Identification of the Apical Membrane-targeting Signal of the Multidrug Resistance-associated Protein 2 (MRP2/cMOAT)*

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The human canalicular multispecific organic anion transporter (cMOAT), known as the multidrug resistance-associated protein 2 (MRP2), is normally expressed in the liver and to a lesser extent in the kidney proximal tubules. In these tissues MRP2 specifically localizes to the apical membrane. The construction of MRP2 fused to the green fluorescent protein, and subsequent site-directed mutagenesis enabled the identification of a targeting signal in MRP2 that is responsible for its apical localization in polarized cells. The specific apical localization of MRP2 is due to a C-terminal tail that is not present in the basolaterally targeted MRP1. Deletion of three amino acids from the C-terminal of MRP2 (ΔMRP2) causes the protein to be localized predominantly in the basolateral membrane in polarized Madin-Darby canine kidney cells. Interestingly, MRP2 expressed in a mouse leukemia cell line (L1210 cells) predominantly accumulates intracellularly with minimal cell membrane localization. In contrast, ΔMRP2 was shown to predominantly localize in the cell membrane in L1210 cells. Increased transport of 2,4-dinitrophenyl glutathione from L1210 cells expressing ΔMRP2 showed that the re-targeted protein retains its normal function.

The canalicual multispecific organic anion transporter (cMOAT) is a member of the ATP binding cassette (ABC) transporter family of membrane proteins (designated ABCB2). cMOAT is a member of a subfamily of transporters called multidrug resistance-associated proteins (MRPs) (recently reviewed in Refs. 1 and 2). There are six known members, MRP1 to MRP6, of which cMOAT is known as MRP2. These proteins function as export “pumps” and extrude a broad range of compounds from the cell. MRP1 was the first (3) and most extensively characterized member and has 49% sequence identity with MRP2 (4–7). The function of MRP2 was initially characterized and shown to be distinct from MRP1 by the use of MRP2-deficient rats GY/TR (8–10) and EHBR (11). Many studies have shown that MRP1 and MRP2 have similar substrates, which include glutathione conjugates, glucuronide conjugates, reduced glutathione, and chemotherapeutic drugs. Although these two proteins have similar functions, their tissue distribution would indicate that they have different roles. MRP1 is found throughout the body in many tissues, including the hematopoietic system, the blood brain barrier, lungs, and at lower expression levels in the liver and kidneys. In contrast, MRP2 is only found at significant levels in the liver and to a lesser extent in the kidneys. In these two tissues, where both proteins are expressed, they differ in their specific cellular localization. MRP1 is found in the basolateral (sinusoidal) membrane and thus may serve to redirect potential excretion products back into the bloodstream. Conversely, MRP2 is solely found in the apical membrane, and this defines its function as an export pump of compounds destined for terminal excretion from the body. Although both proteins can be found in the hepatocyte, higher expression levels of MRP2 than MRP1 create the vectorial transport of excretion products from the blood into bile.

The polarization of cells, such as epithelial cells lining the bile duct or the kidney proximal tubules, is created largely by the differential localization of specific proteins. To use the hepatocyte as an example, the proteins required for the excretion of products are concentrated solely in the membrane that creates the canalicular border of the cells. To create the polarization of cells, proteins destined for specific membranes have been shown to contain molecular targeting signals. Such signals include glycosylation, phosphorylation, transmembrane domains, or cytoplasmic motifs (12). The recent identification of a PDZ-interacting domain in the cystic fibrosis transmembrane regulator (CFTR) is the first report of a targeting motif in mammalian ABC transporters (13). This domain interacts with PDZ domain containing proteins, which are commonly involved in scaffolding and stabilization at the plasma membrane (14). The signals responsible for the differential targeting of the MRP proteins to particular domains have not been determined.

Initially we aimed to further characterize the function of MRP2 by expressing the protein in a hematopoietic cell line. However, these transfected cells did not show MRP2 function due to intracellular accumulation of the protein and minimal cell membrane localization. Similar results have been reported by others (15). In contrast, MRP1 shows total cell membrane localization in these cells. We were therefore interested in the sorting signals responsible for the difference in localization of these two proteins in both epithelial cell lines and cells with a hematopoietic lineage. Using green fluorescent protein (GFP) fusion proteins and site-directed mutagenesis we have been...
able to identify a sequence motif responsible for exclusive apical localization of MRP2 in polarized MDCK cells. Deletion of the motif results in lateral localization of MRP2 in polarized MDCK cells and allows cell membrane localization in L1210 cells. The mutated protein was able to transport 2,4-dinitrophenyl glutathione (DNP-GS), a known substrate of MRP2.

