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Mutation of proline residues in the NH_2-terminal region of the multidrug resistance protein, MRP1 (ABCC1): effects on protein expression, membrane localization, and transport function

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Abstract

The Multidrug Resistance Protein, MRP1 (ABCC1) confers drug resistance and transports organic anions such as leukotriene C_4 (LTC_4) and 17β-estradiol 17-(β-D-glucuronide) ($E_217\beta G$). Previous studies showed that portions of the first membrane spanning domain (MSD1) and the cytoplasmic loop (CL3) connecting it to MSD2 are important for MRP1 transport function. We have replaced 12 prolines in MSD1 and CL3 with alanine and determined the effects of these substitutions on MRP1 expression and transport activity. All singly substituted MRP1-Pro mutants could be expressed in HeLa cells, except MRP1-P104A. The expressed mutants also transported LTC_4 and $E_217\beta G$, and their K_m (LTC_4) values were similar to wild-type MRP1. Expression of the double mutant MRP1-P42/51A was reduced by >80% although it localized to the plasma membrane and transported organic anions. MRP1 expression was also reduced when the first transmembrane helix (amino acids 37–54) was deleted. In contrast, the phenotypes of the multiply substituted CL3 mutants MRP1-P196/205/207/209A and MRP1-P235/255A were comparable to wild-type MRP1. However, Pro^{255}-substituted MRP1 mutants showed reduced immunoreactivity with a monoclonal antibody (MAb) whose epitope is located in CL3. We conclude that certain prolines in MSD1 and CL3 play a role in the expression and structure of MRP1.

Keywords: Multidrug resistance protein, MRP; Proline substitution; Transmembrane helix; Cytoplasmic loop; Monoclonal antibody epitope

1. Introduction

The ATP-binding cassette (ABC) transporter proteins comprise one of the largest known superfamilies of proteins [1,2]. ABC proteins are found in bacteria, archaea, and eukaryotes, and more than 500 have been sequenced to date. These proteins are responsible for the ATP-hydrolysis-driven transport of a wide variety of molecules across biological membranes including sugars, amino acids, ions, drugs, antibiotics, vitamins, iron complexes, peptides, proteins, and complex carbohydrates. Current annotation of the human genome sequence indicates that there are 48 human ABC genes and these have been assigned to one of seven subfamilies, designated A through G, based on the relative similarities of their deduced amino acid sequences [3]. A number of these proteins play a prominent role in essential physiological processes, and mutations in several ABC genes are now known to be the underlying cause of certain genetic disorders. For example, conjugated hyperbilirubinemia (Dubin–Johnson syndrome) is caused by mutations in the *ABCC2* gene encoding the canalicular multispecific organic anion transporter cMOAT/MRP2 [4], and cystic fibrosis is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR; *ABCC7*) gene that encodes an ATP-gated cAMP-regulated chloride channel [5,6].

Abbreviations: ABC, ATP-binding cassette; MSD, membrane spanning domain; NBD, nucleotide binding domain; LTC_4, leukotriene C_4; $E_217\beta G$, 17β-estradiol-17-β-(D-glucuronide); HEK, human embryonic kidney; TM, transmembrane; MAb, monoclonal antibody; CFTR, cystic fibrosis transmembrane conductance regulator

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In addition to their involvement in genetic disorders, ABC proteins are increasingly being implicated as important determinants of drug efficacy and toxicity because of their role in the absorption, disposition, and elimination of xenobiotics and their metabolites. Moreover, when overexpressed in tumor cells, some ABC proteins, most notably the 170-kDa P-glycoprotein (gene symbol *MDR1* or *ABCB1*) and the 190-kDa multidrug resistance protein 1, MRP1 (gene symbol *ABCC1*), can cause resistance to a wide variety of clinically important chemotherapeutic agents [7–11].

Almost all known ABC transporters are characterized by a structure that includes at least one hydrophobic polytopic membrane spanning domain (MSD) with up to six transmembrane (TM) segments, and one hydrophilic cytosolic nucleotide binding domain (NBD) that contains three highly conserved sequence motifs, viz., the Walker A and B motifs and the so-called 'active transport family' signature [1,12]. In bacteria, these structural domains are usually encoded as separate polypeptides which then associate with one another to form an active transporter. Eukaryotic ABC transporters, on the other hand, typically contain four domains configured either as two alternating MSDs and NBDs encoded as a single large polypeptide (e.g. CFTR and P-glycoprotein), or as two independently encoded 'half-molecules' which associate to form a functional dimer with both the NH_2 and COOH termini located in the cytoplasm.

Exceptions to this four domain structure include several members of the "C" subfamily of ABC transporters. These proteins, which include MRP1, contain a fifth domain, MSD1, with five TM helices. This configuration places the NH_2 terminus of these proteins on the extracellular face of the membrane [13–15]. Previous studies have shown that portions of the first 280 amino acids of MRP1 encompassing the NH_2-terminal MSD1 (sometimes referred to as MSD_o) and the cytoplasmic loop (CL3 or L_o) connecting it to the second MSD are important for transport activity in insect and mammalian cell membranes [16–18]. For example, we found that NH_2-terminally truncated MRP1 polypeptides in which the first 66, 228, or 282 amino acids were eliminated did not show detectable LTC_4 transport activity when expressed in insect cell membranes [16]. On the other hand, Bakos et al. [17] demonstrated that the first 204 amino acids of MRP1 can be removed without loss of LTC_4 and vinblastine transport activity. Thus, the precise function of this region in MRP1 and related ABCC proteins is not yet fully understood.

Proline residues located in the MSDs and cytosolic domains of transport proteins, including P-glycoprotein and CFTR [19–21], have frequently been shown to play an important role in ensuring proper protein folding and function in mammalian cells. To investigate whether any of the Pro residues within the first 280 amino acids of MRP1 play a similar role in this ABC transporter, we have replaced them with Ala residues, singly, and in some cases, in combination, by site-directed mutagenesis. The effects of these mutations on MRP1 expression levels, membrane localization, and organic anion transport activity were then determined using HeLa cells stably transfected with the mutant proteins.

2. Materials and methods

2.1. Materials

[6,7-^{3}H]$E_2$17βG (55 Ci mmol^{-1}) was purchased from NEN Life Science Research Products (Boston, MA) and [14,15 (n)-^{3}H]LTC_4 (115.3 Ci mmol^{-1}) was from Amersham Pharmacia Biotech (Buckinghamshire, UK). LTC_4 was purchased from CalBiochem (La Jolla, CA) and nucleotides and $E_2$17βG were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Site-directed mutagenesis and vector construction

The expression vector pcDNA3.1(−)-$MRP1_K$ containing wild-type MRP1 with an optimized Kozak sequence at the ATG codon has been described previously [22]. The template for mutagenesis was prepared by cloning the *Xba*I/*Bam*HI fragment from pcDNA3.1(−)-$MRP1_K$ (containing nucleotides −24 to +840 of the MRP1 sequence encoding the first 280 amino acids of the protein) into pGEM7-3Z (Promega, Madison, WI). Mutagenesis was performed using the Transformer™ site-directed mutagenesis kit (Clontech Laboratories Inc., Palo Alto, CA) according to the manufacturer's instructions.

