein, routing to the apical membrane and transport activity. Our results suggest that the TMD0 region is required for routing to or stable association with the apical membrane.
Introduction

Several members of the ATP-binding cassette superfamily of transporter proteins are able to confer multidrug resistance to tumor cells. Examples are MDR1 P-glycoprotein (1) and members of the multidrug resistance protein (MRP) family MRP1 and MRP2 (2, 3). Whereas MDR1 Pgp preferably transports large amphipathic molecules (4), MRP1 and MRP2 transport in addition acidic compounds with a large hydrophobic moiety such as drugs conjugated with glutathione, glucuronide or sulfate (5-7). MRP1 and MRP2 mediated transport of cytotoxic drugs not known to be conjugated to negatively charged ligands is most likely due to co-transport with reduced glutathione (8-12). Besides MRP1 and MRP2, there are seven other MRP homologs expressed in humans, called MRP3-9 (13-15). MRP3-5 can confer resistance against some anti-cancer drugs and are able to transport certain organic anions (16-21).

The predicted membrane topology of MDR1 Pgp and MRPs is different. Whereas MDR1 Pgp and MRPs share a similar core region consisting of two times six transmembrane regions and two intracellular ATP-binding cassettes, MRP1 and MRP2 and several other members of the MRP family contain an extra amino-terminal domain of about 280 amino acids (22-26; see also Figure 1). This latter domain is thought to consist of five transmembrane segments and is bound to the core region via an intracellular loop (L0) of approximately 80 amino acids. By producing various mutants of MRP1 in baculo virus infected insect cells, others and we previously showed that the MRP1 core, called ΔMRP1, was inactive. Co-expression of the core (-MRP1) with
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TMD0L0, however, resulted in an active transporter (24, 27). Interestingly, extending the core region with the intracellular loop L0 alone also resulted in transport activity, indicating that the TMD0 region is dispensable for the function of MRP1 (27). In a recent report, we demonstrated that co-expression of the L0 peptide with ∆MRP1 in insect cells resulted in an active transporter, suggesting that L0 forms a distinct domain within MRP1 that specifically interacts with the core region (28).

In polarized monolayers of Madin-Darby canine kidney (MDCKII) cells MDR1 Pgp and MRP2 are localized in the apical membrane, whereas MRP1 is found in the lateral membrane (29, 30, 27, 31). Interestingly, the MRP1 molecule containing L0 but lacking the TMD0 segment (L0-MRP1) which showed activity in insect cells was also active in MDCKII cells and routed to the lateral plasma membrane (27).

To examine whether the TMD0 segment is dispensable for the routing of MRP2 to the apical membrane, we have constructed similar mutants as described above for MRP1. Our results show that the TMD0 region of MRP2 is required for apical routing in MDCKII cells and/or for the stabilization of the protein in the apical plasma membrane, in contrast to what was previously found for MRP1.
Experimental Procedures

Materials

\(^{3}\text{H}\)Vinblastine (9.4 Ci/mmol), inulin-\(^{14}\text{C}\)carboxylic acid (6.4 mCi/mmol; average molecular weight 5175) and \(^{14}\text{C}\)-1-chloro-2,4-dinitrobenzene ([\(^{14}\text{C}\)]CDNB; 10 mCi/mmol) were from Amersham Pharmacia Biotech (Little Chalfont, England).

\(^{3}\text{H}\)LTC\(_4\) (135 Ci/mmol) was from Dupont NEN (USA). Lactacystin and N-acetyl-L-leucil-L-leucil-L-norleucinal (ALLN) were from Calbiochem (Bad Soden, Germany), and tunicamycin from Sigma (Deisenhofen, Germany). pECFP-Endo and pECFP-Golgi vectors were from Clontech Laboratories, Inc. (Palo Alto, U.S.A). Alexa Fluor 532 goat anti-mouse Ig (H+L) conjugate was from Molecular Probes (Eugene, U.S.A). FITC labeled, sheep anti-mouse IgG was from Chemicon International, Inc. (Temecula, CA, U.S.A.).