EXPERIMENTAL PROCEDURES

Green Fluorescent Protein Fusion Constructs—GFP was fused to the C-terminal of MRP1 and MRP2 and utilized to detect the localization of the fusion proteins. Human MRP2 cDNA was amplified by polymerase chain reaction using PfuTurbo DNA polymerase (Stratagene) to remove the stop codon and introduce restriction enzyme sites suitable for cloning. The cDNA was amplified using the sense primer (5′-AGCGGTAGCGATGCGTAGGAGGAGG) and antisense primer (5′-TACCGTACCGGATCCGCGTGCTCTGACAG), which adds an NheI site immediately adjacent to the start codon, and the antisense primer (5′-TACCGTACCGGATCCGCGTGCTCTGACAG) adds an AgeI site and removes the stop codon of MRP1.

The constructs were transiently transfected into MDCK cells and L1210 cells using a LipofectAMINE transfection kit (Life Technologies, Inc.) using 1 μg of DNA. Transfections of MDCK cells were carried out using Transwell plates (Costar, 24 mm × 3 μm polycarbonate membrane) to enable cell polarization. Cells were imaged using a Nikon TE300 inverted microscope linked to a Radiance 2000 Laser Scanning System for confocal microscopy and Lasersharp 2000 imaging software (Bio-Rad).

Site-directed Mutagenesis—All mutations were achieved using the QuickChange site-directed mutagenesis kit (Stratagene). Successful mutagenesis was confirmed by sequencing through the site of the mutations using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Two clones from separate mutagenesis reactions were selected for all mutants and transfected separately to eliminate the chance that an unintended mutation that would interfere with the targeting of the protein would be incorporated during the amplification reaction.

ΔMRP2 Function—A construct that expressed MRP2 with the C-terminal TKF motif deleted (ΔMRP2) and without the GFP tag was prepared using the QuickChange site-directed mutagenesis kit. A construct of the MRP2 cDNA in the mammalian expression vector pcDNA3 (Invitrogen) (7) was used as the template for site-directed mutagenesis. The sense primer (5′-GAAGTGACAGCTAGCAGAAGGCC) and antisense primer (5′-GGCTTCGTCCTAGTCTCCCTT) deleted the nine nucleotides that coded for the amino acid residues Thr1543, Lys1544, and Phe1545. Successful mutagenesis of two clones from separate reactions was confirmed by sequencing. Stable transfectants in L1210 cells were selected for further study.

2,4-Dinitrophenyl Glutathione Transport—DNP-GS was generated in L1210 cells by exposure to 1-chloro-2,4-dinitrobenzene and its efflux determined as described previously (16).

Immunofluorescence—Detection and localization of untagged mutant MRP2 (ΔMRP2) was achieved by immunofluorescence using the antibody M₃H16 (Kamiya Pty. Ltd.). 2 × 10⁵ cells were washed with PBSF (phosphate-buffered saline supplemented with 2.5% fetal bovine serum). The cells were permeabilized using digitonin (5 μg/ml) and incubated at room temperature for 15 min. The cells were then washed three times with PBSF and then incubated with the primary antibody (2 μg) for 1 h at room temperature before being washed twice with PBSF. The cells were incubated with fluorescein isothiocyanate-conjugated F(ab')₂ (Silenus, Hawthorn, Victoria, Australia) (1:50 dilution) for 30 min at room temperature. Finally the cells were washed three times and resuspended in PBSF ready for immediate confocal microscopy.

Alignments and Molecular Modeling—The protein sequence of the C-terminal cytoplasmic domains of 37 ABC transporters from the P-glycoprotein and MRP subfamilies were aligned with the histidine permease (HisP) sequence using the ClustalW alignment program. The multiple sequence alignment was used with the coordinates of the HisP crystal structure (17) to generate a homology model of the C-terminal cytoplasmic domain from MRP1 and MRP2 using BioNavigator at the ANGIS Internet site (BioNavigator by eBioinformatics Pty. Ltd.). The models were generated using the Rigorous Models software (18) and presented using Swiss PdbViewer (v3.6b3) (19).