For the single substitutions of Pro with Ala in MSD1, the following sense mutagenic primers were used (substituted nucleotides are underlined): MRP1-P42A (5′-C GTG TGG GTG GCT TGT TTT TAC CTC TGG-3′), MRP1-P51A (5′-G GCC TGT TTC GCC TTC TAC TTC C-3′), MRP1-P69A (5′-CAG ATG ACA GCT CTC AAC-3′), MRP1-P104A (5′-C ATA TTC CTG GCC GCA GTG TTT CTG G-3′), MRP1-P110A (5′-GT TTC TGG TCA GCG CAA CTC TCT TGG-3′). For substitution of Pro residues in the CL3 loop connecting MSD1 to MSD2, the mutagenic primers were: MRP1-P196A (5′-CA GAT CGC TCA GCC CTG TTC TC-3′), MRP1-P205A (5′-C ATC CAC GAC GCT AAT CCC TG-3′), MRP1-P207A (5′-C GAC CCT AAT GCC TGC CCA GAG-3′), MRP1-P209A (5′-CTA ATC CCT GCG CAG AGT CCA G-3′), MRP1-P235A (5′-C TAC CGC CAG GCC CTG GAG GG-3′), MRP1-P255A (5′-CG GAA CAA GTC GTG GCT GTT TTG G-3′), MRP1-P272A (5′-CT AGG AAG CAG GCG GTG AAG G-3′).

A selected number of multiply substituted MRP1 constructs and a construct eliminating amino acids 37–54 corresponding to the predicted first TM segment of MRP1 were also created using the same mutagenesis protocol as above with the following primers: MRP1-P42/51A (5′-C GTG TGG GTG GCT TGT TTT TAC CTC TGG GCC

TGT TTC GCC TTC TAC TTC C-3′); MRP1-P104/110A (5′-GGC ATA TTC CTG GCC GCA GTG TTT CTG GTC AGC GCA ACT CTC TTG GGC-3′); MRP1-P205/207/209A (5′-C ATC CAC GAC GCT AAT GCC TGC GCA GAG TCC AG-3′), and MRP1-Del37-54 (5′-GCT TTC AGA ACA CGC TCT ATC TCT CCC G-3′). The quadruple MRP1-P196/205/207/209A construct was generated by introducing the P205/207/209A mutations into the MRP1-P196A construct. MRP1-P235/255A was generated by introducing P255A into the MRP1-P235A construct. After confirming all mutations by sequencing or diagnostic restriction enzyme digests, the 0.87 kb *Xba*I/*Bam*HI fragment was subcloned back into pcDNA3.1(−)-MRP1$_K$ and the integrity of the inserts and the cloning boundaries in the full-length constructs was verified by DNA sequencing.

Construction of the pcDNA3.1(−)-MRP1$_K$-GFP vector has been described previously [22,23]. Selected mutant MRP1-GFP fusion proteins were generated by replacing the *Xba*I/*Bam*HI fragment in pcDNA3.1(−)-MRP1$_K$-GFP with the comparable fragment containing the desired mutation from the vectors described above and the integrity of the subcloning was again confirmed by DNA sequencing.

2.3. Transfections of MRP1 expression vectors

To obtain stably transfected cell lines, wild-type and mutant MRP1 pcDNA3.1(−) expression vectors or the empty vector were transfected into HeLa cells using FuGENE6 (Roche Biochemicals, Laval, PQ, Canada) as described [22]. Briefly, approximately $1.5–2 \times 10^6$ cells were seeded in each well of a six-well plate and 24 h later, 1-μg DNA mixed with FuGENE6 according to the manufacturer's protocol was added. After 48 h, the cells were subcultured 1:12 and the medium replaced with fresh medium containing G418 (geneticin) (1000 μg ml^{-1}). After approximately 2 weeks, cell colonies were individually removed using cloning cylinders and subcultured in 600 μg ml^{-1} G418. Levels of MRP1 protein in G418-resistant cell populations were then determined by dot blotting and/or immunoblotting as described below. The proportion of cells expressing the mutant MRP1 proteins was determined by flow cytometry using the MRP1-specific MAb QCRL-3 which recognizes a conformation-dependent epitope in the first NBD of MRP1 as described previously [22,24,25]. Several HeLa cell populations containing the greatest number of MRP1 positive cells were then cloned by limiting dilution and expanded to obtain clonal cell lines with >90% of the cells expressing wild-type or mutant MRP1. For some mutants, transient transfections were carried out with SV40-transformed human embryonic kidney cells (HEK293T). Briefly, $1.5–2 \times 10^6$ cells were seeded in each well of a six-well plate and, after 24 h, were exposed to 1 μg of DNA with FuGENE6. Cells were harvested 48–60 h later [22].

2.4. Determination of MRP1 protein levels in transfected cells

The levels of wild-type and mutant MRP1 proteins were determined by immunoblot and/or dot blot analysis of membrane protein fractions from transfected cells essentially as described [25]. The antibodies used were the murine MAb QCRL-1, which recognizes a linear heptapeptide epitope comprised of amino acids 918–924 in the linker region of MRP1 [24,26], and the rat MAb MRPr1, which recognizes a linear epitope in the cytosolic region of MRP1 connecting MSD1 to MSD2 (amino acids 238–247) [27]. Dot blots were prepared on Immobilon P membrane in a 96-well manifold as described previously [25]. For immunoblotting, proteins were resolved on a 6% or 7% polyacrylamide gel and electrotransferred to a nylon membrane. MAb QCRL-1 and MAb MRPr1 binding to the dot blots and immunoblots was detected with horseradish peroxidase-conjugated goat anti-mouse and goat anti-rat IgG (H+L) (Pierce, Edmonton, Alberta, Canada), respectively, followed by enhanced chemiluminescence detection (NEN Life Science Research Products). Relative levels of MRP1 expression were estimated by densitometric analysis using a ChemiImagerJ 4000 (Alpha Innotech, San Leandro, CA). In some cases, the glycosylation state of selected Pro-MRP1 mutants was determined by subjecting membrane proteins to PNGaseF treatment as before [25] and then immunoblotted as described above.