Generation of MRP2 variants and their expression in MDCKII cells

All MRP2 (ABCC2) mutants used in this study (see Figure 1) were generated by PCR using the human MRP2 sequence as the template (32, 2GenBank). The HA-TMD\(_0\) fragment encompasses amino acids 2-189 with a hemagglutinin tag (amino acids MAYPYDVPDYA) fused to the amino terminus. TMD\(_0\)L\(_0\) encompasses amino acids 1-305, ΔMRP2 amino acids 306-1545, and L\(_0\)ΔMRP2 amino acids 189-1545. PCR fragments were verified by sequence analysis. Further details are available upon request. MRP2, ΔMRP2, and L\(_0\)ΔMRP2 were inserted into the retroviral vector pCMV-neo (33);
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HA-TMD0 and TMD0L0 were inserted into the retroviral vector pBabe-CMV-Puro (kindly provided by Dr. J. Wijnholds, The Netherlands Cancer Institute, Amsterdam, The Netherlands; see also Bakos et al., (28)). Retroviral transductions of MDCKII cells were performed as described previously (30). After selection of transduced cells with either G418 (800 µg/ml) or puromycin (2 µg/ml), single clones were isolated. Detection of the various mutant MRP2 molecules was performed by Western blotting. Proteins were detected using the appropriate monoclonal antibodies (mAbs) (see Figure 2A).

Expression of MRP2 and L0-MRP2 in Insect cells and vesicle uptake experiments

L0-MRP2 cDNAs was removed from the pCMV-L0-MRP2 construct and subcloned into the pAcUW21 plasmid (InVitrogen, San Diego, CA). PAcUW21-MRP2 was described before (28). Recombinant baculovirus was prepared and cultured as described by Bakos et al., (27) by using the BaculoGold Transfection Kit (Pharmingen, San Diego, CA). [3H]LTC4 uptake experiments were performed as described before (27).

Immunocytochemistry

Cells were grown on microporous polycarbonate membrane filters (3µm pore size, 24 mm diameter, Transwell™ 3414; Corning Costar Corp., Cambridge, MA) at a density of 5 x 10⁵ cells per well as described previously (30). Antibody incubations were as described before (30). Cells were examined with a Leica TCS SP confocal laser scanning microscope (CLSM; Leica Microsystems Heidelberg GmbH, Mannheim, Germany)
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equipped with a x63 objective. For the co-localization experiments (Figure 7), cells were examined with a Zeiss Laser Scanning Microscope LSM410 equipped with a x40 objective. ECFP was excited with a Krypton 413 laser line, Alexa 532 with a HeNe 543 laser line.

***Transfection of pECFP-vectors in MDCKII cells***

MDCKII-MRP2 and MDCKII-L0ΔMRP2 cells were seeded in 12 well plates (3µm pore size, 12mm diameter, Transwell TM 3402; Corning Costar Corp., Cambridge, MA) at a density of 1.5 x 10⁵ cells per well. The next day, cells were transiently transfected with pECFP-Endo (0.8 Tg) or pECFP-Golgi (1.25 Tg) using Lipofectamine™ 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. Five hours after the transfection, the medium was replaced with D-MEM. Cells were analyzed after culturing for 24 h. Longer incubation times resulted in toxic effects of the pECFP vectors.

***Transport assays with MDCKII cells***

Cells were grown for 3 days on microporous polycarbonate filters at a density of 2 x 10⁶ cells per well. Export of [¹⁴C]DNP-GS from cells was determined by incubating cells with [¹⁴C]CDNB as described previously (30). [³H]Vinblastine transport assays were carried out exactly as described (30).

***Deglycosylation experiments and treatment with proteasome inhibitors***
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Cells were seeded in petridishes and grown overnight. The next day tunicamycin (1.5 µg/ml) was added to the medium and cells were harvested at the time points indicated. For treatment with proteasome inhibitors, cells were seeded as mentioned above. The next day lactacystin (50 µM) or ALLN (50 µg/ml) was added to the medium. Cells were harvested after an incubation time of 8h.
Results

Generation of MDCKII cells expressing MRP2 constructs

To investigate the role of the TMD0L0 segment in the routing and function of MRP2, the following mutants were generated (Figure 1): (i) ΔMRP2 (amino acids 306 to 1545), (ii) HA-TMD0 (HA-tag followed by amino acids 2-189) (iii) TMD0L0 (amino acids 1 to 305), and (iv) L0ΔMRP2 (amino acids 189-1545). The TMD0L0 and TMD0 fragments were cloned into the retroviral pBabe-CMV-puro vector. ΔMRP2 and L0ΔMRP2 were cloned into the pCMV-neo vector. The use of the pBabe-Puro-CMV and pCMV-neo vectors enabled the selection of two different constructs in one cell. The ΔMRP2 and L0ΔMRP2 proteins were detected with monoclonal antibody (mAb) M2III-6, TMD0L0 and L0ΔMRP2 with mAb M2I-4, and HA-TMD0 with mAb 12CA5 (recognizing the HA-epitope), as shown in Fig. 2A. Monoclonal antibody M2III-6 was raised against amino acids 1339-1545 from rat Mrp2 (3) but also recognizes human MRP2 (32), mAb M2I-4 was raised against amino acids 215-310 of human MRP2 (34).

