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A Functional Role of Intracellular Loops of Human Multidrug Resistance Protein 1

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Multidrug resistance protein 1 (MRP1) is a human ATP-binding cassette (ABC) transporter in the plasma membrane. It confers multidrug resistance to tumor cells by actively effluxing intracellular drugs. To examine the functional significance of intracellular loops (ICLs) in MRP1, we determined the effect of mutation of the amino acid sequence EXXXG, which is conserved in ICL5 and ICL7 of human MRP1, 2 and 3, sulfonylurea receptor (SUR) 1 and 2, and mouse MRP1 and 2. E and G in the ICLs of human MRP1 were mutated to L and P, respectively, and the N-terminal (including ICL5) and C-terminal (including ICL7) wild type or mutant halves of MRP1 were co-expressed in insect cells. The mutation of either ICL5 or ICL7 considerably decreased ATP-dependent LTC_4 uptake into vesicles of insect cells expressing mutated MRP1. GSH-dependent photolabeling of MRP1 with an ^{125}I-labeled photoaffinity analog of azido agosterol A (azido AG-A) was abolished by the mutations in ICL5 and ICL7. Mutations in ICL5 of MRP1 almost completely inhibited the labeling of NBD2, but not NBD1, by 8-azido-α-[^{32}P]ATP. In contrast, mutations in ICL7 of MRP1 abolished the labeling of both NBDs. Mutation of either ICL5 or ICL7 of MRP1 almost completely inhibited vanadate trapping with 8-azido-α-[^{32}P]ATP by both NBD1 and NBD2 domains. These findings indicate that the intramolecular signaling between NBD and ICL_s in MRP1 is vital for MRP1 function.

Key words: ICL, MRP1, structure-function.

Abbreviations: P-gp, P-glycoprotein; MRP1, multidrug resistance protein 1; ABC, ATP-binding cassette; MDR, multidrug resistance; NBDs, nucleotide binding domains; CFTR, cystic fibrosis transmembrane conductance regulator; LTC_4, leukotriene C_4; AG-A, agosterol A; azido AG-A, azidophenyl agosterol A; GSH, glutathione; VCR, vincristine; TM, transmembrane segment; SUR, sulfonylurea receptor; CF, cystic fibrosis; PMP70, 70-kDa peroxisomal membrane protein; ICL, intracellular loop; ALDP, adrenoleukodystrophy protein; ALD, adrenoleukodystrophy.

ATP-binding cassette (ABC) transporters are members of a superfamily of membrane proteins involved in the transport of a variety of molecules across membranes (*1*). A typical ABC transporter is composed of two homologous halves. Each half of the protein comprises six transmembrane segments and one nucleotide binding domain (NBD) (*1*). Multidrug resistance protein 1 (MRP1), which confers multidrug resistance (MDR) on tumor cells, belongs to this superfamily (*2*). MRP1 is characterized by an extra N-terminal extension of five transmembrane segments that is connected to the typical 12 transmembrane segments by a cytoplasmic linker (*2*).

EAA motifs are conserved sequences of approximately 30 amino acid residues in intracellular loop 2 (ICL2) of prokaryotic ABC transporters (*3, 4*). Alignment of prokaryotic ABC transporters reveals several residues that are highly conserved in the core of this motif (*3, 4*). Mutation of a central conserved glycine residue results in loss of function in bacterial transporters (*3*). A 15 amino acid EAA-like motif is also found in eukaryotic ABC transporters (*5*). Mutations in a conserved glutamic acid residue in the EAA-like motif of the gene encoding adrenoleukodystrophy protein (ALDP) have been reported in four unrelated adrenoleukodystrophy (ALD) patients (*6–8*). A deletion of 19 amino acids in ICL2 of the cystic fibrosis transmembrane regulator (CFTR) influences the stability of Cl^- channel conductance (*9*). Conserved motifs in ICL_s therefore appear to play critical roles in the functions of transporters.