RESULTS

MRP2-gfp and MRP1-gfp Localization in MDCK Cells—To conveniently detect the localization of the proteins under investigation, we constructed GFP fusion proteins and used confocal microscopy to visualize the fluorescent product. Using an MRP2-specific antibody, native MRP2 was previously shown to localize to the apical membrane of MDCK cells (15, 20). In the present study, human MRP2 with GFP fused to its C terminus was localized to the apical membrane in polarized MDCK cells, consistent with the localization of the native protein (Fig. 1). The apical membrane of polarized MDCK cells grown on Transwell membranes is the surface facing the media as opposed to the surface adhering to the membrane (basolateral).

MRP1 has been previously immunolocalized to the basolateral membrane of a pig kidney epithelial cell line (LLC-PK1) (21). In the present study, human MRP1 with GFP fused to its C terminus also demonstrated basolateral localization in polarized MDCK cells (Fig. 2). These studies establish that fusion of MRP1 and MRP2 to GFP does not interfere with the normal targeting of these proteins to the basolateral and apical membranes.

Expression of ΔMRP2-gfp in MDCK Cells—Recently a specific apical targeting signal was identified in CFTR (13). The signal consisted of a three-residue PDZ-interacting motif at the C terminus. The alignment of C-terminal sequences of MRP1 and MRP2 revealed a similar C-terminal motif in MRP2 (TKF) that was absent in MRP1 and has recently been shown to function as a PDZ-interacting domain (22). To determine whether the TKF motif influenced the apical localization of MRP2, we constructed ΔMRP2-gfp in which the three C-termi-
nal residues were deleted. When expressed in polarized MDCK cells, ΔMRP2-gfp was found to localize predominantly in the lateral membranes rather than the apical localization of MRP2-gfp (Fig. 3).

MRP2-gfp Alanine Mutants in MDCK Cells—To further characterize the TKF motif of ΔMRP2-gfp and to determine the relative importance of each residue, individual alanine mutations were introduced into each of the residue positions 1543–1545 of the MRP2-gfp construct. Since residues 1542–1544 (STK) form a predicted phosphorylation site, residue 1542 was also mutated to alanine.Fig. 4, A—E, show the localization of each of these mutants in MDCK cells. The effects of the substitutions were determined by visualizing the change in localization of the mutant compared with the native protein. The T1543A and K1544A mutants had both apical and basolateral targeting (nonpolarized distribution) with an increase in protein accumulation in intracellular vesicles. The F1545A mutant did not have altered targeting. Mutation of all three residues to alanines caused the protein to be distributed in a nonpolarized manner.

Expression of MRP2 in L1210 Cells—In initial unpublished
studies we found little evidence for the transport of DNP-GS by MRP2 expressed in the mouse leukemia cell line L1210 (data not shown). This lack of function suggests that the protein did not localize to the cell membrane in these cells. To confirm this observation, MRP2-gfp was expressed in L1210 cells and was found to localize predominantly in intracellular vesicles with minimal plasma membrane localization. In contrast, the majority of ΔMRP2-gfp was detected in the cell membrane. An antibody to MRP2 (M II6) was used to detect MRP2 and ΔMRP2 localization in stably transfected L1210 cells. MRP2 was detected in intracellular vesicles surrounding the nucleus (N). This localization is consistent with MRP2-gfp localization. ΔMRP2 was detected in the cell membrane confirming the effects of the TKF motif deletion found with ΔMRP2-gfp.

FIG. 5. A, L1210 cells were transiently transfected with MRP2-gfp and ΔMRP2-gfp. i, the majority of MRP2-gfp accumulated in intracellular vesicles with minimal plasma membrane localization. ii, in contrast, the majority of ΔMRP2-gfp localized to the cell membrane. B, an antibody to MRP2 (M II6) was used to detect MRP2 and ΔMRP2 localization in stably transfected L1210 cells. i, MRP2 was detected in intracellular vesicles surrounding the nucleus (N). This localization is consistent with MRP2-gfp localization. ii, ΔMRP2 was detected in the cell membrane confirming the effects of the TKF motif deletion found with ΔMRP2-gfp.

had a vesicular localization within the cell (Fig. 5B, (ii)), the same distribution as in Fig. 5A. ΔMRP2 was detected in the cell membrane (Fig. 5B, (ii)), the same localization as ΔMRP2-gfp shown in Fig. 5A, (ii). These results suggest that the deletion of the TKF motif from MRP2 allows the successful targeting of the protein to the membrane of nonpolarized L1210 cells. However, some L1210 cells expressing ΔMRP2 or ΔMRP2-gfp demonstrated a degree of intracellular accumulation, suggesting that localization of ΔMRP2 in the cell membrane of L1210 cells is not as stable as in MDCK cells.