We previously characterized MDCKII derived cell lines stably producing the MRP2 protein (30). Using retroviral transductions, we generated MDCKII derived clones stably expressing the ΔMRP2 or L0ΔMRP2 constructs. By Western blot analysis, we identified several clones that produced relatively high amounts of L0-MRP2 (Fig. 2B, lane 7). We also succeeded in isolating clones producing -MRP2, but the protein was difficult to detect in total cell lysates (not visible on the exposure of the Western blot shown in Fig. 2B, lane 3, but see Fig. 3). To investigate whether this was due to
proteasome-mediated degradation, cells were incubated in the presence of the proteasome inhibitors ALLN and lactacystin and this resulted in a clear increase in the amount of ΔMRP2 (Figure 3).

The MDCKII-MRP2 and MDCKII-L0-MRP2 cells were retrovirally transduced with plasmids containing either TMD0L0 or HA-TMD0. Cells were selected with puromycin and single clones were analyzed by Western blotting. As documented in Fig. 2B and C, clones producing both the -MRP2 and TMD0L0 protein were isolated. Remarkably, whereas -MRP2 produced alone was hardly detectable, co-production with TMD0L0 resulted in a strong increase in the amount of -MRP2 (Fig. 2B, lane 3 versus lanes 4 and 5). In the clone containing both -MRP2 and TMD0 (without the L0 part), the amount of -MRP2 was not increased (Fig. 2B, lane 6). Cells producing L0-MRP2 and TMD0L0 or TMD0, respectively were also isolated (Fig. 2C and 2E, lanes 8-10). In these clones no significant changes in the level of L0-MRP2 was observed (Fig. 2B). TMD0 appeared as a discrete band with an apparent molecular weight of 24 kD and a number of bands around 40 kD (Fig. 2E, lanes 6 and 10). These are most likely explained by differential glycosylation as shown below. While clones producing the TMD0L0 region alone were isolated (Fig. 2D, lane 2), we only detected TMD0 if combined with -MRP2 (Fig. 2E, lane 6) or L0-MRP2 (Fig. 2E, lane 10).

The glycosylation sites present in MRP2 have not been mapped but, like in MRP1 (35), N-glycosylation sequences are present in the N-terminus (amino acids 7 and 12)
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and between putative transmembrane segments 12 and 13 (amino acid 1011). To investigate whether the various fragments of MRP2 were glycosylated, cells were grown in the presence of tunicamycin, a drug preventing N-linked glycosylation. As expected, full length MRP2 was glycosylated as illustrated by the appearance of a smaller band, around 150 kD in the presence of tunicamycin (Figure 4A). L₀ΔMRP2 was glycosylated both in the presence and absence of TMD₀ (Figure 4B and C). Part of ΔMRP2 was core glycosylated if produced alone, as illustrated by the band visible just above the unglycosylated protein (Figure 4D, lane 2). Interestingly, ΔMRP2 became fully glycosylated if co-produced with the TMD₀L₀ polypeptide and this glycosylation was inhibited by treatment with tunicamycin (Figure 4D). The amounts of unglycosylated MRP2, L₀ΔMRP2 and ΔMRP2 were strongly reduced compared to the glycosylated forms, suggesting that the unglycosylated proteins are rapidly degraded. Both the TMD₀ and TMD₀L₀ fragments were also glycosylated. An increased mobility of these proteins was observed in the presence of tunicamycin (Figure 4E and F). For MRP2 and TMD₀L₀ intermediate bands were observed between the glycosylated and deglycosylated proteins after t = 24 and 36 hours (Figure 4A and F, lanes 3 and 4). These could either be due to proteolysis or partial deglycosylation by endogenous endoglycosidases.