The ICL5 and ICL7 regions of MRP1 correspond to ICL2 and ICL4 in CFTR. We presumed that the MRP1 family of transporters may also have conserved amino acids within these regions that are important for function, and found amino acids in ICL5 and ICL7 of MRP1 that are conserved among human MRP isoforms as well as in sulfonylurea receptor (SUR) 1 and 2. In view of the importance of understanding MRP1 molecular mechanisms for tumor therapy we therefore examined the effects of mutations of these conserved amino acids on the function of human MRP1.

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MATERIALS AND METHODS

Materials—[^{125}I]NaI [3.7 GBq (100 μCi)/ml] and [14,15, 19,20-^{3}H(N)]LTC_4 (leukotriene C_4) (146 Ci/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA). 8-Azido-α-[^{32}P]ATP was from ICN Biomedicals (20 Ci/mmol) (St. Laurent, Quebec, Canada). Unlabeled LTC_4 was from Calbiochem (La Jolla, CA). The synthesis and use of [^{125}I]11-azidophenyl agosterol A ([^{125}I]azido AG-A) were described in our previous paper (*10*). CELLFECTAMINE and competent DH10Bac *E. coli* cells were purchased from Invitrogen Corp. (Carlsbad, CA). Anti-MRP1 monoclonal antibodies, MRPr1 (epitope amino acids 238–247), and MRPm6 (epitope amino acids 1511–1520) were obtained from Progen Biotechnick (Heidelberg, Germany).

Cell Culture and Membrane Vesicle Preparation—Sf21 insect cells were cultured in serum-free Sf-900 II SFM medium (Invitrogen Corp.). Membrane vesicles were prepared from Sf21 insect cells infected with various recombinant baculoviruses as previously described (*10, 11*). Membrane vesicles were suspended in a buffer comprising 10 mM Tris-HCl (pH 7.5) and 250 mM sucrose. Protein concentrations were determined by the method of Bradford (*12*).

Generation of Constructs and Viral Infection—The construction of pFastBac1 for dual expression of both the N- and C-terminal halves ($N_{1–932}$ and $C_{932–1531}$) of MRP1 and the strategy for mutagenesis were previously described (*10*).

The strategies employed for site-directed mutagenesis of E507L/G511P and E1157L/G1161P in MRP1 cDNA were previously described (*10*). The primers used to generate E507L/G511P mutations were forward and reverse primers: 5′CTCAATCCGATCAAAGTGCTAAAG3′ and 5′AATTAAGTTCATCAGCTTGATCCG3′ (The underlining indicates mismatched bases the encoding E507L and G511P mutations, respectively). We also used a pair of forward and reverse primers to generate E1157L/G1161P mutations. The primers were: Forward: 5′TTGCTGCCGGTCAGCGTCATTCGA3′, and reverse: 5′GGTCAAGTTGAAATGGGAATA3′ (The underlining indicates mismatched bases encoding the E1157L and G1161P mutations, respectively). Baculoviruses expressing the wild type (wt) and mutant MRP1s described above were generated using the Bac to Bac expression system (Invitrogen Corp.) as described previously (*10, 11*).

[^{3}H]LTC_4 Uptake by Membrane Vesicles—The extent of [^{3}H]LTC_4 uptake was measured using a rapid filtration technique as previously described (*10*). Briefly, isolated membrane vesicles (25 μg of protein) were incubated in the presence or absence of 4 mM ATP in 50 μl of transport buffer [0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM phosphocreatine, and 100 μg/ml creatine phosphokinase] with 100 nM [^{3}H]LTC_4 for the indicated times at 37°C. The reaction was stopped with 3 ml of an ice-cold stop solution [0.25 M sucrose, 0.1 M NaCl, and 10 mM Tris-HCl (pH 7.5)]. Samples were passed through Millipore filters (GVWP, 0.22 μm pore size) under light vacuum. Following three rinses with 3 ml of the cold stop solution, the filters were dried and immersed in liquid scintillation fluid, and their radioactivity was measured.