2,4-Dinitrophenyl Glutathione Transport—L1210 cells are nonadherent and nonpolarized and could be potentially used as a convenient cell line for assessing the transport function of MRP2. Although deletion of the TKF motif allowed expression of MRP2 in the L1210 cell membrane, it was not clear if the motif was also necessary for its transport function. As shown in Fig. 6, L1210 cells stably expressing ΔMRP2 or ΔMRP2-gfp demonstrated significantly higher efflux of DNP-GS compared with control L1210 cells or L1210 cells expressing native MRP2.

Alignments and Homology Modeling—To gain an overview of the potential differences in structure of the MDR and MRP proteins targeted to apical membranes, an alignment was made of the sequences encoding the C-terminal cytoplasmic domains of 37 ABC transporter proteins. Part of the alignment is represented in Fig. 7, which shows that those MRP proteins that localize to the apical membrane (MRP2 from four species) have a C-terminal extension when compared with MRP1, MRP3, MRP5, and MRP6, which are targeted to the basolateral membrane. MRP4 also appears to have a potential PDZ-interacting domain at its C terminus, but its site of expression has not been described.

The structural coordinates for the ATP binding subunit of histidine permease from Salmonella typhimurium (17) and the full ABC transporter sequence alignment allowed the construction of homology models of the equivalent regions of MRP1 and MRP2 (Fig. 8). Since the C-terminal motif of MRP2 extends beyond the alignment with HisP, the exact position of the TKF residues cannot be predicted. However, the models predict that the TKF motif is positioned on the outside of the protein, away from the ATP binding cassette and regions involved in the cytoplasmic subunit interface. In addition, the external position of the motif would favor interactions with other proteins involved in the targeting process.
Human MRP2 specifically localizes to the apical membrane of polarized epithelial cells in the liver and kidney. This localization can be replicated experimentally in MDCK cells (15, 20) and LLC-PK1 cells (23, 24), and we demonstrate in this study that an MRP2-gfp fusion protein also localizes to the apical membrane (Fig. 1). This allowed us to undertake mutational analysis to determine targeting signals for apical localization.

Deletion of the three amino acids from the C terminus of MRP2 (D-MRP2-gfp) caused a dramatic change in the targeting of the protein to the basolateral membrane in MDCK cells. The mutant's localization in a polarized cell now mimics MRP1 (Fig. 2), which does not have the deleted motif. This observation indicates that the three-amino acid motif targets MRP2 to the apical membrane and dominates any (as yet unidentified) basolateral targeting signals. Conversely, the motif could exclude the protein from the basolateral domain thus only allowing apical localization. Targeting and/or exclusion are usually mediated by a secondary protein(s) responsible for the recognition of such sorting signals. Interestingly, the motif responsible for apical targeting of MRP2 is a predicted PDZ-interacting domain and has been reported to associate with a PDZ protein, PDZK1 (22). This is an analogous mechanism to that of CFTR, which is also targeted apically by a PDZ-interacting domain at its C terminus (13, 30). CFTR was shown to associate with EBP-50, a PDZ protein, and both EBP-50 and PDZK1 were shown to localize specifically to the apical domains in polarized cells (13, 22). Whether PDZK1 and EBP-50 are responsible for sorting and targeting of their respective ABC transporters is yet to be determined. Alternatively these PDZ proteins may be responsible for stabilizing transporters such as CFTR and MRP2 in the membrane, which could also explain their localization solely at the apical domain.

To further characterize the motif, alanine was introduced into the position of each residue separately, and an additional mutant was made in which all three residues were replaced by alanine. The T1543A mutant did produce a change in targeting compared with the native protein, allowing both basolateral and apical targeting, i.e. nonpolarized targeting, and also an increased accumulation in vesicles, suggesting some instability in the targeting mechanism. This is consistent with the motif being a PDZ-interacting domain characterized by the motif Ser/Thr-X-hydrophobic residue, where X represents any amino acid (26). This was consistent with the results obtained by the TKF-AAA mutant. The F1545A mutant did not alter normal targeting, suggesting that alanine is a sufficiently hydrophobic residue. The canonical PDZ domain is reported to tolerate any residue (X) at the -2 position, but the K1544A caused nonpolarized targeting, suggesting some flexibility in the constraints determining functional PDZ domains. Interestingly, the serine residue at position 1542 forms a predicted phosphorylation site, and mutation of the serine caused the fusion protein to localize in subapical vesicles. This suggests that the serine may be phosphorylated and could regulate recruitment into the apical membrane.