2.5. Localization of MRP1 by confocal microscopy

The localization of the MRP1 proteins in the transfected HeLa cells was determined by indirect immunofluorescence with MAb QCRL-3. Briefly, cells were seeded at 1.5×10^6 cells per well in six-well plates on coverslips coated with 0.1% gelatin in 2 ml of serum-containing medium. The following day, the coverslips were washed once with PBS and the cells fixed with 4% paraformaldehyde for 10 min at room temperature. After washing twice with PBS, the cells were permeabilized by incubation in 0.2% Triton X-100 in PBS for 5 min. The coverslips were then washed 3×5 min in blocking solution (0.1% Triton X-100/1% BSA in PBS). MAb QCRL-3 [diluted 1:2500 in blocking solution with 10 μg ml^{-1} RNaseA (Roche Molecular Biochemicals)] was added and the cells incubated with gentle shaking for 60 min at room temperature. After washing with blocking solution, 500-μl Alexa Fluor 488 conjugated goat anti-mouse IgG (H+L) (Fab′)$_2$ fragment (Molecular Probes, Eugene, OR) (diluted 1:1000 in blocking solution with 10 μg ml^{-1} RNaseA) was added and the coverslips incubated 30 min in the dark. The coverslips were washed again and cell nuclei stained by incubation in 1-ml propidium iodide (2 μg ml^{-1} in PBS) for 45 min in the dark. Finally, the coverslip was placed

on a slide containing 1 drop of Antifade Solution (Molecular Probes) and cells examined and photographed using a Meridien InSight confocal microscope equipped with an air-cooled argon laser. Fluorescent signals were collected with a 530/30 nm bandpass filter and propidium iodide signals were collected with a 620/40 nm bandpass filter. Images obtained with 488-nm excitation were pseudo-colored using Maxim DL software.

Confocal microscopy was also used to examine the cellular localization of several GFP-tagged MRP1 constructs expressed in transiently transfected cells. Briefly, HEK293T cells were seeded at 3×10^5 cells per well in a six-well plate on gelatin-coated coverslips and, 24 h later, transfected with the MRP1-GFP cDNA constructs as described above for the stably transfected HeLa cells. Forty eight hours later, the coverslips were washed and the cells fixed with 4% paraformaldehyde for 10 min and permeabilized by adding 0.2% Triton X-100 in PBS for 5 min. The coverslips were then incubated in RNaseA followed by nuclear staining with propidium iodide and viewed as before. Propidium iodide signals were collected with a 620/40 nm bandpass filter and GFP signals were collected with a 530/30 nm bandpass filter.

2.6. MRP1-mediated LTC_4 and $E_2 17\beta G$ transport in inside-out membrane vesicles

Membrane vesicles were prepared from transfected cells and used to measure ATP-dependent [^{3}H]LTC_4 and [^{3}H]$E_2 17\beta G$ transport according to a rapid filtration technique described previously [28]. Briefly, [^{3}H]LTC_4 transport assays were performed at 23 °C in a 50-μl reaction containing 50 nM substrate (40 nCi per reaction), 4 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg ml^{-1} creatine kinase, and 2–4 μg of membrane vesicle protein. Uptake was stopped at selected times by rapid dilution in ice-cold buffer, and then the reaction was filtered through glass fiber (Type A/E) filters presoaked in transport buffer. Radioactivity was quantitated by liquid scintillation counting and all data were corrected for the amount of [^{3}H]LTC_4 that remained bound to the filter, which was usually $<10\%$ of the total radioactivity. Transport in the presence of AMP was subtracted from transport in the presence of ATP to determine ATP-dependent uptake of [^{3}H]-labelled substrate. After preliminary time courses, kinetic parameters (K_m and V_{max}) were determined by measuring initial rates of [^{3}H]LTC_4 uptake into membrane vesicles for 45 s at several substrate concentrations. Eadie–Hofstee plots were generated from initial rate measurements using GraphPad Prism7 software (San Diego, CA).

[^{3}H]$E_2 17\beta G$ uptake was also measured in a similar fashion except that membrane vesicles were incubated at 37 °C for a single time point (120 sec) in a total reaction volume of 50 μl at an initial $E_2 17\beta G$ concentration of 400 nM (40 nCi per reaction). The remaining components were as described above for LTC_4 transport. All transport assays were carried out in triplicate and were repeated at least once.

3. Results

3.1. Most MRP1-Pro mutants can be stably expressed in transfected HeLa cells

Pro residues in MRP1 MSD1 (at positions 42, 51, 69, 104, and 110) and the cytosolic loop (CL3) connecting MSD1 to MSD2 (at positions 196, 205, 207, 209, 235, 255 and 272) (Fig. 1) were replaced singly, and in some cases, in combination, with Ala. The resulting mutant MRP1 constructs were transfected into HeLa cells and clonal HeLa cell lines established. Despite multiple attempts, stable HeLa cell lines expressing MRP1-P104A and MRP1-P104/110A could not be established.

Immunoblots of membrane vesicles prepared from the cell lines expressing MSD1 MRP1 mutants containing single (P42A, P51A, P69A and P110A) and double (P42/51A) Pro substitutions detected with MAb QCRL-1 are shown in Fig. 2A. The relative expression levels of the different mutants ranged from $<20\%$ to approximately 150% that of wild-type MRP1. The double TM1 mutant, MRP1-P42/51A, was consistently expressed at very low levels ($<20\%$ wild-type MRP1) in multiple independent transfections, suggesting that biogenesis of this mutant MRP1 was impaired or that the mutant protein was not very stable. Treatment of membrane proteins prepared from cells expressing this mutant MRP1 with PNGaseF showed that the small amount of MRP1 made was fully glycosylated (not shown).

The seven single (P196A, P205A, P207A, P209A, P235A, P255A, and P272A) and two multiply substituted CL3 mutants (P196/205/207/209A and P235/255A) could also be stably expressed in HeLa cells. The relative expression levels of these mutants as determined by immunoblotting with MAb QCRL-1 ranged from approximately 50% to 160% that of wild-type MRP1 (Fig. 2B).

3.2. MRP1 lacking TM1 (amino acids 37–54) cannot be expressed in HeLa cells and is poorly expressed in HEK293T cell

Because the MRP1-P42/51A mutant was so poorly expressed, we further explored the contribution of TM1 to the expression and activity of MRP1 by creating a mutant which did not contain this transmembrane segment (MRP1-Del37-54). Stable HeLa cell lines expressing significant levels of this deletion mutant could not be established. However, the low level of MRP1-Del37-54 expressed in transiently transfected HEK293T cells migrated as a tight band with a significantly faster electrophoretic mobility than the diffuse band observed for wild-type MRP1, indicating that glycosylation of the MRP1-Del37-54 mutant was impaired (Fig. 3).

