Cytoplasmic Retraction of the Amino Terminus of Human Multidrug Resistance Protein 1 †

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Cytoplasmic Retraction of the Amino Terminus of Human Multidrug Resistance Protein 1†

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ABSTRACT: Human multidrug resistance protein 1 (MRP1) is a member of the ATP-binding cassette (ABC) transport superfamily which also includes human multidrug resistance 1 (MDR1) gene product P-glycoprotein (Pgp). Overexpression of MRP1 or Pgp causes multidrug resistance in cancer cells. Different from Pgp, MRP1 contains an extra membrane-spanning domain (MSD1) with a putative extracellular amino terminus in addition to the core structure of two MSDs and two NBDs (nucleotide-binding domains). The structural and functional significance of the additional MSD1 in MRP1 remains elusive. In this study, we generated an IgG_1 subclass monoclonal antibody, IU2H10, specific to the amino terminus of human MRP1 and mapped its epitope to 10 amino acids (S^{8}ADGSDPLWD^{17}). It can be used for Western blot, immunoprecipitation, and indirect immunofluorescence studies of human MRP1. However, surprisingly we found that IU2H10 cannot react with MRP1 unless cells are permeabilized. Furthermore, the IU2H10 epitope is exposed extracellularly when the carboxyl-terminal core domain of human MRP1 is deleted. Examination of the amino-terminal sequence of human MRP1 suggests that it consist of mainly coiled structures. These observations provide evidence for a model that is different from the prevailing extracellular location of the amino terminus of human MRP1. It is possible that part of the amino terminus of human MRP1, following exposure to the lumen of the endoplasmic reticulum, is retracted to the cytoplasm.

Multidrug resistance (MDR)[1] is a serious problem for successful chemotherapy of human cancers. Overexpression of drug efflux membrane transporters such as P-glycoprotein (Pgp) (*1*) and multidrug resistance protein (MRP) (*2*) in cancer cells is one of the main causes of MDR. Although MRP and Pgp share a low degree of homology (*2*), both MRP and Pgp belong to the ATP-binding cassette (ABC) membrane transport superfamily which includes various transporters with a wide variety of substrates (*3*−*6*). MRP and Pgp each has its own subfamily of transporters. Human MRP1 (also named ABCC1; refer to http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html for complete nomenclature of human ABC transporters) has been shown to have clinical relevance to MDR of some human cancers (*7*).

Different from Pgp and most of other members of the ABC transport superfamily, human MRP1 has an extra membrane-spanning domain (MSD) in addition to the core structure consisting of two nucleotide-binding domains (NBDs) and two MSDs (Figure 1A) (*2*). This additional MSD (MSD1) consists of five transmembrane (TM) segments with a putative extracellular amino terminus. The amino terminus has been suggested to be extracellular by determining the glycosylation status (*8*, *9*), by epitope insertion (*10*), and by cell-free expression in microsomal membranes (*11*). The functional significance of MSD1 is currently in dispute with one study showing that deletion of MSD1 (as little as the 66 amino-terminal amino acids, including the first TM segment) reduced 90% of the LTC_4 transport activity of human MRP1 (*12*), although the cause for this observation is not known. On the other hand, Bakos et al. (*13*) found that human MRP1 lacking the entire MSD1 is still functional in transporting substrates such as LTC_4. However, both groups found that the loop (L_0) linking MSD1 and MSD2 is functionally important (*12*−*14*).

To investigate further the structural and functional significance of the putative extracellular amino terminus of human MRP1, we generated a monoclonal antibody (IU2H10) directed against the amino terminus of human MRP1. Using IU2H10 as a probe, we found that the amino terminus of human MRP1 is not accessible unless the cells are permeabilized. Deletion of the carboxyl-terminal core domain (from P^{282} to V^{1531}) revealed the extracellular accessibility of the amino terminus. These findings imply that part of the amino terminus of human MRP1 may retract into the cytoplasm following exposure to the lumen of the endoplasmic reticulum (ER).

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[1] Abbreviations: MDR, multidrug resistance; MRP, multidrug resistance protein; Pgp, P-glycoprotein; MSD, membrane-spanning domain; NBD, nucleotide-binding domain; ABC, ATP-binding cassette; TM, transmembrane.

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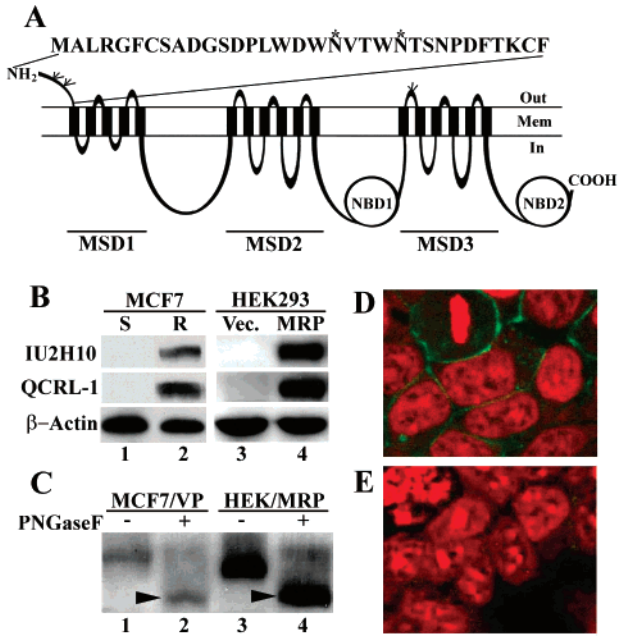

FIGURE 1: Generation and characterization of monoclonal antibody IU2H10. (A) Schematic drawing of the human MRP1 topological structure with the sequence of the putative extracellular amino terminus. The glycosylation sites are denoted with branched symbols and asterisks. (B) Western blot analysis of human MRP1 with IU2H10 and QCRL-1 (Centocor). Membranes from MCF7 (lane 1), MCF7/VP (lane 2), vector-transfected (lane 3), and MRP1-transfected (lane 4) HEK293 cells were used for Western blot analysis as described in Materials and Methods. β-Actin was used as a loading control. (C) Deglycosylation analysis of human MRP1. Membranes from MRP1-expressing cells were treated without (lanes 1 and 3) or with (lanes 2 and 4) PNGase F before being separated on SDS−PAGE for Western blot analysis. Arrowheads indicate the deglycosylated human MRP1. (D and E) Indirect immunofluorescence staining of human MRP1- (D) and vector-transfected (E) HEK293 cells. The nuclei are counterstained with propidium iodide.

MATERIALS AND METHODS

Materials and Cell Lines. FITC-, peroxidase-, and alkaline phosphatase-conjugated goat anti-mouse IgG, the ECL reagent, and PVDF membranes were purchased from Sigma, Amersham, and Bio-Rad, respectively. QCRL-1 was obtained initially from Centocor (Malvern, PA) and later from ID Labs (London, ON). All other chemicals were purchased from either Sigma or Fisher Scientific. Murine myeloma cell line SP2/0-AG14 was purchased from ATCC. HEK293 cells transfected with wild-type and mutant human MRP1 and the MCF7/VP drug resistant cell line were used as previously described by Yang and Zhang[2] and Schneider et al. (*15*), respectively. Cell culture media were obtained from Biowhittaker, Biofluids, and Invitrogen.