Photoaffinity Labeling of MRP1—[^{125}I]Azido AG-A (7.2 μCi/nmol) was used for photolabeling studies that were carried out as previously described (*10*). For photolabeling of NBD1 and NBD2 of MRP1 with 8-azido-α-[^{32}P]ATP, membrane vesicles (50 μg of protein) were incubated for 5 min on ice with 5 μM 8-azido-α-[^{32}P]ATP in the presence of 5 mM $MgCl_2$ and 1 μM LTC_4 as indicated. Following continuous irradiation of samples with a short wavelength (366 nm) UV lamp for 10 min on ice, the samples were solubilized in sodium dodecyl sulfate (SDS) sample buffer as described by Debenham *et al.* (*13*), and then subjected to SDS-PAGE. Autoradiograms were developed after 6–24 h exposure at room temperature.

Orthovanadate Trapping of 8-Azido-α-[^{32}P]ADP by MRP1—Membrane vesicles (100 μg of protein) were incubated in 20 μl of transport buffer containing 5 μM 8-azido-α-[^{32}P]ATP, 5 mM $MgCl_2$ and 1 mM sodium orthovanadate at 37°C for 15 min in the presence or absence of 1 μM LTC_4. Trapping in the absence of orthovanadate was used as a negative control. The reactions were stopped by the addition of 1 ml of ice-cold Tris-EGTA buffer [50 mM Tis-HCl (pH 7.4), 0.1 mM EGTA, and 5 mM $MgCl_2$]. The membranes were collected by centrifugation at 25,600 × *g* for 30 min at 4°C. The pellets were washed again with the same buffer and then resuspended in 20 μl of transport buffer. The samples were then irradiated with UV and subjected to SDS-PAGE as described (*14*). Autoradiograms were developed after 10–24 h exposure at –70°C.

RESULTS

Expression and Membrane Topology of MRP1 with Mutations in ICL5 or ICL7—A comparison of the amino acid sequences of the human and mouse MRP isoforms (human MRP1, 2 and 3, and mouse MRP1 and 2) and SUR1 and 2a revealed that amino acids EXXXG are conserved in both ICL5 and ICL7 of these proteins (Fig. 1). To determine the functional significance of these amino acids we constructed the N- and C-terminal halves of MRP1 in which the conserved E and G in ICL5 or ICL7 were mutated to L and P, respectively. The mutant N- or C-terminal halves of MRP1 were simultaneously expressed in insect cells with the wild type C- or N-terminal halves, respectively, using a dual expression vector. The expression levels of the reconstituted fragments were similar for the mutant and wild type combinations, as judged on immunoblot analysis (Fig. 2).

Effects of ICL Mutations in MRP1 on ATP-Dependent LTC_4 Uptake—To determine the effects of the mutations in ICL5 and ICL7 on drug transport, membrane vesicles were prepared from insect cells co-expressing the N- or C-terminal halves with mutations in ICL5 or ICL7, respectively, with the wild type C- or N-terminal halves, respectively, and then the ATP-dependent LTC_4 uptake into the vesicles was examined. The LTC_4 uptake activitity of membrane vesicles expressing the mutated N- or C-terminal fragments was considerably decreased compared with those expressing the wt N+C fragments (Fig. 3). These results suggest that both ICL5 and ICL7 of MRP1 play important roles in drug transport.

Effects of ICL Mutations on Drug and ATP Interactions of MRP1—We next investigated the potential mechanism by which EXXXG mutations in the ICL regions modulate

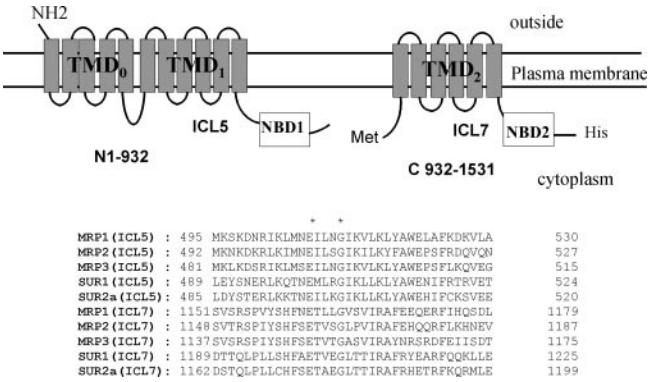

Fig. 1. **A conserved ExxxG region in the ICL5 and ICL7 domains of MRP1-related transporters.** A schematic diagram depicting the location of the ICL5 and ICL7 domains in MRP1 is shown at the top. Alignment of sequences within ICL5 and ICL7 of human MRP isoforms 1, 2 and 3 with those of the homologous regions of mouse MRP1 and 2, and those of SUR 1 and 2 is shown at the bottom. The conserved E and G residues are asterisked.