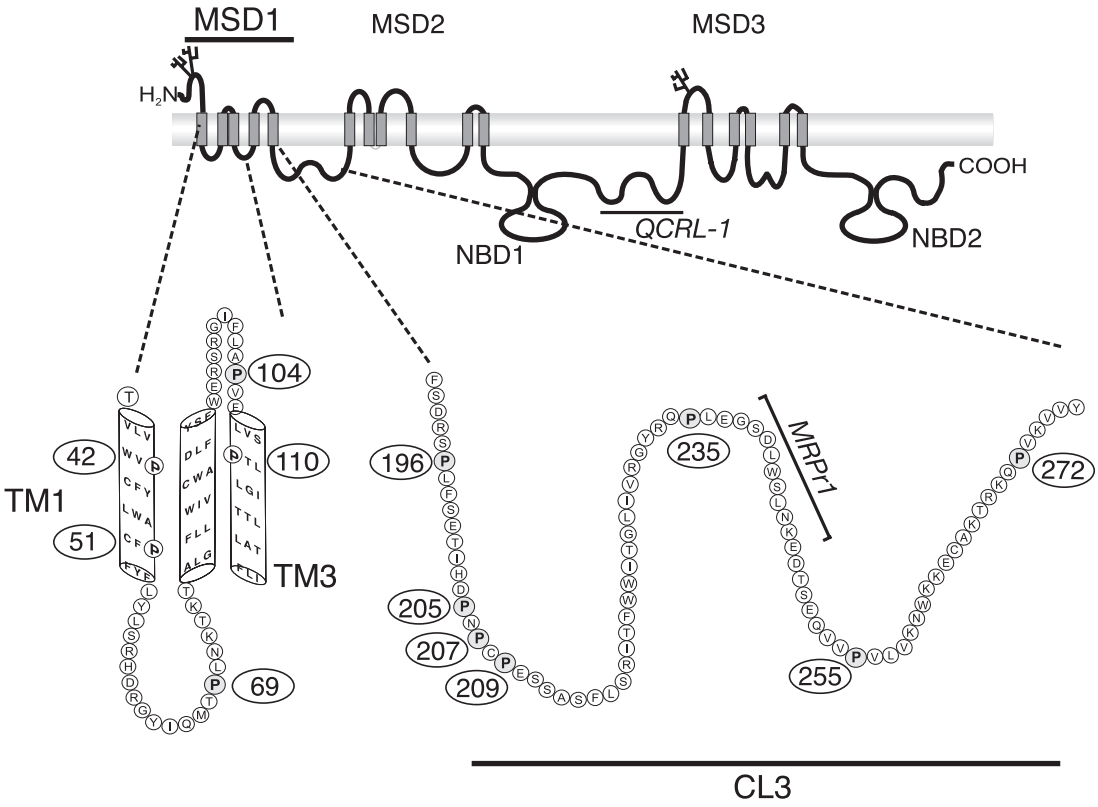

Fig. 1. Schematic diagram of a predicted secondary structure of MRP1 showing the positions of the Pro residues in MSD1 and CL3 replaced with Ala residues in this study. The MAb MRPr1 epitope is located between amino acids 238 and 247 in CL3 and the MAb QCRL-1 epitope between amino acids 918 and 924 are indicated [26,27].

3.3. Plasma membrane trafficking of MRP1-P42/51A and MRP1-Del37-54 is impaired

To examine the cellular localization of the different MRP1-Pro mutant proteins, the transfected HeLa cells were grown to confluence and stained with the MRP1-specific MAb QCRL-3, and then viewed by confocal microscopy [22,24]. Representative confocal micrographs of wild-type and MSD1 MRP1-Pro mutant proteins are shown in Fig. 4A. Wild-type MRP1 localized strongly to the plasma membrane as expected (Fig. 4A, first panel). Similarly, the singly substituted MRP1-Pro mutants P42A, P51A, P69A, and P110A localized strongly to the plasma membrane, similar to wild-type MRP1 (MRP1-P42A and MRP1-P51A shown in Fig. 4A, middle two panels). In marked contrast, cells transfected with the double mutant MRP1-P42/51A showed very weak plasma membrane staining and

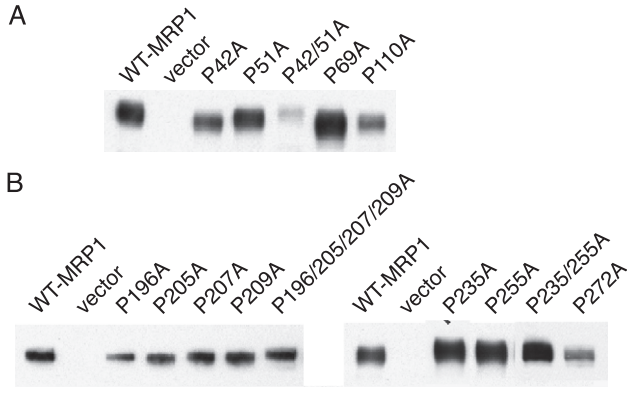

Fig. 2. Immunoblot analysis of mutant MRP1 molecules containing MSD1 and CL3 Pro substitutions expressed in transfected HeLa cells. Relative levels of wild-type and MRP1-Pro mutant proteins in HeLa cell membranes were determined by immunoblotting with the MRP1-specific murine MAb QCRL-1 as described in Materials and methods. (A) Immunoblot of MSD1 (aa 1–195) MRP1-Pro mutant proteins. (B) Immunoblot of CL3 (aa 196–281) MRP1-Pro mutant proteins.

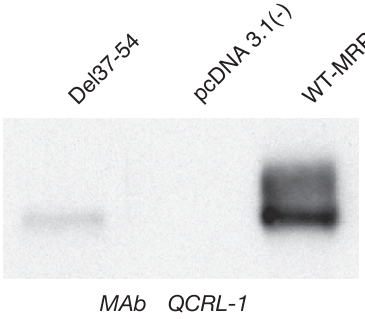

Fig. 3. Immunoblot analysis of TM1 deletion mutant MRP1-Del37-54. Expression of wild-type MRP1 and MRP1-Del37-54 in membranes prepared from transiently transfected HEK293T cells was determined by immunoblotting with the MRP1-specific murine MAb QCRL-1 as described in Materials and methods.