Generation and Purification of GST Fusion Protein and Synthetic Peptides. cDNA encoding the 33 amino acids of the amino terminus of human MRP1 was amplified by PCR using full-length human MRP1 as a template (*16*) and the following two primers containing *Bam*HI and *Eco*RI sites, respectively: 5′-CGGGATCCATGGCGCTCCGGG-3′ and 5′-GGAATTCTAAAAGCACTTGGT-3′. The PCR products were digested with *Eco*RI and *Bam*HI and inserted into GST expression vector pGEX-4T-1 (Pharmacia Biotech Inc.) for producing the GST−MRP fusion protein in *Escherichia coli* DH5α. Purification of the GST−MRP protein was conducted using a glutathione−Sepharose 4B column (Pharmacia), and the amino-terminal peptide of human MRP1 was further purified by cleaving off GST with thrombin. The purified peptide (SMALRGFCSADGSDPLWDWNVTWNTSNPDFTKCF) has an extra serine at the amino terminus derived from the vector. Peptides with sequences of MALRGFCSADGSDPLWDWNV and SADGSDPLWD were synthesized commercially, purified by HPLC, and confirmed by mass spectrometry. The peptide was quantified by determining the OD_{280} using extinction coefficients of 11380 for MALRGFCSADGSDPLWDWNV and 5690 for SADGSDPLWD which were calculated using online software (http://www.expasy.ch/tools/protparam.html).

Immunization and Hybridoma Generation. Adult female Balb/c mice were first immunized subcutaneously with 50 μg of GST−MRP1 fusion protein in complete Freund's adjuvant. Subsequent immunizations were made at approximately 3-week intervals using incomplete Freund's adjuvant. Three days before the fusion procedure, the mice were boosted intravenously with 20 μg of the purified recombinant peptide. The mice were then euthanized, and spleen cells were collected and fused with the SP2/0-AG14 myeloma cell line at a ratio of 1:10 (*17*). Hybridoma clones were first tested for the production of antibody against the purified recombinant peptide with an ELISA and then tested for their ability to detect human MRP1 in crude membranes of multidrug resistant cells by Western blot analysis. A positive clone (IU2H10) was obtained following two rounds of limited dilution and finally expanded. A hybridoma clone (IU15H6) of which supernatant does not react with human MRP1 was also isolated and used as a negative control for immunological assays.

Purification of Antibodies. The hybridoma clone (IU2H10) producing the anti-MRP1 antibody was cultured in serum-free SFM medium (Invitrogen) in T175 flasks. The supernatants were harvested at the 24 h time point and were loaded onto a protein A-G column (Pierce). Following extensive washing, the antibody was eluted with ImmunoPure elution buffer (Pierce), and 1 mL fractions were collected. All fractions were neutralized with 100 μL of binding buffer (Pierce) and desalted using Excellulose columns (Pierce) equilibrated in PBS at pH 7.4. The antibody concentration was measured by determining OD_{280} (1 OD_{280} = 0.7 mg/mL IgG).

ELISA. Microtiter plates (Labsystems) were coated overnight at 4 °C with solutions of 2 μg/mL peptides in 0.1% Na_2CO_3 and blocked with 3% BSA in PBS. Hybridoma supernatants or purified mAbs were added and incubated for 2 h followed by incubation with the alkaline phosphatase-conjugated goat anti-mouse IgG antibody for 1 h both at room temperature. Following extensive washing, substrate pNPP (Sigma) was added and the reaction was developed for 30 min before it was stopped by addition of 2 N NaOH and OD_{405} was determined.

Western Blot. Cells harboring full-length and truncated human MRP1 were generated in a study by Yang and Zhang.[2] Western blot analysis was performed as described previously (*18*). Briefly, 20 μg of proteins of crude membrane fractions or membranes treated with PNGase F as described previously (*18*) was separated by SDS−PAGE and transferred to PVDF membranes. The blot was then blocked in

[2] Y. Yang and J.-T. Zhang, manuscript in preparation.

10% milk and probed with IU2H10 (supernatant or purified antibody) or QCRL-1 followed by HRP-conjugated goat anti-mouse IgG. The signal was detected using ECL. For peptide epitope competition analysis, synthetic peptides were added to the IU2H10 supernatant to final concentrations of 10, 1, and 0.1 μM and incubated for 30 min at room temperature. The mixture was then used to probe a blot containing human MRP1.

Indirect Immunofluorescence Staining. For confocal microscopy, cells on the coverslip were washed twice with cold PBS and fixed and permeabilized with an acetone/methanol mixture (1:1) for 10 min and blocked at 4 °C for 30 min with a blocking solution (1% BSA and 1% normal horse serum in PBS). The coverslip was then probed with the primary antibody (hybridoma supernatant or purified IU2H10) for 30 min at 4 °C followed by incubation with the FITC-conjugated goat anti-mouse IgG $F(ab')_2$ fragment and washed twice before viewing on a confocal microscope.

For FACS analysis, all the procedures were carried out at 4 °C. Cells were aliquoted into a microtiter plate at a density of 0.5×10^6 cells/well and were washed twice with wash buffer (1% BSA in PBS). Cells were then blocked in a blocking solution for 30 min followed by incubation with the hybridoma supernatant or purified IU2H10 and QCRL-1 for 30 min either in the absence or in the presence of 0.2% saponin. The cells were then washed twice with wash buffer and incubated with the FITC-conjugated goat anti-mouse IgG $F(ab')_2$ fragment in a blocking solution for 30 min and then washed twice with wash buffer. Finally, the cells were resuspended in 1% paraformaldehyde in PBS for 30 min and analyzed by FACS analysis. For the saponin study, 0.03% saponin was present in all the subsequent washing and staining steps. For the saponin dose−response study, the concentration of saponin in the washing buffer is the same as in the permeabilization buffer. For testing the effect of chaotropic agents on the IU2H10 reaction, cells were first treated with 1 M urea for 20 min followed by fixation with paraformaldehyde in the presence of urea for 20 min prior to primary antibody incubation. For testing the effect of chelating agents on the IU2H10 reaction, cells were first treated with 10 mM EGTA or 10 mM EDTA in wash buffer (see above) for 30 min followed by antibody incubations in the presence of 1 mM EGTA or 1 mM EDTA.

Energy depletion prior to antibody labeling was carried out as previously described (*19*). Briefly, the cells were pretreated at 37 °C for 15 min with 1% BSA in PBS containing 4 μM potassium cyanide and 7 μM sodium azide or 1 μM oligomycin and 7 μM sodium azide. These treatments have been shown to deplete 98−100% of the ATP level (*19*). The treated cells were then used for antibody labeling as described above, but all buffers contain 7 μM sodium azide.