Immunolocalization of MRP2 mutants in MDCKII monolayers

The subcellular localization of the various MRP2 mutants was determined by indirect immunofluorescence using confocal laser scanning microscopy. The proteins were
detected with the same mAbs as used in the Western blot experiments. Cells were analyzed at the horizontal plane (X/Y) and at the plane perpendicular to the monolayer (X/Z). The apically localized wild-type MRP2 was used as a control in these experiments (Figure 5A). The results for ∆MRP2 can be summarized as follows: (i) ∆MRP2 alone was present in low concentrations in an intracellular compartment, probably the endoplasmic reticulum, but we have not verified this (Figure 5B). (ii) ∆MRP2 in cells co-expressing TMD$_0$L$_0$ together with ∆MRP2 was mainly present in the apical membrane (Figure 5C). A substantial amount of the TMD$_0$L$_0$ protein was localized intracellularly. Some protein, however, was also detected in the apical membrane. (iii) ∆MRP2 was detected intracellularly in cells co-expressing ∆MRP2 and TMD$_0$ (data not shown). (iv) The TMD$_0$L$_0$ polypeptide alone was localized in an intracellular compartment (Figure 5D).

As we previously found that L$_0$∆MRP1 was properly routed to the lateral membrane, we investigated whether L$_0$∆MRP2 similarly routed to the apical membrane. The results shown in Figure 6 document the following: (i) The L$_0$∆MRP2 protein produced alone is localized intracellularly (Figure 6A). (ii) In cells co-expressing L$_0$∆MRP2 and TMD$_0$, the L$_0$∆MRP2 protein was in the apical membrane although also some intracellular staining was observed (Figure 6C). In these cells, we could not visualize the TMD$_0$ polypeptide with mAb 12CA5 in immunofluorescence experiments. Full-length, HA-tagged MRP2 was also not detectable by immunofluorescence, although the protein was functional and visualized in Western blots with mAb 12CA5 (data not shown). The HA-epitope is probably not accessible in these proteins as we also did not succeed in
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detecting it with other anti-HA mAb or polyclonal antibodies using different fixation
methods. (iii) In cells co-expressing L0ΔMRP2 and TMD0L0, both proteins were
detected intracellularly (Figure 6B). Taken together, these data suggest that the TMD0
and TMD0L0 polypeptides interact with L0ΔMRP2 and ΔMRP2, respectively, to form a
protein routed to the apical membrane.

Since L0-MRP2 is glycosylated, it most likely is not a folding mutant associated
with the endoplasmic reticulum. To analyze whether the protein was associated with
endosomes or the Golgi apparatus, we performed co-localization studies with
fluorescently labeled marker proteins. As marker for the Golgi apparatus, we used a
fusion protein consisting of enhanced cyan fluorescent protein (ECFP) and a sequence
encoding the N-terminal 81 amino acids of human J-1,4-galactosyl transferase
(pECFP-Golgi; 36). As marker for endosomes, we used the pECFP-Endo vector, which
encodes a fusion protein between ECFP and the human RhoB GTPase (37). Stable
MDCKII-MRP2 and MDCKII-L0-MRP2 cells were transiently transfected with the
pECFP constructs. The co-localization of proteins in these assays resulted in a yellow
signal after merging the ECFP signal (shown in green in Fig. 7, left panels) with the
signal obtained with mAb M2III-6 (shown in red, middle panels). No overlap was
detected between MRP2 and the ECFP-Golgi protein, whereas some overlap was found
between MRP2 and the ECFP-Endo protein (Fig. 7A and B, right panels). Like MRP2,
LoΔMRP2 was not co-localizing with the ECFP-Golgi marker (Fig. 7C), but overlap
was found in the merge with the pECFP-Endo marker (Fig. 7D), suggesting that at least
part of this protein is associated with endosomes.

*Transport of \[^{3}H\]vinblastine and \[^{14}C\]dinitrophenyl glutathione by MDCKII derived monolayers*

To verify whether the co-produced proteins detected in the apical membrane were properly folded, we tested their biological activity. We have shown that wild-type MRP2 is able to transport the *Vinca* alkaloid \[^{3}H\]vinblastine to the apical side of a cell monolayer (30, 11). Using the same assay conditions, we analyzed the transport of vinblastine by the MDCKII derived cell lines expressing the various regions of MRP2. Figure 8A shows that wild-type MDCKII cells transported little vinblastine under the experimental conditions employed, whereas MDCKII-MRP2 cells demonstrated a clearly increased transport of vinblastine to the apical side of the cell monolayer. Neither in MDCKII-L\(_0\)\(\Delta\)MRP2 nor in MDCKII-\(\Delta\)MRP2 cells was a significantly increased transport rate detected to either side of the monolayer. In contrast, transport to the apical side of the monolayer was clearly increased in cells co-expressing \(\Delta\)MRP2 and TMD\(_0\)L\(_0\), or L\(_0\)\(\Delta\)MRP2 and TMD\(_0\), respectively (Figure 8B and C, right hand panels versus left hand panels).