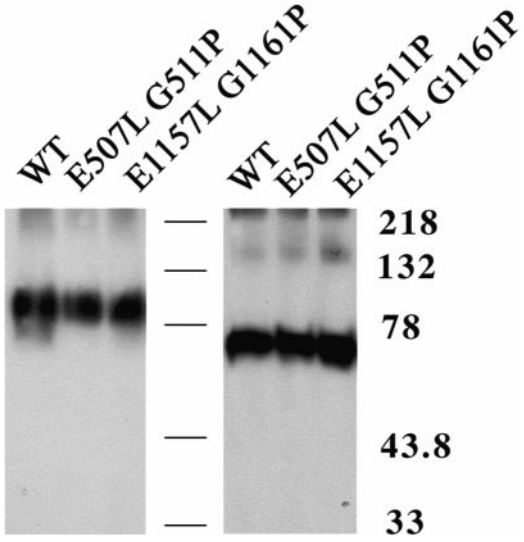

Fig. 2. **Expression of MRP1 mutant fragments in insect cells.** Membrane vesicles (20 μg of protein) from insect cells infected with recombinant baculovirus dual expression vectors encoding both N- and C-termini of either wild type or mutant MRP1 in the indicated combinations were separated by 8.5% SDS-PAGE, followed by immunoblotting with either anti–N-terminal antibody r1 (left) or anti–C-terminal antibody m6 (right), respectively. Molecular weight markers are shown on the right. N, $N_{1–932}$ of MRP1. C, $C_{932–1531}$ of MRP1.

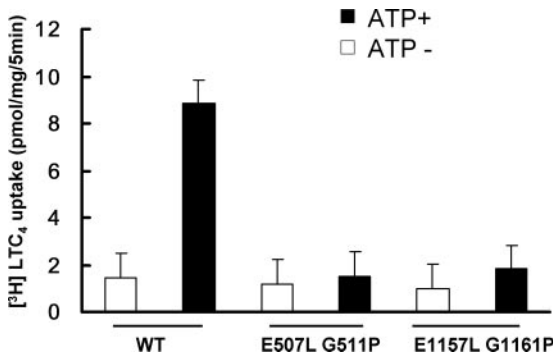

Fig. 3. **Effect of mutations in ICL5 and ICL7 on ATP-dependent uptake of [^{3}H]LTC_4 by membrane vesicles.** Membrane vesicles (25 μg of protein) co-expressing both N- and C-terminal wt or mutant fragments of MRP1 were incubated with 50 nM [^{3}H]LTC_4 in 50 μl of transport buffer [0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM phosphocreatine, and 100 μg/ml creatine phosphokinase] in the absence (open) or presence (filled) of 4 mM ATP at 37°C for 5 min. The uptake of [^{3}H]LTC_4 over five minutes was calculated as pmol/mg protein. The data represent the means ± SE for three separate experiments.