In Vitro Translation and Immunoprecipitation. In vitro transcription and translation of MRP1-N2R and MRP1-N2ΔNE(C18) were performed as described previously (*11*). Translation products (25 μL) were treated with the same volume of 1% SDS for 10 min at room temperature and then mixed with 1 mL of immunoprecipitation buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, and 0.5% NP-40] containing 4 μg of purified IU2H10. The reaction was performed for 1 h at 4 °C followed by addition of 20 μL of a 50% slurry of protein A-conjugated Sepharose (Sigma). The reaction was then continued at 4 °C for an additional 2 h with rocking. The Sepharose beads were pelleted (500*g* for 1 min) in a microfuge, and the supernatant was carefully removed. The Sepharose beads were washed five times with immunoprecipitation buffer and collected by pelleting the beads (500*g* for 1 min). The pellet was then solubilized by boiling in 20 μL of electrophoresis sample buffer for SDS−PAGE followed by fluorography analysis.

RESULTS

Generation and Characterization of Monoclonal Antibody IU2H10. Murine hybridomas were generated from spleens of mice immunized with the recombinant peptide of the 33 amino-terminal amino acids of human MRP1 (MALRGFCSADGSDPLWDWNVTWNTSNPDFTKCF) (Figure 1A) and were screened for their ability to react with the peptide immunogen using an ELISA and subsequently with MRP1 of isolated membranes using Western blot analysis. In the end, a positive clone with high affinity, designated IU2H10, was obtained, and it was determined to be an IgG_1 subclass. IU2H10 reacted strongly with a protein that was the same size as full-length human MRP1 expressed in both drug-selected MCF7/VP and human MRP1-transfected HEK293 cells, as confirmed by a known MRP1-specific monoclonal antibody QCRL-1 (Figure 1B). Deglycosylation analysis with endoglycosidase PNGase F further suggests that the protein detected by IU2H10 in MCF7/VP and MRP1-transfected HEK293 cells is human MRP1 (Figure 1C). There appeared to be no difference in IU2H10 reactivity between glycosylated and deglycosylated MRP1, suggesting that the sugar chains in the amino-terminal end of MRP1 do not constitute the IU2H10 epitope or affect its reactivity to IU2H10. IU2H10 stained mostly plasma membranes of fixed and/or permeabilized MRP1-transfected (Figure 1D) but not vector-transfected (Figure 1E) HEK293 cells.

Epitope Mapping. To map the IU2H10 epitope, a few experiments were performed. First, a synthetic peptide consisting of the 19 amino-terminal amino acids (Figure 2A) was used to test its reactivity to IU2H10 using an ELISA. As shown in Figure 2B, this synthetic peptide has the same reactivity with respect to IU2H10 as the immunogen of the 33-amino acid recombinant peptide. Thus, the epitope is likely located in the first 19 of the 33 amino acids. Second, an immunoprecipitation experiment was performed using in vitro-translated MRP-N2ΔNE(C18) which lacks the 17 amino-terminal amino acids in comparison with MRP-N2R which has an intact amino terminus (*20*) [see also Figure 2A for ΔNE(C18)]. As shown in Figure 2C, both MRP-N2R and MRP-N2ΔNE(C18) were produced in a cell-free system (lanes 1 and 2), but only MRP-N2R with an intact amino terminus can be immunoprecipitated by IU2H10 (lanes 3 and 4). To confirm the in vitro study, the same 17-amino acid deletion was engineered into full-length human MRP1 and transfected into HEK293 cells. Plasma membranes were prepared from the transfected cells and used for Western blot analysis. As shown in Figure 2D, MRP1 lacking the 17 amino-terminal amino acids [MRP-ΔNE(C18)] did not react with IU2H10 (lane 3). However, human MRP1 lacking seven amino-terminal amino acids [MRP-ΔNE(C8)] was reactive with IU2H10 (lane 2, Figure 2D). Thus, the IU2H10 epitope likely constitutes 10 amino acids with the sequence of

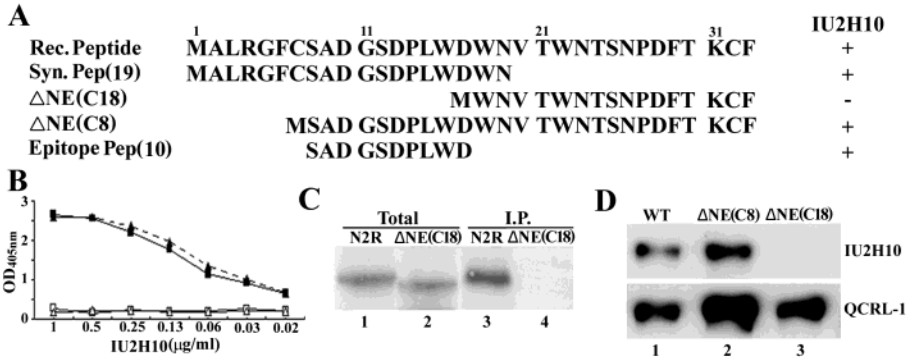

FIGURE 2: Mapping of the IU2H10 epitope. (A) Sequence of peptides and the truncated amino terminus of human MRP1. The reactivities of IU2H10 to these sequences are indicated by a plus (+) or minus (−). (B) Titration of IU2H10. An ELISA was used to titrate IU2H10 reactivity to recombinant peptide (■), synthetic peptide(19) (▲), or no peptide (△). Irrelevant antibody IU15H6 does not react with synthetic peptide(19) (□). (C) In vitro translation and immunoprecipitation of MRP-N2R and MRP-N2ΔNE(C18). MRP-N2R (lanes 1 and 3) and MRP-N2ΔNE(C18) (lanes 2 and 4) were translated in a cell-free system (lanes 1 and 2) as described previously (*11*) and immunoprecipitated (lanes 3 and 4) with IU2H10 for SDS−PAGE and fluorography. (D) Reactivity of IU2H10 to truncated human MRP1 on the Western blot. Membranes from cells expressing wild-type (lane 1) and truncated MRP-ΔNE(C8) and MRP-ΔNE(C18) were isolated for Western blot analysis by IU2H10. QCRL-1 (Centocor) was used as a control.

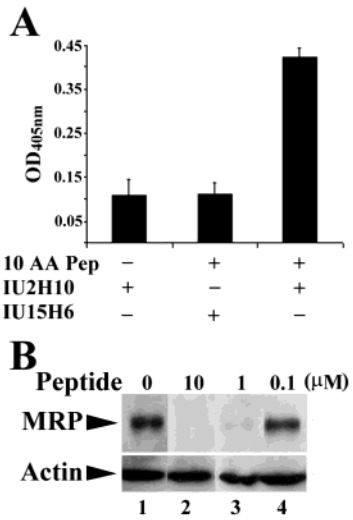

FIGURE 3: Reactivity of IU2H10 to epitope synthetic peptides. (A) Reactivity of IU2H10 to the 10-amino acid synthetic peptide. (B) Peptide inhibition of the IU2H10 reaction. The IU2H10 antibody supernatant was preincubated without (lane 1) or with 10 (lane 2), 1 (lane 3), and 0.1 μM (lane 4) synthetic peptides of 10 amino acids before probing a Western blot. Note that all lanes were from the same gel but the lanes were separated for exposure to different antibody−peptide mixture probes.