An important characteristic of MRP2 is its ability to transport certain organic anions. A model substrate to measure MRP-mediated transport of an organic anion from intact cells is dinitrophenyl glutathione (DNP-GS). DNP-GS is hydrophilic and therefore only slowly diffuses over the plasma membrane. To measure transport of this compound, cells are incubated with the hydrophobic precursor \[^{14}C\]CDNB. This
compound rapidly diffuses across membranes and is conjugated intracellularly to glutathione by glutathione-S-transferases. The $[^{14}\text{C}]\text{DNP-GS}$ formed can only leave the cells via an active transporter (38). As shown before, apical DNP-GS transport by MDCKII-MRP2 monolayers was 3.2-fold higher than in the wild type cells (Table 1). In MDCKII-L0ΔMRP2 monolayers, the apical transport activity was 1.8-fold lower than in wild-type cells. This might be due to a down regulation of the endogenous canine MRP2 that is present in these cells (11). Basolateral transport of DNP-GS was somewhat variable among the different clones, probably due to variation in expression levels of endogenous basolaterally localized organic anion transporters. This variation did not correlate with the expression of MRP2 variants. In two clones tested, co-expression of ΔMRP2 with TMD0L0 resulted in a 1.8-2.5-fold higher apical transport of DNP-GS than in cells containing ΔMRP2 alone. Co-expression of L0ΔMRP2 with TMD0L0 did not result in an increased transport to the apical side of the monolayer. In contrast, apical transport was increased 2.5-fold in the cells containing both L0ΔMRP2 and TMD0, compared to cells producing L0ΔMRP2 alone.

Taken together these data show a correlation between the presence of L0-MRP2 plus TMD0 and -MRP2 plus TMD0L0, respectively, in the apical membrane and transport of both vinblastine and DNP-GS.

Uptake of LTC4 in membrane vesicles containing L0-MRP2

The experiments shown above indicate that L0-MRP2 did not transport drugs into the
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medium in MDCKII cells and was localized intracellularly. To investigate whether L₀-MRP2 is able to transport leukotriene C₄, a typical substrate for MRP2, we expressed MRP2 and L₀-MRP2 in baculovirus infected insect (Sf9) cells. Fig. 9A shows that we obtained similar levels of MRP2 and L₀-MRP2 in membrane vesicles prepared from these cells. To examine the functionality of L₀-MRP2, ATP-dependent uptake of LTC₄ was determined in isolated membrane vesicles. The apparent transport rate of L₀ΔMRP2 for LTC₄ (240 nm) was approximately 50% of the transport of wild-type MRP2 (Fig. 9B). These transport rates were corrected for the differences in protein levels and for the transport-competent inside-out vesicles content, as determined by measuring active Ca²⁺ uptake. The transport rate of L₀ΔMRP2, measured in the presence of higher concentrations of LTC₄ (720 nM) for 30 seconds, was also found to be about 50% of wild type MRP2 indicating that deletion of the TMD₀ region did not cause a major change in the apparent Kᵡ of L₀ΔMRP2 for LTC₄.
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Discussion

By co-expressing various mutants of MRP2 in polarized MDCKII cells, we searched for regions that are important for routing of MRP2 to the apical plasma membrane. In MDCKII cells the L0-MRP2 protein was localized intracellularly and mainly associated with endosomes. Uptake experiments with L0-MRP2 containing vesicles that were isolated from baculovirus infected Sf9 cells demonstrated that this mutant protein is in principle functional as it mediated the transport of the MRP2 substrate LTC4. Co-expression in MDCKII cells of the N-terminal fragments TMD0L0 and TMD0 with the core fragments ∆MRP2 and L0ΔMRP2, respectively, resulted in routing to the apical plasma membrane. Co-produced mutants localized in the apical membrane are functional as they transport both the Vinca alkaloid vinblastine and the organic anion DNP-GS. This strongly suggests that the co-produced MRP2 fragments are properly folded and that routing is most likely determined by the same signals that are functional in wild-type MRP2. Our data suggest that the complete TMD0L0 region of MRP2 is required for routing although it is also possible that TMD0L0 is required to remain stably localized in the apical membrane. These findings are in contrast to our previous experiments with MRP1 mutants, which showed that the TMD0 region of MRP1 is not required for routing to the lateral membrane (27, 28).