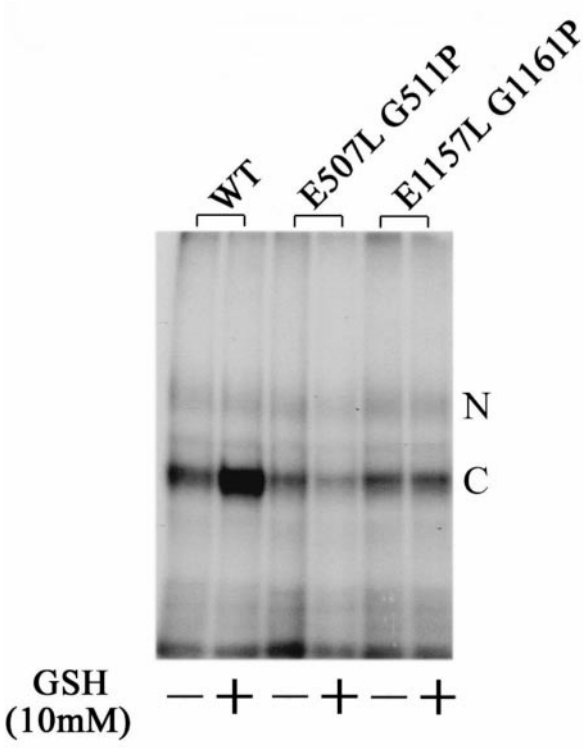

Fig. 4. **Effects of mutations in ICL5 and ICL7 on MRP1 drug binding activity.** Membrane vesicles (50 μg of protein) co-expressing both N- and C-terminal wt or mutant fragments of MRP1, as indicated at the right, were incubated with 5 μM [^{125}I]azido AG-A in the absence (–) or presence (+) of GSH (10 mM). Samples were then separated by 8.5% SDS-PAGE. Autoradiograms were developed after 8 h exposure at room temperature.

solute transport. One possibility was that these mutations inhibit drug transport by affecting drug binding and/or ATP binding to MRP1. We therefore assayed drug binding to membrane vesicles expressing wt or mutated MRP1 by photolabeling of the vesicles with [^{125}I]azido AG-A in the presence of 10 mM GSH (Fig. 4). Quantification of the signal intensities for the N- and C-terminal halves of MRP1 showed that mutation of either ICL5 or ICL7 completely abrogated the GSH-stimulated azido AG-A binding to the C-terminal half of MRP1, this indicating that these mutations directly or indirectly affect the drug binding ability of MRP1.

The effects of the mutations on ATP binding to MRP1 were investigated by photoaffinity labeling of membrane vesicles expressing both half molecules of MRP1s on ice using 8-azido-α-[^{32}P]ATP. The N-terminal and C-terminal

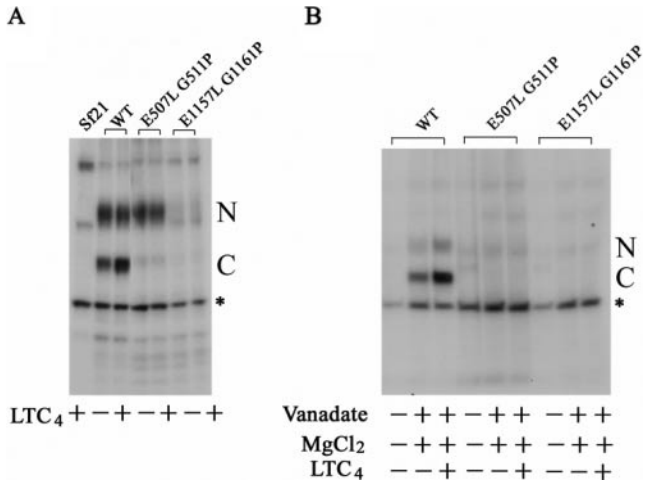

Fig. 5. **Effects of mutations in ICL5 and ICL7 on ATP binding and ATPase activity of MRP1.** A: Membrane vesicles (50 μg of protein) from insect cells (Sf21) co-expressing both N- and C-terminal wt or mutant fragments of MRP1 were incubated on ice with 5 μM 8-azido-α-[^{32}P]ATP and 5 mM $MgCl_2$ in the presence (+) or absence (–) of 1 μM LTC_4 as described under "EXPERIMENTAL PROCEDURES." Bound 8-azido-α-[^{32}P]ATP was detected by autoradiography. A photolabeled endogeneous protein is indicated by an asterisk. B: Membrane vesicles (100 μg of protein) from insect cells co-expressing both N- and C-terminal wt or mutant fragments of MRP1 were incubated with 5 μM 8-azido-α-[^{32}P]ATP and 1 mM sodium orthovanadate in the absence (–) or presence (+) of 1 μM LTC_4, followed by the trapping procedures described under "EXPERIMENTAL PROCEDURES" in the absence (–), or the presence (+) of $MgCl_2$ and vanadate.