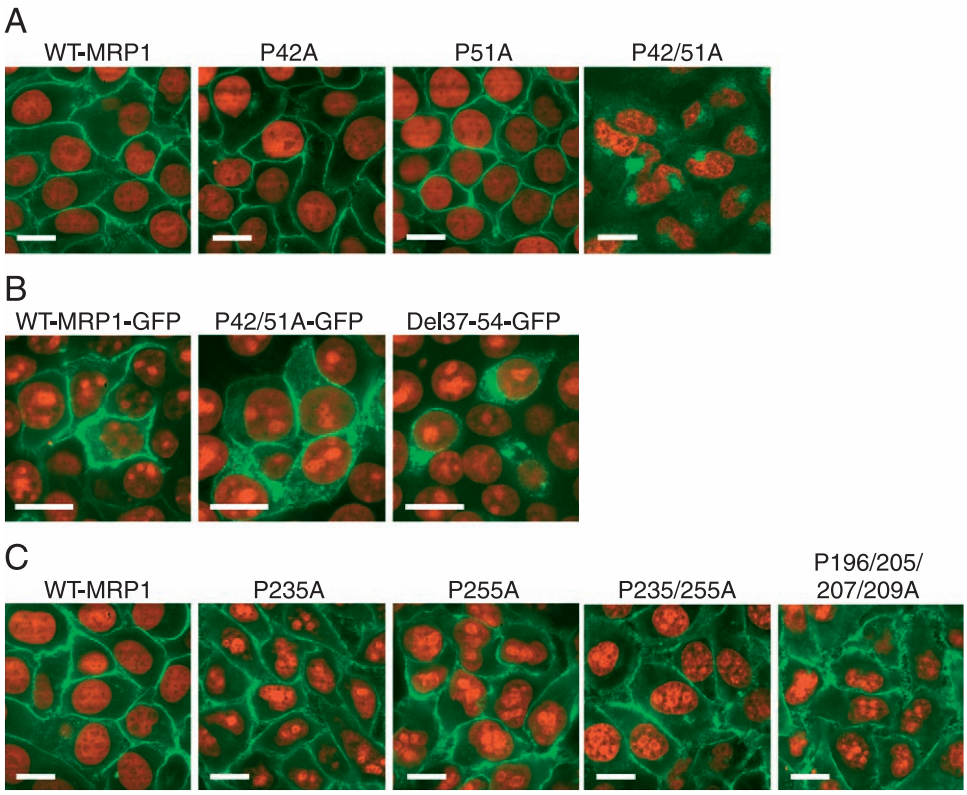

Fig. 4. Confocal laser scanning micrographs of cells expressing wild-type MRP1, TM1 and CL3 MRP1-Pro mutant proteins. (A) HeLa cells expressing wild-type MRP1, MRP1-P42A, MRP1-P51A, and MRP1-P42/51A were seeded at 1.5×10^6 cells per well and processed 24 h later for indirect immunofluorescence and confocal microscopy. MRP1 proteins were detected using MAb QCRL-3, visualized with Alexa 488-tagged secondary antibody, and are shown in green. Nuclei were stained with propidium iodide and are shown in red. No green fluorescence was detected in HeLa cells transfected with pcDNA3.1(−) vector alone (not shown). (B) HEK293T cells were seeded at 3×10^5 cells per well and 24 h later, transfected with cDNAs encoding GFP-tagged wild-type MRP1, MRP1-P42/51A, and TM1 deletion mutant, MRP1-Del37-54. Cells were viewed by confocal microscopy 48 h later. GFP-tagged MRP1 proteins are shown in green; nuclei were stained with propidium iodide and are shown in red. (C) HeLa cells stably expressing wild-type MRP1, MRP1-P235A, MRP1-P255A, MRP1-P235/255A, and MRP1-P196/205/207/209A were cultured and processed for immunofluorescence and confocal microscopy as described in panel A. The horizontal white scale bar in the photographs represents 20 μm.

the mutant MRP1 was localized primarily intracellularly as is evident by the densely fluorescent spots observed (Fig. 4A, fourth panel).

To confirm that the plasma membrane trafficking of the MRP1-P42/51A mutant was impaired, a COOH-terminally GFP-tagged derivative of P42/51A was created and transiently expressed in HEK293T cells. When cells were viewed under the confocal microscope, GFP-tagged wild-type MRP1 localized to the plasma membrane as expected (Fig. 4B, left panel). On the other hand, MRP1-P42/51A-GFP (Fig. 4B, middle panel) showed significant intracellular fluorescence similar to the indirect immunofluorescent staining observed with MAb QCRL-3 in the stably transfected HeLa cells (Fig. 4A, fourth panel). The GFP-tagged mutant showed more plasma membrane localization in the HEK cells than in the stable HeLa cell transfectants, likely due to the higher levels of expression achieved in the transiently transfected HEK cells. Taken together, these results indicate that Ala substitution of the two Pro residues in TM1 at the same time alters MRP1 such that both its expression and plasma membrane localization are impaired.

HeLa cells transfected with the TM1 deletion mutant MRP1-Del37-54 were also examined by indirect immunofluorescence and showed that in the few cells expressing this mutant, its plasma membrane trafficking was severely disrupted (not shown). Similarly, when a GFP-tagged construct (MRP1-Del37-54-GFP) was transiently expressed in HEK293T cells and examined by confocal microscopy, very little of the protein was observed in the plasma membrane despite the fact that the N-glycosylation sites at positions 19 and 23 were still present (Fig. 4B, right panel).

3.4. Mutation of Pro^{255} alters the conformation of CL3

For initial immunoblotting experiments, the well-characterized human MRP1-specific MAb QCRL-1, which detects an epitope in the linker region between NBD1 and MSD3, was used. Subsequently, membranes from HeLa cells expressing the MRP1 mutants with Ala substitutions of P235, P255, and P272 were also immunoblotted with MAb MRPr1 since the epitope of this MAb is located in

CL3 (Fig. 1A). Duplicate immunoblots of the P235A, P255A, P235/255A, and P272A MRP1 mutants probed with MAb QCRL-1 and MAb MRPr1 indicated that detection of the P255A and P235/P255A mutant MRP1 proteins relative to wild-type MRP1 by MAb MRPr1 was lower than that by MAb QCRL-1 (not shown). To quantitate these differences in immunoreactivity more accurately, dot blot analyses of serial dilutions of membrane vesicles were carried out with these two MRP1-specific MAbs (Fig. 5).

When MRP1-P235A was detected with MAb QCRL-1, the signal intensity was approximately 1.8-fold higher than that for wild-type MRP1, while the signal intensity for MRP1-P235/P255A was approximately 2.3-fold higher (Fig. 5A). When MRP1-P235A was detected with MAb MRPr1, the signal intensity was also higher than that of wild-type MRP1 (approximately 1.4-fold) but the signal intensity for P235/255A was less than that of wild-type MRP1 (approximately 75%) (Fig. 5B). Thus, MRP1-P235A