In cells producing ∆MRP2 and TMD0L0 only a fraction of the latter protein was detectable in the apical membrane whereas all ∆MRP2 was in the apical membrane. We hypothesize that the TMD0L0 region is required for the sorting of ∆MRP2 from the ER.
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to the Golgi apparatus where it becomes glycosylated. The TMD\textsubscript{0}L\textsubscript{0} protein probably is
expressed in molar excess in our cells, resulting in routing of only those TMD\textsubscript{0}L\textsubscript{0}
molecules that interact with ΔMRP2. This hypothesis is in line with the observation that
TMD\textsubscript{0}L\textsubscript{0} in the absence of ΔMRP2 is localized intracellularly.

The ΔMRP2 protein in the absence of TMD\textsubscript{0}L\textsubscript{0} is most likely degraded by
proteasomes as the protein was only detectable at very low concentrations and was only
core glycosylated. Moreover, treatment with two different proteasome inhibitors resulted
in a substantially increased amount of the protein. Co-expression with TMD\textsubscript{0}L\textsubscript{0} clearly
increased the stability of ΔMRP2 and resulted in glycosylation. These findings are not
specific for -MRP2 as we found the same in cells containing ΔMRP1 and its N-terminal
TMD\textsubscript{0}L\textsubscript{0} fragment (G. Calenda and R.E., unpublished data). We do not know at present
whether ΔMRP2 is a misfolded protein and therefore recognized by the quality control
system of the cell, resulting in proteasome-mediated degradation or whether this
molecule lacks signals that are required for sorting from the ER to the Golgi apparatus.
Such signals should then be present in L\textsubscript{0} as the L\textsubscript{0}ΔMRP2 protein is glycosylated and
functional, at least \textit{in vitro}. Remarkably, the L\textsubscript{0}ΔMRP2 mutant was glycosylated in the
absence of TMD\textsubscript{0} but was not detected in the plasma membrane. L\textsubscript{0}ΔMRP2 was in part
co-localizing with the ECFP-Endo- and not with the ECFP-Golgi protein. An
interesting question is whether L\textsubscript{0}-MRP2 is routed from the Golgi apparatus to the
plasma membrane and subsequently rapidly endocytosed or directly sorted from the
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Golgi to endosomes. The implication of the former hypothesis is that TMD\textsubscript{0} is required for MRP2 to remain stably localized in the apical membrane. Alternatively, it is tempting to speculate that TMD\textsubscript{0} is required for routing of L\textsubscript{0}ΔMRP2 and that this region contains an apical routing signal. At steady state, however, we see only intracellular staining of the TMD\textsubscript{0}L\textsubscript{0} protein produced alone. This indicates that a possible routing signal only functions in the context of the complete molecule.

It is possible that MRP2 contains multiple signals required for routing. That routing signals in polytopic apically localized transporters may be complex is illustrated by recent experiments in which the routing behavior of chimeric proteins containing part of the Na/K-ATPase α-subunit (basolateral localization) and the H,K-ATPase α-subunit (apical localization) was studied (39). Both the extracellular and intracellular loops flanking transmembrane segment four of the H,K-ATPase, but not transmembrane four itself, were sufficient to redirect the normally basolaterally localized Na,K-ATPase to the apical membrane. Following a similar approach for MRP1 and MRP2 may be difficult, as most of the chimeric molecules we analyzed remained associated with the endoplasmic reticulum (R.E and P.B, unpublished observations).