S[8]ADGSDPLWD[17] (see Figure 2A). We next synthesized a peptide with this sequence and performed an ELISA. As shown in Figure 3A, this peptide reacted with IU2H10. Furthermore, this peptide inhibited the reaction of IU2H10 to MRP1 on Western blot analysis at 1 μM (Figure 3B), like the 19-amino acid synthetic peptide (data not shown). Thus, the sequence S[8]ADGSDPLWD[17] likely constitutes the IU2H10 epitope.

Topological Orientation of the IU2H10 Epitope. The above results suggest that monoclonal antibody IU2H10 reacts specifically with human MRP1, and the epitope has been mapped within 10 amino acid residues. Because the amino terminus of human MRP1 has been suggested to be extracellular, we next tested if IU2H10 could label human MRP1 in live cells using FACS analysis. As shown in Figure 4A, we found no staining of live cells, suggesting that the amino terminus of human MRP1 in live cells is not accessible to

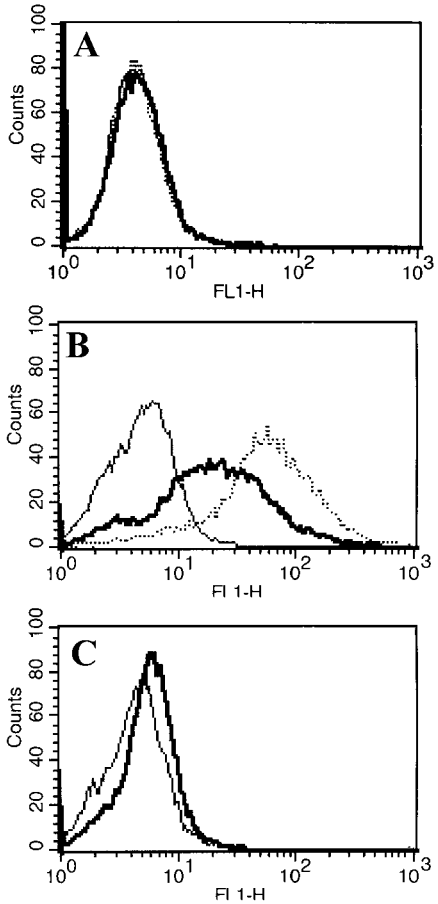

FIGURE 4: FACS analysis of human MRP1-transfected HEK293 cells with IU2H10. Human MRP1- (A and B) and vector-transfected (C) HEK293 cells were probed with IU15H6 (thin line), IU2H10 (thick line), and QCRL-1 (dotted line) (Centocor) in the absence (A) or presence (B and C) of 0.2% saponin followed by a secondary antibody conjugated with FITC for FACS analysis.

IU2H10. Treatment of cells with tunicamycin to inhibit glycosylation did not reveal the IU2H10 epitope and did not interfere with MRP1 trafficking to the cell surface (data not shown), suggesting that the oligosaccharide chains in the amino terminus are not responsible for the amino terminus being unable to be detected by IU2H10. This conclusion is

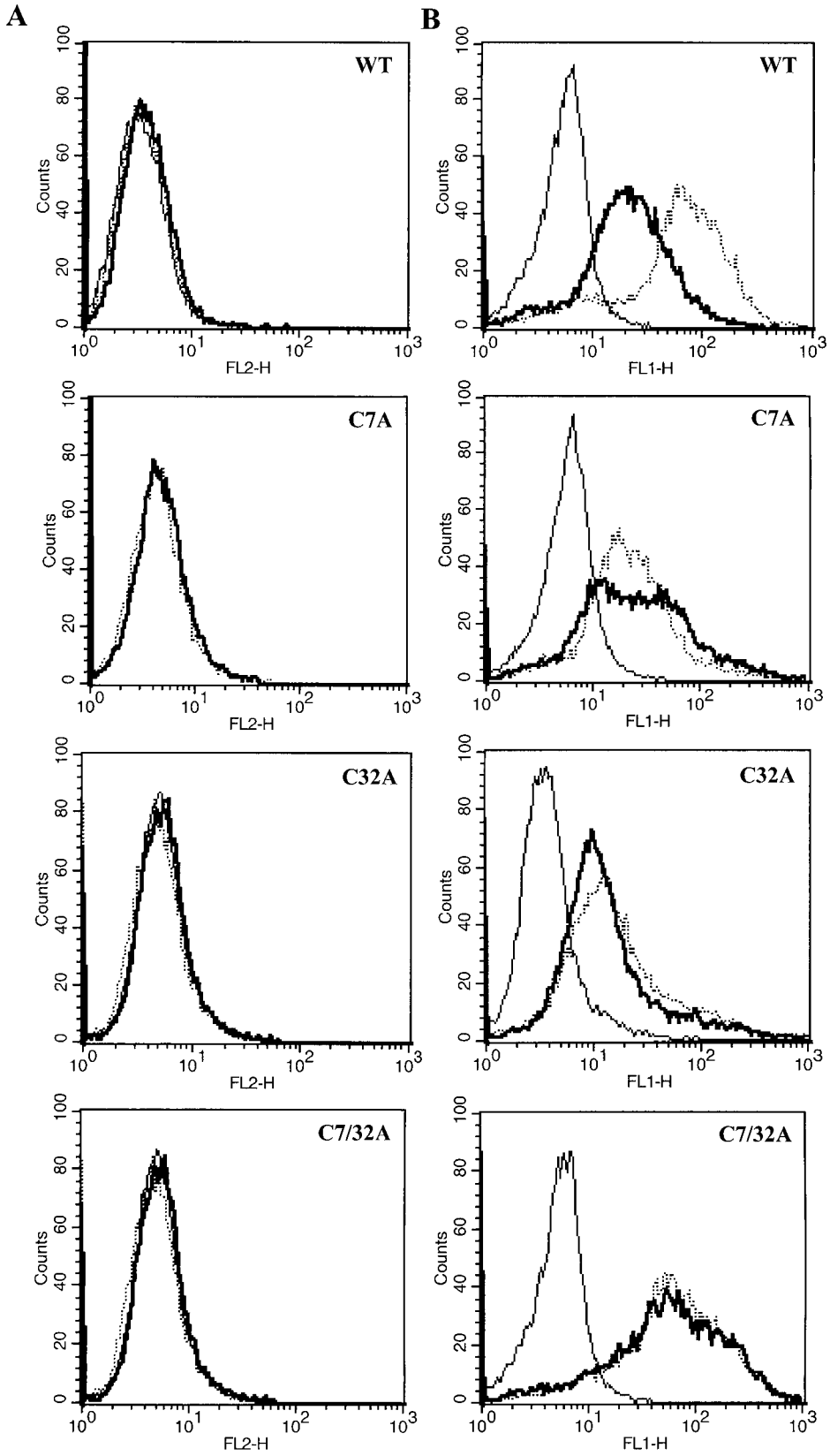

FIGURE 5: FACS analysis of wild-type and mutant human MRP1-transfected HEK293 cells with IU2H10. Wild-type and mutant (C7A, C32A, and C7/32A) human MRP1-transfected HEK293 cells were probed with IU15H6 (thin line), IU2H10 (thick line), and QCRL-1 (dotted line) (Centocor) in the absence (A) or presence (B) of 0.2% saponin followed by incubation with FITC-conjugated anti-mouse IgG for FACS analysis.