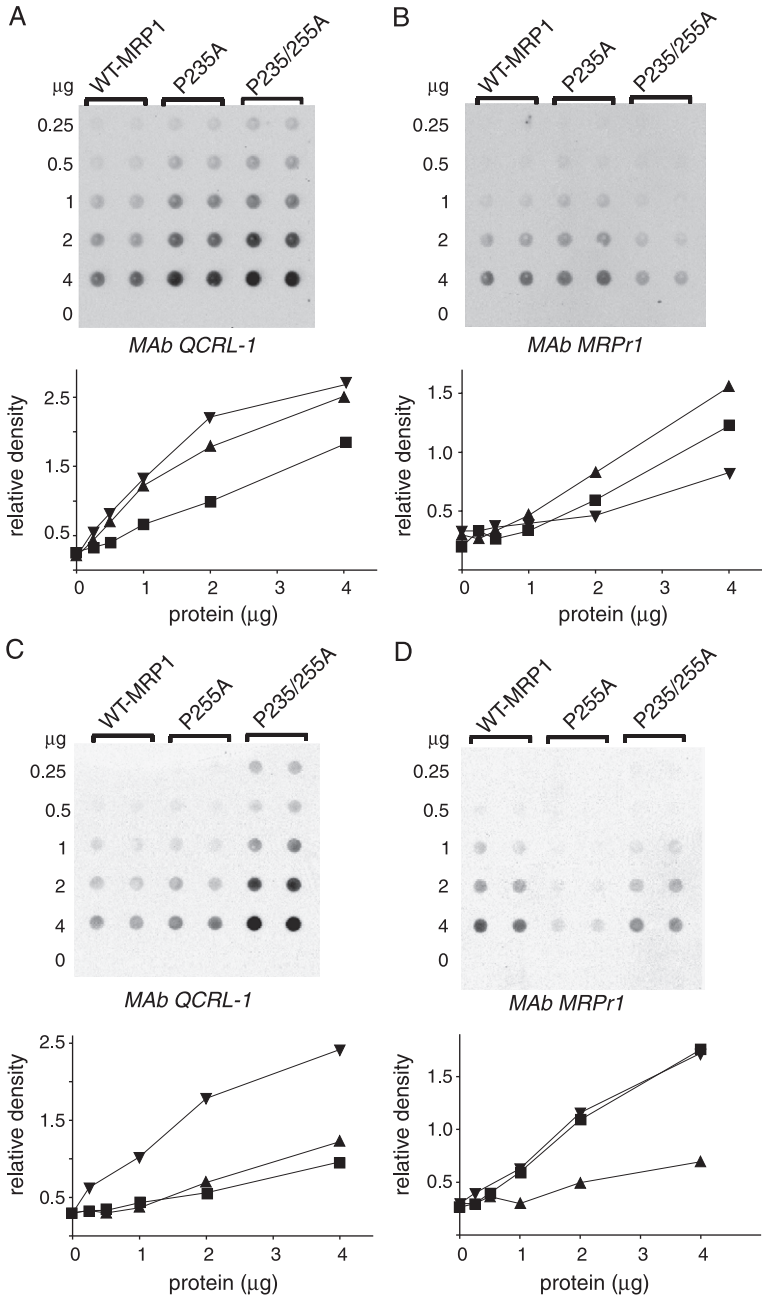

Fig. 5. Dot blot analyses of MRP1 expression in membranes from HeLa cells expressing CL3 mutants MRP1-P235A, MRP1-P255A, and MRP1-P235/255A. Serial dilutions of membrane proteins (0, 0.25, 0.5, 1, 2, and 4 µg) were spotted in duplicate onto nylon membranes as described [25,27]. MRP1-P235A and MRP1-P235/255A mutants probed with (A) MAb QCRL-1 and (B) MAb MRPr1. MRP1-P255A and MRP1-P235/255A mutants probed with (C) MAb QCRL-1 and (D) MAb MRPr1. Graphic representations of densitometric analyses are shown beneath each of the dot blots. (A and B) Wild-type MRP1 (■); MRP1-P235A (▲); MRP1-P235/255A (▼). (C and D) Wild-type MRP1 (■); MRP1-P255A (▲); MRP1-P235/255A (▼).

appeared to be detected equally well by both monoclonal antibodies whereas the double mutant MRP1-P235/255A was not.

We next compared the immunoreactivity of the double mutant MRP1-P235/255A and the single mutant MRP1-P255A. When detected with MAb QCRL-1, the signal intensity for MRP1-P255A was approximately 1.3-fold higher than that for wild-type MRP1 (Fig. 5C). In contrast, the signal intensity of MRP1-P255A detected with MAb MRPr1 was only 50% that of wild-type MRP1 detected with the same MAb (Fig. 5D). Overall, these findings suggest that substitution of Pro^{255} with Ala might introduce a change in the structure of this region of MRP1 such that the MRPr1 epitope is less accessible to the MAb.

3.5. Plasma membrane localization of CL3 MRP1-Pro mutants is unchanged in transfected HeLa cells

The changes in immunoreactivity of the MRP1-P255A and MRP1-P235/255A mutant proteins suggested that the Pro^{255} to Ala substitution introduced a conformation change in CL3. To determine whether this change might also affect plasma membrane trafficking of MRP1-P255A, transfected HeLa cells expressing this mutant as well as the MRP1-P235A and MRP1-P235/255A mutants were examined by confocal microscopy as before. Like wild-type MRP1, MRP1-P255A as well as MRP1-P235A and MRP1-P235/255A localized mostly to the plasma membrane of confluent HeLa cells (Fig. 4C). Thus, despite the apparent change in conformation of MRP1 proteins containing the Pro255-Ala substitution, their expression at the plasma membrane remained unchanged. Plasma membrane staining was also observed in cells expressing the quadruple MRP1-P196/205/207/209A mutant.

3.6. LTC_4 and $E_217\beta G$ transport activity of MSD1 and CL3 MRP1-Pro mutants

To determine whether any of the Pro substitutions affected organic anion transport by MRP1, the activity of the various Pro mutants was examined using membrane vesicles prepared from the transfected HeLa cell lines and LTC_4 as substrate. The kinetic parameters of LTC_4 transport obtained for the wild-type and MRP1-Pro mutants are summarized in Table 1. Two sets of experiments are shown because it was technically not feasible to measure kinetic parameters of all mutants in a single experiment. The differences in the K_m values obtained for wild-type MRP1 in the two sets of experiments are within the range reported in the literature. V_{max} values that were normalized to take into account differences in expression levels of the various MRP1 mutants relative to the wild-type protein are also shown.

In the first set of experiments, the K_m (LTC_4) values for the four single MSD1 mutant proteins (range 103–150 nM) were found to be comparable to that of wild-type MRP1 (128 nM). When normalized for relative MRP1 expression levels, the V_{max} values of these mutants ranged from 41 to 124 pmol mg protein^{-1} min^{-1} versus 80 pmol mg protein^{-1} min^{-1} for wild-type MRP1. Thus, all of the MSD1 Pro mutants retained the ability to transport LTC_4 with TM1 mutants P42A, P51A, and P69A showing an approximately 30–50% decrease in relative LTC_4 transport efficiency, while P110A (TM3) showed an increase of approximately 50%. Expression levels of the double P42/51A TM1 mutant were so low that reliable estimates of V_{max} for LTC_4 transport could not be obtained; the K_m was estimated to be similar to wild-type MRP1.

Table 1
Kinetic parameters of [^{3}H]LTC_4 uptake by membrane vesicles prepared from transfected HeLa cells expressing MRP1-Pro mutants

Transfected cell line	K_m (nM)	V_{max} (pmol min^{-1} mg^{-1})	Normalized V_{max}
MSD1 mutants			
WT-MRP1	128	80	80
P42A	139	35	41
P51A	109	55	50
P42/51A	103	15	48
P69A	142	46	42
P110A	150	73	124
CL3 mutants			
WT-MRP1	88	57	57
P196A	143	25	52
P205A	63	14	88
P207A	81	75	110
P209A	112	121	85
P196/205/207/209A	90	87	94
P235A	91	45	30
P255A	99	110	67
P235/255A	97	81	35
P272A	80	59	109

The kinetic parameters of [^{3}H]LTC_4 uptake were determined by regression analysis of the Eadie–Hofstee transformed data. The normalized V_{max} values were obtained by correcting determined V_{max} values for differences in MRP1 expression levels as determined by immunoblot analysis. Results shown are means of triplicate determinations within a single experiment. Similar results were obtained in a second independent experiment.