Another ABC-transporter that shows some structural homology to MRPs is the cystic fibrosis transmembrane conductance regulator (CFTR). The C-terminus of CFTR contains sequences important for routing to the apical membrane. The last C-terminal three amino acids form a PDZ-binding motif that interacts with proteins containing PDZ domains (40, 41). Interestingly, a CFTR with a truncation of the last three amino acids had lost its apical localization and was distributed between the apical and basolateral...
membrane (42). Subsequent work showed that additional C-terminal residues are required for localizing CFTR to the apical membrane (43). As one of the PDZ domain containing proteins, PDZK1 (also called CAP1), which binds to CFTR, also interacts with the C-terminus of MRP2 (43), it has been speculated that PDZ domain containing proteins play a role in the localization of MRP2 (45). Although we formally can not exclude that sequences present in the C-terminal tail of MRP2 play an additional role in routing, the last three amino acids are not required in the MDCKII cell line we use, as a MRP2 mutant lacking these last three amino acids localizes to the apical membrane and is functional (S.B.M.F and R.E., unpublished results). For the time being we have no evidence for other routing information in MRP2 than in TMD0.

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References


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Footnotes

1 Abbreviations: ABC, ATP binding cassette; ALLN, N-acetyl-L-leucil-L-leucil-L-norleucinal; ECFP, enhanced cyan fluorescent protein; DNP-GS, dinitrophenyl S-glutathione; GPI, glycosyl phosphatidylinositol; MRP2, human MRP2; LTC4, leukotriene C4; MDCK, Madin-Darby canine kidney; MDR, multidrug resistance; MRP, human multidrug resistance (-associated) protein.

2 GenBank = GenBank Accession Number U49248
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**Figure legends**

Fig. 1. Schematic representation of the MRP2 mutants used in this study. Putative glycosylation sites are indicated by branched motifs.

Fig. 2. Detection of MRP2 variants in MDCKII cells by Western blot analysis. Panel A. Schematic representation of MRP2. The regions recognized by the mAbs used are indicated. Panels B-E. Western blot analysis with total cell lysates from MDCKII derived clones. Twenty µg of protein was size fractionated in a 7.5% (panel B) or 11% (panel C-E) denaturing polyacrylamide gel. After immunoblotting MRP2 fragments were visualized with mAbs M2III-6 (panel B), M2I-4 (panels C and D), or 12CA5 (panel E). The relevant protein bands are marked with arrows. Protein–antibody interactions were detected using the enhanced chemiluminescence technique.

Fig. 3. Effect of proteasome inhibitors on the degradation of ΔMRP2. MDCKII-ΔMRP2 cells were treated without inhibitor (lane 1), lactacystin (50 µM; lane 2), or ALLN (50 µg/ml; lane 3). Cells were incubated with inhibitors for 8 hours. Cell lysates (20 µg) were size fractionated in a 7.5% polyacrylamide gel (see Figure 2). ΔMRP2 was detected with mAb M2III-6.

Fig. 4. Tunicamycin treatment of MDCKII derived clones. Cells were treated with tunicamycin (1.5 µg/ml) for 0, 12, 24 or 36 hours. Lysates were size fractionated in 7.5% (panels A-D) or 11% (panels E, F) polyacrylamide gels. Blots were incubated with mAbs
M_{III-6} (panels A-D), 12CA5 (panel E) or M_{I-4} (panel F). The lane marked with an asterisk in panel D, lane 2, contains a lysate from MDCKII-ΔMRP2 cells treated with lactacystin. Glycosylated proteins are marked with arrows, deglycosylated proteins with stars.

Fig. 5. Immunolocalization by CLSM of MRP2 and ΔMRP2 plus N-terminal fragments in MDCKII monolayers. Panel A. Wild-type MRP2. Panel B. ΔMRP2. Panel C. TMD_{0}L_{0} plus ΔMRP2. Panel D. TMD_{0}L_{0}. The various proteins were detected by indirect immunofluorescence (green signal) with mAb M_{III-6} or M_{I-4}. Antibody-antigen interactions were detected with a FITC-labeled secondary antibody. Nucleic acids were detected by counterstaining with propidium iodide (red signal). The upper part of each panel shows a top (x/y) view of the cell monolayer, the lower part a vertical (x/z) section. The constructs stably expressed in the various cell lines are indicated on the left side of the panels. Arrows indicate the position where the x/z section was made.

Fig. 6. Immunolocalization of L_{0}ΔMRP2 and N-terminal fragments in MDCKII derived monolayers. Panel A. L_{0}ΔMRP2. Panel B. TMD_{0}L_{0} plus L_{0}ΔMRP2. Panel C. TMD_{0} plus L_{0}ΔMRP2. For details see legend Figure 5.