The deletion of the TKF motif increases the sequence similarity of MRP2 to MRP1 and results in the same basolateral targeting. To investigate the tertiary structure of the subunit and the position of the motif, homology models of both MRP1 and MRP2 were created based on crystal structure of HisP. Comparisons of the homology models clearly show the difference in length of the C terminus of MRP1 and MRP2. It is not clear whether it is the interaction with the PDZ-interacting domain that is solely responsible for the apical localization or whether it is the spatial arrangement of the extension and the apical membrane.
predicted motif that allows binding/modification to another part of the protein. From the homology model of MRP2 it appears likely that the motif is available for interaction and not buried within the subunit. Also, the position of the C terminus in this model suggests that the motif does not interact with functionally significant areas such as the ATP binding sites. This is further supported by the ability of the deletion mutant ΔMRP2 to transport 2,4-dinitrophenyl glutathione when expressed in L1210 cells (Fig. 6). Alignment of MRP2 with the basolateral MRP proteins MRP1, MRP3, MRP5, and MRP6 shows the absence of the motif in the basolateral transporters. Based on this alignment the MRP2 residues 1539–1545 may play a role in the targeting mechanism as this is the full length of the extension of the C terminus compared with the basolateral proteins. If this motif is the sole targeting signal, then one could predict that MRP4 will be found to be apically targeted in polarized cells. The P-glycoprotein homologues MDR2 (or MDR3) and sPgp (all apically targeted in polarized cells) also have a difference in length compared with the basolaterally localized proteins (alignment not shown). However their overall similarity to MRP2 is very low and does not allow a prediction based on the MRP2 motif. Further studies are required to determine the targeting signals of the Pgp subfamily.

There is a notable difference in the sorting of MRP2 in L1210 cells compared with the MDCK cells. When expressed in the L1210 cells, MRP2 transport function was minimal (results not shown). Immunofluorescence studies of cells expressing MRP2 and confocal imaging of cells expressing MRP2-gfp both confirm the intracellular localization of the protein in L1210 cells (Fig. 5). Deletion of the apical targeting motif (the PDZ-interacting domain) from MRP2 and MRP2-gfp allows the mutant protein to be expressed in the plasma membrane. Therefore it is the apical targeting motif that excludes the native MRP2 from the membrane. The stability of the protein in the membrane also differs between MDCK cells and L1210 cells. In MDCK cells ΔMRP2-gfp has basolateral localization in the majority of cells. In L1210 cells, the majority of cells have intracellular localization of ΔMRP2-gfp or ΔMRP2 with the remainder of the cells having cell membrane targeting. This suggests an instability of the mutant protein in the membrane, and it is possible that an additional protein responsible for stabilizing MRP2 in the membrane is not present in L1210 cells.

The attachment of GFP to the C terminus of MRP1 or MRP2 did not interfere with correct targeting in this study nor in a previous study of MDR1 (27). Based on the homology models in Fig. 8, the position of the C-terminal helix indicates that GFP therefore would suggest that the PDZ-interacting domain does not need to be freely exposed and carboxylated to function. In support of this, rabbit MRp2 has a predicted PDZ-interacting domain in the same position as human, mouse, and rat MRP2 but is followed by a further 21 amino acids (Fig. 7).

The GFP fusion proteins were expressed at consistent levels under the CMV promoter of the EGFP-N1 vector. MRP2-gfp localized apically in the majority of polarized MDCK cells as represented in Fig. 1. However, a minority of cells had very high levels of MRP2-gfp expression and exhibited both apical and lateral localization. This situation could occur in vivo in cancer cells where a number of multidrug-resistant proteins are up-regulated and thus “over”expressed. MRP2 has been found to be expressed in ovarian cancer cells lines (28), renal clear cell carcinomas (29), lung, gastric, and colorectal cancer cells (25). Depending on the expression levels of MRP2 in these cells, the localization may not be solely apical but may also be lateral and basolateral. Interestingly, P-glycoprotein is specifically apical in polarized cells but can also be expressed in the membrane of hematopoietic cells without the intracellular accumulation observed with MRP2 in L1210 cells (results not shown). Furthermore, Pgp is normally expressed in polarized cells of the gastrointestinal tract but is expressed in MDR tumors in other tissue types that are not necessarily polarized. This indicates a difference in the targeting signals between MRP2 and P-glycoprotein.

This study demonstrates that the PDZ-interacting domain of MRP2 functions as the apical targeting signal in MDCK cells yet excludes the protein from the cell membrane in L1210 cells. This motif is not present in the basolaterally targeted MRP proteins 1, 3, 5, and 6.

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