In the second set of experiments, the K_m (LTC_4) values for the seven singly substituted CL3 mutant MRP1 proteins were determined to range from 63 to 143 nM compared to 88 nM for wild-type MRP1. The normalized V_{max} values for these mutants ranged from 30 to 110 pmol mg protein^{-1} min^{-1} compared to 57 pmol mg protein^{-1} min^{-1} for wild-type MRP1. Thus, the level of LTC_4 transport by these mutants ranged from approximately 0.5- to 2-fold that of wild-type MRP1. The transport kinetics for the multiply substituted MRP1 CL3 mutants were also determined. MRP1-P196/205/207/209A displayed LTC_4 transport activity comparable to that of wild-type MRP1 whereas transport by the MRP1-P235/255A mutant was reduced by approximately 25%.

To determine whether $E_217\beta G$ uptake was affected by Ala substitution of the Pro residues in MSD1 and CL3, transport of this substrate was also measured in membrane vesicles enriched for the various mutant proteins at a single time point. Results were expressed relative to wild-type

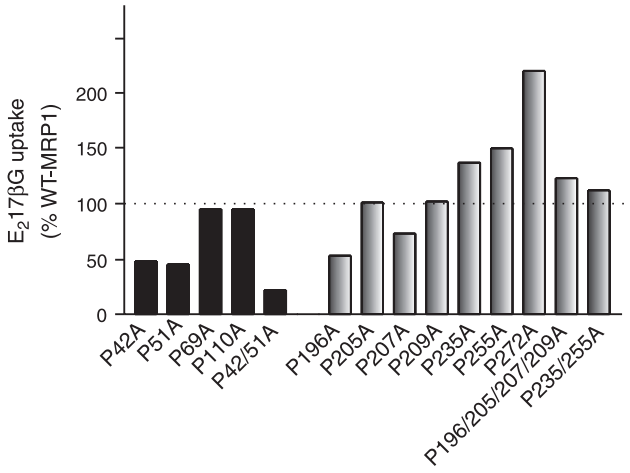

Fig. 6. [^{3}H]$E_2$17βG uptake by membrane vesicles prepared from HeLa cells expressing MRP1-Pro mutant proteins. The levels of [^{3}H]$E_2$17βG uptake are expressed relative to wild-type MRP1 transport activity after normalization of the data to take into account differences in MRP1 expression of the various mutants. Bars represent the means of results obtained in two independent experiments.

MRP1 after normalization of the data to take into account differences in relative MRP1 expression and are shown in Fig. 6. $E_2$17βG uptake levels by the four single Pro MSD1 mutants P42A, P51A, P69A, and P110A were 48%, 43%, 95%, and 95% those of wild-type MRP1, respectively. $E_2$17βG uptake by the double P42/51A TM1 mutant was approximately 25% that of wild-type MRP1 although, again, expression levels of this mutant were so low that this estimate may not be reliable.

All seven single CL3 mutants transported $E_2$17βG, with levels of uptake ranging from approximately 0.5- to 2-fold that of wild-type MRP1. $E_2$17βG uptake by the MRP1-P196/205/207/209A quadruple mutant was comparable to that of wild-type MRP1 as was uptake by the double MRP1-P235/255A mutant. Thus, although there was some variability, all MRP1-Pro mutants retained the ability to transport this glucuronide conjugated substrate as well as LTC_4.

4. Discussion

In this study we have characterized the structural and functional consequences of substituting an Ala residue for each of the five Pro residues in MSD1 and seven Pro residues in CL3 of MRP1. Eleven of the twelve single MRP1-Pro mutants could be stably expressed in HeLa cells; the exception was a mutant containing an Ala in place of Pro^{104}. The double MRP1-P104/110A protein was also not detected in stable transfectants. Exactly why these MRP1-Pro^{104} mutant proteins were not expressed is unclear; however, Pro^{104} is predicted to be located in the relatively short extracellular loop between TM2 and TM3 and thus may be critical for ensuring the proper insertion of TM3 in the membrane during the biosynthesis and folding of MRP1, at least in HeLa cells [29,30]. Misfolding of MRP1 could lead to its rapid degradation and thus explain why mutant MRP1 molecules containing Pro^{104} substitutions could not be detected.

It has previously been noted that Pro residues are often found in the TM segments of ion channels and transporters but not in the TMs of proteins that have no transport function [19]. Presumably because of proline's ability to create a kink in an α-helical structure, this amino acid has frequently been shown to play an important structural and/or functional role when located in the MSDs of certain transport proteins and ion channels including the ABC proteins CFTR and P-glycoprotein [20,21,31,32]. Pro residues present at a given position *i* in α-helices (as is predicted for Pro^{42}, Pro^{51}, and Pro^{110}) cannot form hydrogen bonds to the carbonyl oxygens of amino acids at positions *i*-3 and *i*-4. In this way, proline-containing motifs can distort the helix and may in some cases form so-called 'molecular hinges' [33,34]. Such hinges may be critical for the conformational changes in MSDs required to transduce signals across membranes. However, in the present study, we have shown that single Ala substitutions of the TM residues Pro^{42}, Pro^{51}, and Pro^{110} do not cause any major alterations in MRP1 expression nor its plasma membrane localization or organic anion transport activity. This suggests that none of these residues contribute to a functionally important molecular hinge in MRP1. Whether or not Pro-induced helix distortions or flexibility in other MSDs of MRP1 might play a role in the conformational changes that accompany substrate binding and translocation (and/or nucleotide binding and hydrolysis) is not yet known.

In contrast to the single Pro mutants, expression levels of the double TM1 mutant MRP1-P42/51A were reduced by more than 80%. The exact role of TM1 in the assembly of functional MRP1 at the plasma membrane is not fully understood. Zhang [29] found that TM1 did not have enough activity to initiate membrane translocation in an in vitro translation reporter system and that TM2 was required. In addition, we previously demonstrated that a mutant lacking the first 66 amino acids of MRP1 including TM1 ($MRP1_{67-1531}$) could be expressed in insect cell membranes but could not transport LTC_4 [16]. On the other hand, we have also shown that single substitutions of several other TM1 residues (Trp^{40}, Cys^{43}, Tyr^{45}, Trp^{47}, Cys^{49}, Tyr^{53}) did not significantly affect MRP1 expression and caused no, or only substrate-selective, changes in transport activity [23,35] (C.J. Oleschuk, R.G. Deeley and S.P.C. Cole, unpublished work). Similarly, the expression of the single Pro^{42} and Pro^{51} mutants appeared comparable to wild-type MRP1 and transport activity was only moderately reduced. Thus, a substantial number of single amino acid substitutions can be made in TM1 without a major loss in protein expression levels and transport activity. This raises the possibility that the lack of LTC_4 transport activity observed for the $MRP1_{67-1531}$ truncation mutant might be attributed

to the absence of an amino acid(s) in the first 36 residues of the protein that precede TM1. Consistent with this idea are the recent findings of Yang et al. [36] which showed that replacement of Cys^{7} with Ala caused an almost complete loss of LTC_4 transport activity and a change in conformation of the NH_2 terminus although expression levels and plasma membrane localization of this mutant MRP1 were not diminished compared to wild-type MRP1.