also supported by our deglycosylation analysis of the Western blot (Figure 1C). Treatment of cells with DTT also failed to reveal the IU2H10 epitope in live cells (data not shown). However, IU2H10 clearly stained cells treated with membrane-permeabilizing agent saponin (thick line, Figure 4B). Membrane permeabilization by saponin was confirmed

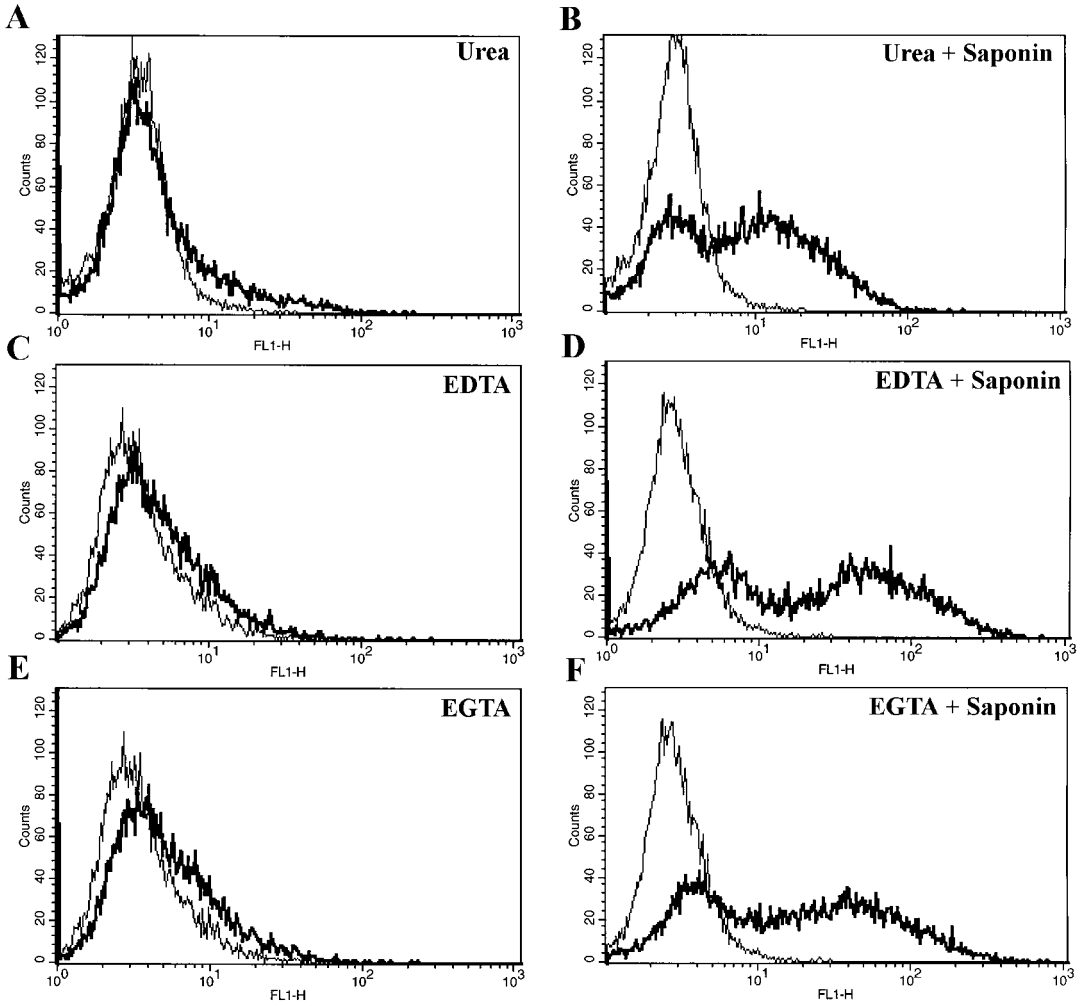

FIGURE 6: Effect of chaotropic and chelating agents on IU2H10 detection of human MRP1. Human MRP1$^{C7/32A}$-transfected HEK293 cells were first treated with 1 M urea (A and B), 10 mM EDTA (C and D), or 10 mM EGTA (E and F) and fixed with 1% paraformaldehyde in the presence of these agents. The cells were then probed with IU15H6 (thin line) or IU2H10 (thick line) in the absence (A, C, and E) or presence (B, D, and F) of 0.2% saponin followed by incubation with FITC-conjugated anti-mouse IgG for FACS analysis.

by the detection of MRP1 using QCRL-1 (dotted line, Figure 4B), a known MRP1-specific monoclonal antibody which reacts with an intracellular epitope (*21*). Similar results were observed when live cells were permeabilized with digitonin and Triton X-100 (data not shown, but see Figure 7). Vector-transfected cells were not stained by IU2H10 after permeabilization with saponin (Figure 4C), suggesting that IU2H10 staining following saponin treatment is not due to its reaction with other cellular proteins. Taken together, these observations suggest that the IU2H10 epitope may be located cytoplasmically.

Effect of Conformational Changes on IU2H10 Reactivity. To rule out the possibility that saponin treatment may disrupt the interaction between the IU2H10 epitope and extracellular proteins or phospholipids by causing conformational changes in human MRP1 which then exposes the IU2H10 epitope, we first treated cells with several known MRP1 substrates such as VP-16, which do not permeabilize cells, and performed FACS analysis. No labeling was found with these drug-treated cells (data not shown). Previously, it has been shown that the reactivity of a monoclonal antibody UIC2 to an extracellular epitope of human Pgp is increased by depleting energy, presumably due to conformational changes (*19*). The conformation of human MRP1 has been shown to change with the binding of MgATP (*22*). We, thus, tested if depletion of ATP affects the reactivity of IU2H10 to human MRP1. However, we found that treatment of cells with sodium azide, potassium cyanide, and oligomycin did not change the IU2H10 reactivity (data not shown).