Fig. 7. Co-localization of L_{0}ΔMRP2 with the ECFP-Endo and ECFP-Golgi proteins in
Apical routing of MRP2

MDCKII-MRP2 and MDCKII-L0-MRP2 cells. After fixing the cells, MRP2 (Panels A and B) and L0ΔMRP2 (Panels C and D) were detected by indirect immunofluorescence using mAb M2III-6 and Alexa 532. The left panels show the signal obtained with mAb M2III-6, the middle panels the ECFP signal detected with pECFP-Golgi (Panels A and C) and pECFP-Endo (Panels B and D). The merge of the Alexa 532 (in red) and ECFP signals (in green) is shown in the panels on the right. Co-localization of both proteins is represented by a yellow signal. An arrow indicates the position where the x/z section was made.

Fig. 8. Vectorial transport of [3H]vinblastine by MDCKII derived monolayers. Panels A-C. At t = 0 [3H]vinblastine (2 µM) was applied either in the apical or in the basal compartment, and the percentage of radioactivity appearing in the opposite compartment was determined. Transport is presented as the fraction of radioactivity added at the beginning of the experiment, appearing in the opposite compartment. PSC833 (0.1 µM) was present in both compartments to inhibit the endogenous MDR1 Pgp present in these cells. Squares: translocation from basal to apical. Circles: translocation from apical to basolateral. The experiments were performed in duplicate and repeated three times. A typical experiment is shown.

Fig. 9. Functional analysis of L0-MRP2 in baculo virus infected Sf9 cells. Panel A. Western Blot of Sf9 cell membranes containing β-galactosidase (lane 1), MRP2 (lane 2)
Apical routing of MRP2

and L0ΔMRP2 (lane 3). The proteins were detected with mAb M2III-6. Panel B. ATP dependent uptake of LTC4 in Sf9 vesicles containing MRP2 (squares), L0-MRP2 (triangles) or J-Galactosidase (diamonds). Membrane preparations were incubated with LTC4 (240 nM) at 23 °C. ATP-dependent uptake was calculated by subtracting the values obtained in the presence of 4 mM AMP form those in the presence of 4 mM ATP. Samples were taken at t = 0.5, 1 and 3 min.
Table 1. Transport into the medium, intracellular accumulation, and total synthesis of $[14C]$DNP-GS by MDCKII-derived monolayers after incubation for 20 min with $[14C]$CDNB.

<table>
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<tr>
<th>Cell line</th>
<th>Apical</th>
<th>Basal</th>
<th>Intracellular</th>
<th>Total</th>
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<td>MDCKII</td>
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<td>706 ± 18</td>
<td>575 ± 74</td>
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<td>MRP2</td>
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<tr>
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<td>304 ± 43</td>
<td>2026</td>
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<td>449 ± 60</td>
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<tr>
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<td>362 ± 16</td>
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<tr>
<td>L0ΔMRP2+TMD0L0# 21</td>
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<td>730 ± 185</td>
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<td>L0ΔMRP2+TMD0# 10</td>
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<td>507 ± 111</td>
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</table>

Transport of DNP-GS by MDCKII-derived monolayers. At t = 0 $[14C]$CDNB (final concentration 2µM; 15nCi ml$^{-1}$) was added to the apical and basal compartment. Samples were taken at t = 1, 3, 6, 12 and 20 min and extracted with ethyl acetate to separate DNP-GS from unconjugated CDNB. Values presented represent the total amount of $[14C]$DNP-GS (in pmoles) transported in the apical or basal medium after 20 min. "Intracellular" represents the amount of radioactivity associated with the cells and "Total" represents the sum of the total amount of DNP-GS transported into the medium and detected intracellularly. The experiments were performed twice in duplicate. A typical experiment is shown and the variation between the measurements is indicated.
Apical routing of MRP2
Figure 1
Figure 2

A. TMD₀ L₀ MDR-like Core

B. MDCKII parental
ΔMRP2 parental
MRP2 + TMDL₀ #3
ΔMRP2 + TMDL₀ #3
L₀ MRP2 + TMDL₀ #12
ΔMRP2 + TMDL₀ # 20
L₀ MRP2 + TMDL₀ #21

C. MDCKII (wild type)
ΔMRP2
ΔMRP2 + TMDL₀ #3
ΔMRP2 + TMDL₀ #20
L₀ MRP2 + TMDL₀ #21

D. ΔMRP2 + TMDL₀ #12

E. αHA(12CA5)
Figure 3

Control Lactacystin ALLN kDa

1 2 3

37 50 75 100 150 250
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8