halves of wild type MRP1 were intensely labeled with 8-azido-α-[^{32}P]ATP (Fig. 5A). LTC_4 enhanced the labeling of the C-terminal half with 8-azido-α-[^{32}P]ATP but not that of the N-terminal half. Mutation of E507L/G511P in ICL5 of MRP1 almost completely inhibited the labeling of 8-azido-α-[^{32}P]ATP in NBD2 but not adjacent NBD1. Moreover, mutation of ICL7 of MRP1 almost completely abolished the labeling of both NBD1 and NBD2. We also examined the effect of the ICL mutations on trapping by determination of 8-azido-α-[^{32}P]ATP labeling of MRP1 fragments at 37°C in the presence of vanadate. No trapping was detected in the absence of vanadate in cells expressing both wt fragments (Fig. 5B). In these cells photolabeling of NBD2 and weak photolabeling of NBD1 were detectable under vanadate trapping conditions with 8-azido-α-[^{32}P]ATP, and the trapping at both NBDs was enhanced by LTC_4. However, mutation of the EXXXG sequence in either ICL5 or ICL7 of MRP1 almost completely inhibited trapping at both the NBD1 and NBD2 domains (Fig. 5B).

DISCUSSION

The molecular mechanism(s) by which ATP hydrolysis is coupled to transport by ABC transporters remains obscure. Although the ICLs of ABC transporters were reported to be important for transporter function (*3–9*), the role of ICLs in the function of MRP1 has not been elucidated.

We investigated the roles of ICL 5 and ICL7 in MRP1 function using a system whereby both the N- and C-terminal halves of MRP1 could be co-expressed in a reconstituted transporter. The N- and C-terminal fragments of MRP1, encoding combinations of wt or mutant ICLs, were co-expressed using a virus with a dual expression cassette. The dual expression vector used in this study was described previously and has successfully demonstrated the functionally distinct properties of NBD1 and NBD2 (*15*), as well as the function of the signature sequences in NBDs of MRP1 (*14*).

ATP-dependent LTC_4 transport by reconstituted E507L G511P/WT MRP1 or WT/E1157L G1161P MRP1 was considerably decreased and GSH-dependent photolabeling of azido AG-A of these MRP1 mutants was abrogated. These findings suggest that E and/or G in both ICLs have important roles in the transport activity of MRP1 and the drug interactions of MRP1. This is consistent with previous findings regarding the roles of ICLs in bacterial and human transporters. The cytoplasmic membrane proteins of bacterial binding protein-dependent transporters belong to the ABC transporter superfamily. These hydrophobic proteins have a conserved region of at least 20 amino acids, $EAAX_3GX_9IXLP$ (the EAA region), that is exposed to the cytosol (*16*). EAA regions have been proposed to constitute a recognition site for the ABC ATPase helical domain and sequence analysis of the EAA region suggested that they determine the substrate specificity (*17*). Previous studies also indicated that the cytoplasmic loops of the hydrophobic domains of prokaryotic ABC transporters interact functionally with NBDs. Among prokaryotic transporters the high-resolution structure of vitamin B_{12} transporter BtuCD has been determined (*18*). BtuCD consists of four subunits, arranged as two homodimers, *i.e.*, a transmembrane BtuC dimer and a nucleotide-binding BtuD dimer. The L-loop of the transmembrane subunits, which is likely to contain a conserved EAA motif, interacts with the Q loop of the nucleotide-binding subunits.