We next determined the IU2H10 reactivity to mutant human MRP1 carrying mutations of the two cysteine residues (Cys^7 and Cys^{32}), which have been shown to be expressed on the cell surface and may have conformations different from the wild-type sequence at the amino terminus.[2] As shown in Figure 5, cells expressing the mutant human MRP1 cannot be stained unless the cells were permeabilized by saponin, the same as wild-type human MRP1. We also tested treatment of cells with chaotropic agents and chelating agents which presumably would disrupt protein−protein or protein−lipid interactions. For this experiment, 1 M urea was used because higher concentrations would permeabilize cells as determined using QCRL-1 (data not shown). As shown in Figure 6, IU2H10 could not stain cells treated with 1 M urea, 10 mM EDTA, or 10 mM EGTA unless the cells were permeabilized by saponin. Furthermore, we found that if cells were first fixed by paraformaldehyde which immobilizes the protein to prevent conformational changes following saponin treatment, the IU2H10 staining again could not be observed

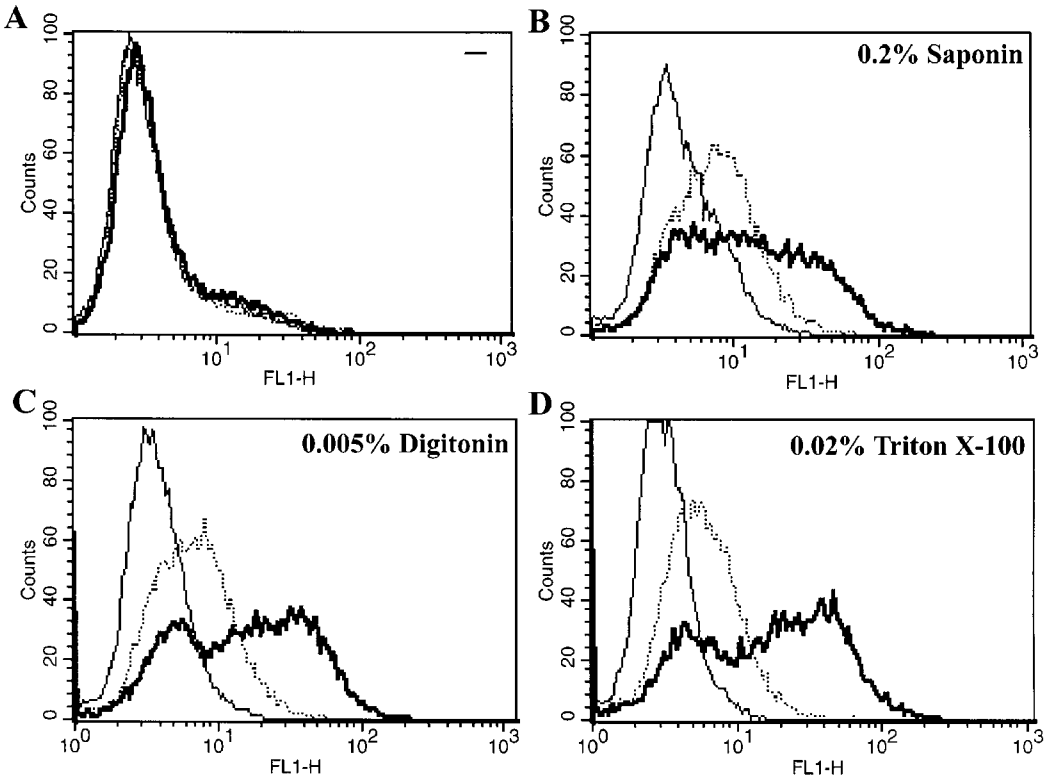

FIGURE 7: Effect of cell permeabilization on IU2H10 detection of human MRP1 following fixation. Human MRP1$^{C7/32A}$-transfected HEK293 cells were first fixed with 1% paraformaldehyde on ice followed by treatment without (A) or with 0.2% saponin (B), 0.005% digitonin (C), and 0.02% Triton X-100 (D). The cells were then probed with IU15H6 (thin line), IU2H10 (thick line), and QCRL-1 (dotted line) (ID Labs) in the presence of different concentrations of saponin followed by incubation with FITC-conjugated anti-mouse IgG for FACS analysis.

unless the cells were permeabilized by saponin, digitonin, or Triton X-100 prior to the antibody reaction (Figure 7). Together, these results suggest that disruption of interactions between the IU2H10 epitope and extracellular proteins or lipids and conformational changes of the amino terminus in human MRP1 due to saponin treatment are not likely to cause the extracellular exposure of the IU2H10 epitope.

Cytoplasmic Exposure of the IU2H10 Epitope. To further show that the IU2H10 epitope is exposed to the cytoplasm, we performed two additional experiments. First, we did a dose−response study by decreasing the concentrations of saponin in the hope that only a concentration of saponin that permeabilizes cells can support IU2H10 staining. As shown in Figure 8, IU2H10 reactivity to the amino terminus decreased when the saponin concentration was decreased to 0.0015% and the reactivity was eliminated at a saponin concentration of 0.0003%. The reactivity of QCRL-1 to a known cytoplasmic epitope of MRP1 was also decreased with 0.0015% saponin and eliminated with 0.0003% saponin. These observations indicate that the reactivity of IU2H10, the same as QCRL-1, correlates with the degree of cell permeabilization.

Second, we determined whether the reactivity of IU2H10 takes place within or outside of cells by taking advantage of the reversibility of saponin permeabilization (*23*). We first treated cells expressing human MRP1 with IU2H10 or QCRL-1 in the presence of 0.0015% saponin. The cells were then washed and sealed by incubating cells in excess saponin-free buffer at 37 °C for 2 h. The secondary antibody was then added to the cells in the absence or presence of 0.004% saponin. We reasoned that if IU2H10 reacts with an extracellular epitope in the presence of saponin, it should be able to be detected by the secondary antibody after the cells are sealed with the removal of saponin. On the other hand, if it reacts with an intracellular epitope in the presence of saponin, it should not be able to be detected by the secondary antibody after the permeabilization is reversed by removing saponin. As shown in Figure 9, we found that the removal of saponin following the primary antibody reaction prevented the detection of the IU2H10 epitope (Figure 9B) unless the cells were kept permeabilized with saponin for the secondary antibody reaction (Figure 9A), the same as our control QCRL-1 epitope which has been shown to be intracellular. The same treatment did not have any effect on extracellular epitopes (data not shown). Thus, we conclude that the IU2H10 epitope is localized inside cells, like the QCRL-1 epitope.

Role of the Carboxyl-Terminal Core Domain in Cytoplasmic Retraction of the Amino Terminus. To determine how the putative extracellular amino terminus of human MRP1 is exposed to cytoplasm, we truncated human MRP1 at amino acid residue 281 (D^{281}) and generated MRP1 fragments lacking the C-terminal core structure domain as previously reported (*13*). Stable clones expressing truncated MRP1 were generated and used to determine its reactivity to IU2H10. As shown in Figure 10, IU2H10 stained live cells expressing truncated MRP1 to the same extent as saponin-treated cells, indicating that the amino terminus in truncated MRP1 is exposed to the extracellular space. Similar results were observed with cells transiently transfected with truncated MRP1 (data not shown). Thus, the cytoplasmic exposure of