The presence of Pro^{42} and Pro^{51} in wild-type MRP1 introduces a significant distortion into the backbone of TM1 which completely disappears when these two residues are both replaced with Ala as in the MRP1-P42/51A mutant (Fig. 7). This straightening of the TM1 α-helical axis would be expected to change the angle between the plane of the helix and the plane of the side chains of the aromatic amino acids found there (Trp^{40}, Tyr^{45}, Trp^{47}, Phe^{50}, Phe^{52}, Tyr^{53}, and Phe^{54}). This change in angle could lead to a disruption of the packing of TM1 with other TM helices of MRP1 which in turn could destabilize the protein or impair its folding, leading to enhanced protein degradation. The small amount of MRP1-P42/51A expressed in the transfected HeLa cells appears fully glycosylated and a portion of it is present in the plasma membrane of HEK cells, indicating that processing was not grossly affected by the mutations. Furthermore, when corrected for relative MRP1 expression levels, organic anion transport activity seemed intact or only modestly altered. Thus, the double Pro substitution in TM1 appears to destabilize MRP1 rather than affect its ability to transport organic anions. MRP1 protein expression was also reduced when the entire TM1 helix (aa 37–54) was deleted, but in contrast to the P42/51A mutant, this deletion mutant MRP1 molecule was not glycosylated. Thus, both our previous and present observations [23,35] indicate that although many single-amino-acid substitutions in TM1 can be tolerated without substantial loss of transport activity

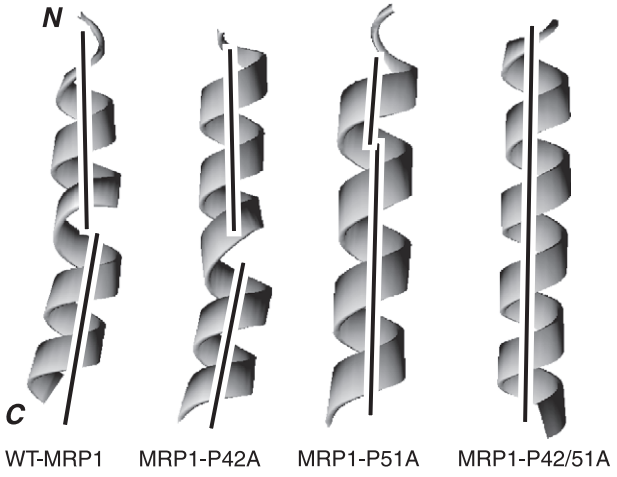

Fig. 7. Models illustrating the predicted differences in the α-helical axis of TM1 in wild-type MRP1 and MRP1-Pro mutants P42A, P51A, and P42/51A. Models were generated using Sybyl (Tripos Inc., St. Louis, MO) and images were prepared using Web Lab viewer Pro42 (Accelrys Inc., San Diego, CA). N, NH_2 terminus; C, COOH terminus.

or plasma membrane localization, TM1 nevertheless appears to play some role in facilitating the stable expression of MRP1 in mammalian cell membranes.

Previous studies have shown that the cytoplasmic linker CL3 connecting MSD1 to MSD2 (i.e. aa 204–281) is important for transport activity as well as for the correct routing of MRP1 to the basolateral plasma membrane of polarized epithelial cells [16–18]. Indeed, this region may be considered a distinct protein domain within MRP1 since at least portions of it can form specific associations with the core four-domain region of the protein [18]. When Ala residues were substituted individually for the seven Pro residues in CL3, the mutant proteins were expressed at levels comparable to those of wild-type MRP1 in most cases. Similarly, all of the single CL3 MRP1-Pro mutants trafficked appropriately to the plasma membrane and exhibited significant levels of organic anion transport activity. Four of the seven CL3 Pro residues (Pro^{196}, Pro^{205}, Pro^{207}, Pro^{209}) are located in a cluster at the beginning of the loop, which might be expected to impose some constraints on the structure of this region. Somewhat surprisingly, however, when all four Pro residues were simultaneously replaced with Ala, the properties of the mutant protein (MRP1-P196/205/207/209A) were similar to those of wild-type MRP1. Previously, Bakos et al. [18] demonstrated that deletion of amino acids 223–232, which are predicted to be within an amphipathic helix in CL3, eliminated MRP1 transport activity but not glycosylation or plasma membrane routing. The four clustered Pro residues mutated in the present study are located NH_2-proximal to this helical domain which may account for the absence of any major phenotypic changes observed in the MRP1-P196/205/207/209A mutant. Nevertheless, three of them are located very close to the NH_2 terminus of the minimal region of CL3 that has been defined thus far to be necessary for LTC_4 binding and transport activity (amino acids 204–281) [17,37]. Interestingly, we have recently shown that Ala- and Ser-substituted MRP1-Cys^{208} mutants also display phenotypes similar to those of the wild-type protein [35].

Two of the more COOH-proximal CL3 Pro residues (Pro^{235} and Pro^{255}) are located near the linear epitope recognized by the MRP1-specific MAb MRPr1 [27]. Consequently, we wondered whether structural changes caused by substitutions of these two Pro residues, alone or in combination, might be detected as a change in reactivity with this MAb. Indeed, substitution of Pro^{255} with Ala caused a significant decrease in MRP1 reactivity with MAb MRPr1 but not MAb QCRL-1, presumably reflecting some conformational change in this region of the protein. Nevertheless, neither the P255A mutant nor the P235/255A double mutant showed major changes in plasma membrane localization or organic anion transport. The change in MAb MRPr1 reactivity caused by the P255A substitution was unexpected. The Pro^{255} residue lies COOH-proximal to the peptide sequence recognized by the MAb MRPr1 and, furthermore, the epitope is linear and its detection would

not normally be expected to change under the denaturing conditions of immunoblotting [27,38]. However, linear epitopes are sometimes known to require some local structural distortion or involve closely spaced but non-contiguous amino acids such as those found on one face of an amphipathic α-helix [39]. Our data suggest that this may be the case for MAb MRPr1 [18] and thus its description as a linear epitope may not be completely accurate.

In conclusion, our studies show that Pro residues in certain regions of the NH_2-terminal MSD of MRP1 and the cytoplasmic loop connecting it to the rest of the molecule can play a role in the expression and structure of this five domain ABC transporter protein. We have demonstrated that the Pro^{42} and Pro^{51} residues in TM1 and possibly the Pro^{104} residue in the extracellular loop connecting TM2 to TM3 are important for stable expression of MRP1 in mammalian cell plasma membranes. In addition, we have shown that certain structural changes in at least two different regions of CL3 can be accommodated without loss of transport activity or plasma membrane localization. Finally, our finding that a mutant MRP1 molecule can show a MAb-selective change in immunoreactivity could be relevant to clinical studies of MRP1 expression and supports the principle that expression levels of MRP-related proteins should be verified with more than one antibody as has been recommended previously for detection of P-glycoprotein and other drug resistance markers [40].

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