Studies on conserved motifs in mammalian transporters have provided further evidence of the functional importance of conserved sequences in the ICL regions of transporters. ALDP is a half-ABC transporter in the human peroxisome membrane, and is defective in X chromosome-linked ALD (*19*). The 70-kDa peroxisomal membrane protein (PMP70) is also a half-ABC transporter in the peroxisomal membrane (*20*). These mammalian transporters have a 14–amino acid EAA-like motif in ICL2 between transmembrane segments 4 and 5. This ICL2 corresponds to ICL5 of MRP1 (*5*). It has been suggested that the EAA-like motif of peroxisomal ABC transporters is important for function but not for stability or targeting (*21*), and that the signature sequence region of the NBD interacts with the TMD through the EAA-like motif. Missense mutations in the EAA-like motif have been detected on analysis of the ALD gene in ALD patients, further suggesting the functional importance of this motif *in vivo* (*22*).

A second membrane transporter, in which mutations of the ICLs are associated with diseased states *in vivo*, is CFTR. The fourth ICL of this transporter has a primary sequence that is highly conserved across species and contains a relatively large number of missense mutations associated with cystic fibrosis (CF) (*23*). Most ICL4 mutants decreased the expression level of CFTR. The effects of ICL4 mutations on CFTR activity were similar to those observed following mutation of the nucleotide binding domains, suggesting that ICL4 might help to

couple the activity of the nucleotide-binding domains to gating of the Cl channel pore (*24*). A deletion mutant of CFTR, in which 19 amino acids were removed from ICL2, exhibited transition to a 6-picosiemens subconductance state, whereas wt CFTR channels rarely exist in this subconductance state. These data suggested that the intracellular loop is involved in stabilization of the full conductance state of the CFTR Cl channel and might interact with other domains such as NBDs (*24*). There is also evidence for an interaction between each NBD and its respective transmembrane domain in P-glycoprotein (*25*). This is similar to the suggestion that the signature sequence of P-glycoprotein interacts with the transmembrane domain (*26*).

In this study, we found a conserved sequence, EXXXG, in both ICL5 and ICL7 of the human and mouse MRP isoforms. Mutation analysis indicated that intact ICL5 was needed for the binding of ATP to NBD2, but not to NBD1, of MRP1. This effect was unlikely to be due to an effect of the ICL5 mutation on the membrane topology and structure of NBD2 since the N- and C-terminal halves of MRP1 were separately expressed. We have previously shown that the signature sequence of NBD1 was involved in the binding of ATP to NBD2. Combining our data with the previous findings concerning the role of ICLs in prokaryotic and other mammalian ABC transporters suggests that the interaction between ICL5 and NBD1 may be indispensable for the binding of ATP to NBD2. Alternatively, the mutations in ICL5 may have affected the structure of the signature sequence and the adjacent region of NBD1. Mutations in ICL7 abrogated the binding of ATP to both NBD1 and NBD2 suggesting that ICL7 is indispensable for the binding of ATP to both NBDs. Although ICL7 appears to have an important role in the transporting activity of MRP1 its role might not be identical to that of ICL5. A previous study suggested that ATP binding to both NBDs of MRP1 was not abrogated when the signature sequence of NBD2 was mutated (G1433D) (*14*). This might suggest the possibility that the mutated NBD2 was still functional and directly or indirectly interacted with the mutated ICL7 and NBD1. It is difficult to conclude whether the observations with the mutant MRP1s with the mutated ICL5 or 7 were due to a conformational change of the ICLs caused by the replacement of amino acid residues or merely due to the replacement of the amino acids. Nevertheless, our findings indicate that the intramolecular signaling between NBD and TMD is vital for MRP1 function. Further study involving MRP1 mutants that have mutations of other amino acids of the signature sequence of NBD2 is required to confirm this possibility. We are now studying the effect of either a single E or G mutation alone with moderate modifications in ICL5 and ICL7. Koike *et al.* demonstrated that mutation of Pro^{1150} in the cytoplasmic loop linking TM15 to TM6 caused substantial increases in 17 β-estradiol-17-β-(D-glucuronide) and methotrexate transport (*27*). It will also be interesting to investigate whether the substrate specificity is also altered in our MRP1 mutants. In this study, we identified conserved amino acids in ICL5 and ICL7 of the MRP1 family, and have shown, through mutational analyses, that ICL5 and ICL7 of MRP1 have important roles in the binding of ATP and drugs to MRP1, and consequently are critical for the transporting activity of MRP1.

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