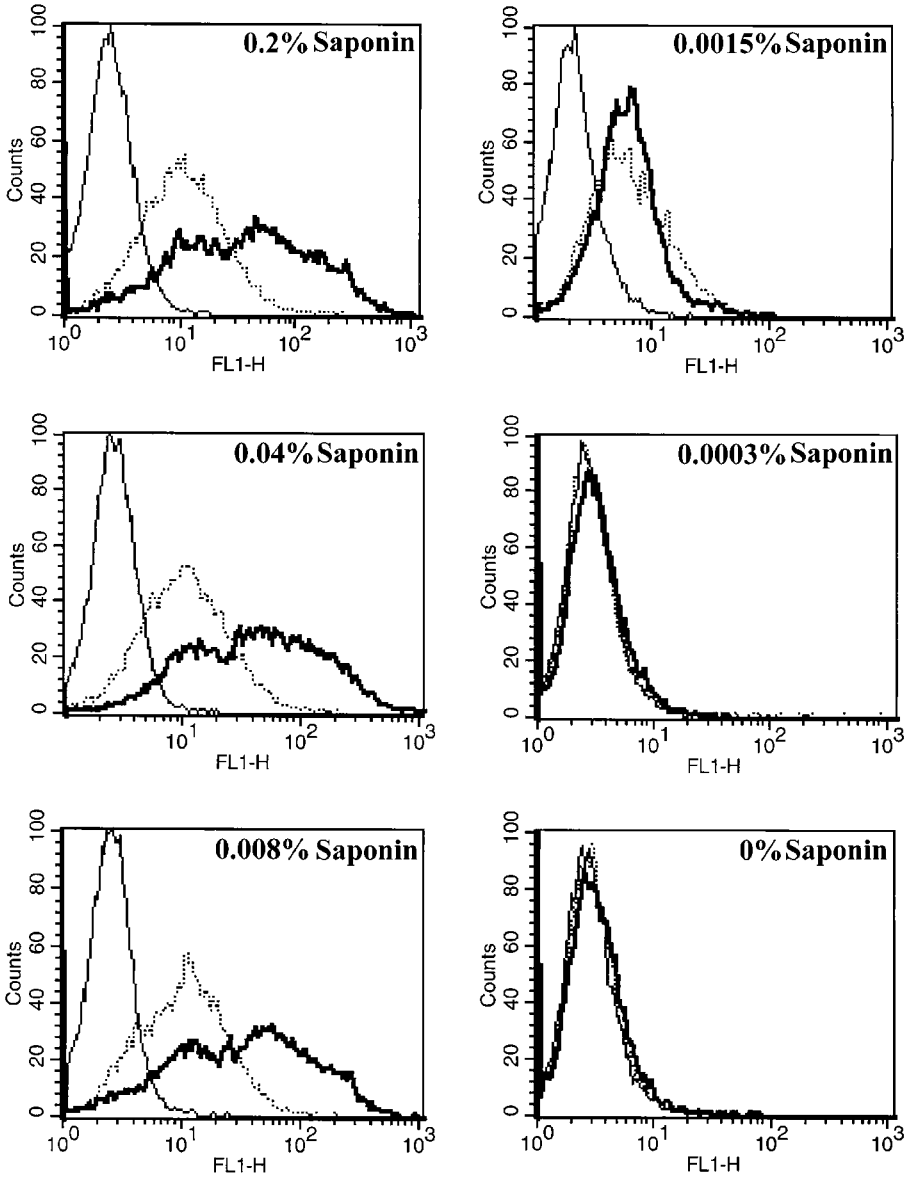

FIGURE 8: Correlation between cell permeabilization and IU2H10 reactivity. Human MRP1$^{C7/32A}$-transfected HEK293 cells were probed with IU15H6 (thin line), IU2H10 (thick line), and QCRL-1 (dotted line) (ID Labs) in the presence of different concentrations of saponin followed by incubation with FITC-conjugated anti-mouse IgG for FACS analysis.

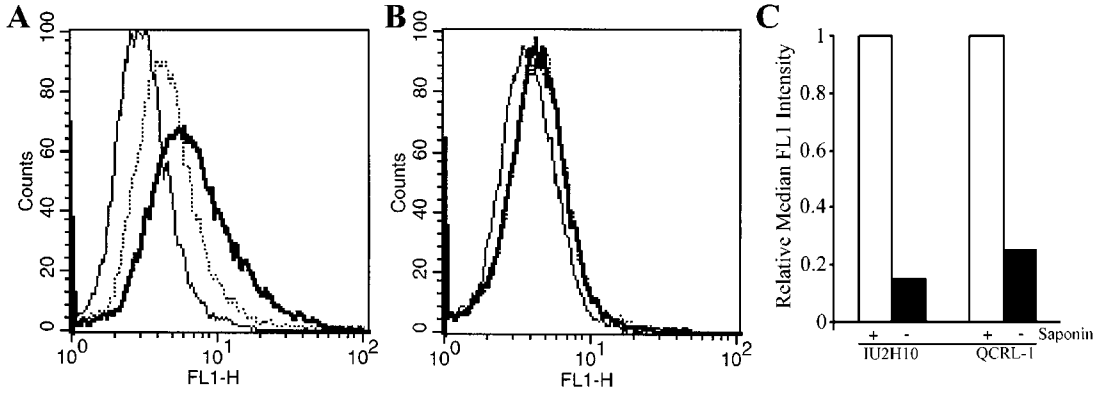

FIGURE 9: Effect of saponin removal on IU2H10 detection. Human MRP1$^{C7/32A}$-transfected HEK293 cells were probed with IU15H6 (thin line), IU2H10 (thick line), and QCRL-1 (dotted line) (ID Labs) in the presence of 0.0015% saponin followed by washing and incubation for 2 h at 37 °C in the absence of saponin. FITC-conjugated anti-mouse IgG was then added in the presence (panel A) or absence (panel B) of 0.004% saponin followed by FACS analysis as described in Materials and Methods. The relative median fluoresence intensity is shown in panel C. The data are a representative of three independent experiments.

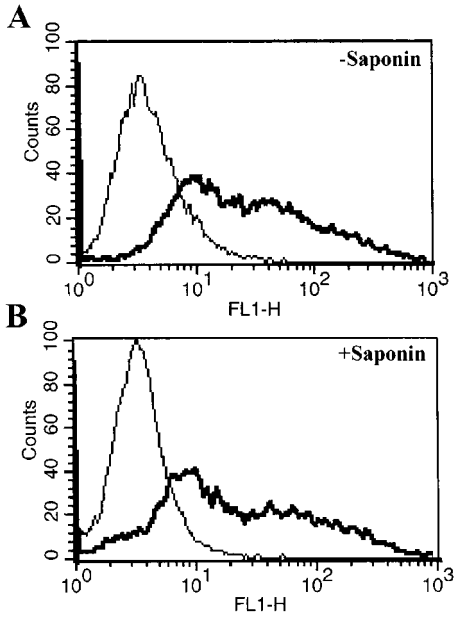

FIGURE 10: FACS analysis of HEK293 cells transfected with truncated MRP1. HEK293 cells transfected with truncated human MRP1$^{C7/32A}$ were probed with IU15H6 (thin line) and IU2H10 (thick line) in the absence (A) or presence (B) of 0.2% saponin followed by incubation with FITC-conjugated anti-mouse IgG for FACS analysis.

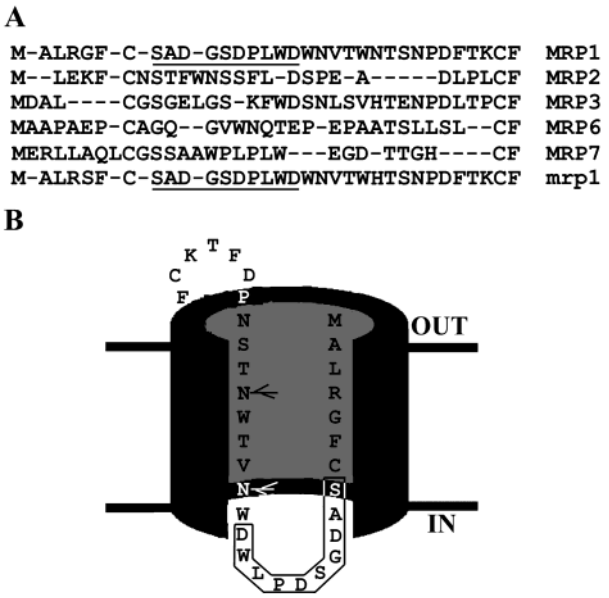

FIGURE 11: (A) Sequence alignment of the amino terminus of human MRP1, MRP2, MRP3, MRP6, MRP7, and mouse mrp1. The IU2H10 epitope is underlined. (B) Schematic model for cytoplasmic retraction of the amino terminus of human MRP1. The position of the amino terminus in the putative channel is arbitrary. The boxed sequence is the IU2H10 epitope. The branched symbols represent oligosaccharides.

the amino-terminal IU2H10 epitope requires the presence of the C-terminal core domains of the protein.

DISCUSSION

In this study, we successfully generated a monoclonal antibody, IU2H10, to the amino terminus of human MRP1. IU2H10 is determined to be an IgG_1 subclass and specific to MRP1. It can be used for Western blot, immunoprecipitation, and indirect immunofluorescence studies of human MRP1. The epitope for this antibody has been mapped to 10 amino acids (S^8ADGSDPLWD^{17}) in the amino terminus of human MRP1. The two oligosaccharide chains of the amino terminus are outside of the epitope region, consistent with the observation that glycosylation status does not affect the reactivity of human MRP1 to IU2H10.

Only five of nine known members of the human MRP subfamily have the amino-terminal membrane-spanning domain (MSD1) with a putative extracellular amino terminus (*2*, *3*, *24*−*29*). The alignment of the putative extracellular amino-terminal sequence among the five members is shown in Figure 11A. On the basis of the epitope mapping data and sequence alignment, it is likely that IU2H10 will react only with MRP1, but not with any other members of the human MRP subfamily. However, it is noteworthy that mouse mrp1 has a sequence very similar to that of human MRP1 at the amino terminus with only two different residues and also has a perfect match with the human IU2H10 epitope sequence. Thus, it is likely that IU2H10 will also react with mouse mrp1. This assumption is interesting because it suggests that the mouse IU2H10 epitope in live animals be not exposed so that the antibody can be produced against the externally introduced immunogen which has the same sequence as endogenous mrp1.

Previously, several studies suggested that the amino terminus of human MRP1 is likely located in the extracellular space. This was inferred from the study of the sites and status of glycosylation (*8*, *9*, *11*) and from an inserted HA epitope (YPYDVPDYA) between Arg^4 and Gly^5 (*10*). On the basis of these observations, it is predicted that monoclonal antibody IU2H10 will stain MRP1 on live cells. Surprisingly, we found that IU2H10 could not label MRP1 in intact cells unless permeabilized by saponin, digitonin, or Triton X-100, suggesting that the IU2H10 epitope may be exposed to the cytoplasmic face of membranes. This finding was confirmed by several experiments delineating the IU2H10 reactivity under permeabilized conditions (Figures 6−9). Furthermore, we found that conformational changes in MRP1 caused by drug binding, nucleotide binding, and mutation do not lead to extracellular exposure of the IU2H10 epitope. Disruption of protein−protein and protein−lipid interaction with chaotropic and chelating agents did not help expose the IU2H10 epitope either.

Saponin is a plant glycoside consisting of a steroid or a triterpene attached to a carbohydrate chain. It has a high affinity for cholesterol in membranes. Thus, in addition to permeabilizing cells, saponin may strip cholesterol from membrane proteins such as human MRP1 and expose the IU2H10 epitope extracellularly. Although it has not been shown that membrane cholesterol interacts with MRP1, it has been found that human MRP1 can transport estrogen in the presence of glutathione (*30*). Thus, it is possible that the amino terminus of human MRP1 interacts with cholesterol within the lipid bilayer and exposes the epitope extracellularly following saponin treatment. Currently, we cannot rule out this possibility. However, since the amino terminus of human MRP1 is hydrophilic, it is less likely to be soluble in the lipid bilayer. Furthermore, results from fixation with paraformaldehyde prior to saponin treatment as well as studies on conformational changes using several approaches all indicate that the possibility that the IU2H10 epitope moves

out of the lipid bilayer and is exposed extracellularly due to stripping of cholesterol by saponin is less likely.

It is possible that part of the amino terminus of human MRP1 is translocated into and glycosylated in the ER and then loops back into the cytoplasm after the full-length protein is synthesized and folded. Such retraction of a domain from one side of the membranes to another has been observed previously with other membrane proteins. The carboxyl-terminal domain of a model protein has been shown to translocate into the lumen of microsomal membranes and to be glycosylated before looping back into the cytoplasm (*31*). A domain of the E2 glycoprotein of alphavirus Sindbis has also been found to reverse into the cytoplasm after being exposed to the ER lumen (*32*). The reversion of E2 glycoprotein occurs after the protein has been processed and exported out of the ER. Interestingly, the IU2H10 epitope in human MRP1 is exposed extracellularly when the majority of the carboxyl-terminal core domain is removed. This observation suggests that the extracellular exposure of the IU2H10 epitope in human MRP1 may be held back into the cytoplasm by the carboxyl terminus of the protein. Thus, it is tempting to propose that the 10 amino acids comprising the IU2H10 epitope (S^8ADGSDPLWD^{17}) within the amino terminus of human MRP1 loop into the cytoplasm possibly through a channel formed by the transmembrane domains of the protein (Figure 11B). However, this model is not intended to be taken literally in the absence of high-resolution structural data. Exactly where each amino acid residue of the amino terminus is located is not known. The model only shows where the IU2H10 epitope is located relative to the membranes and how it is possibly folded in such a fashion.

Analysis of the amino-terminal sequence of human MRP1 using several programs (http://www.expasy.ch/tools/#secondary) predicts that it mainly consists of coiled structures with some β-strand. The potential flexibility of this structural feature makes it possible for the amino terminus to loop into a channel and thus exposes the IU2H10 epitope intracellularly (Figure 11B). It is known that the cytoplasmic sides of transmembrane domains of many polytopic membrane proteins are more positively charged than extracellular loops (*33*−*36*). Thus, attracting and anchoring the IU2H10 epitope (S^8ADGSDPLWD^{17}) which is very acidic into the cytoplasm through ionic interaction is very likely. Recently, the crystal structure of bacterial ABC transporter MsbA is shown to be cone-shaped with the widest opening (25 Å) in the cytoplasm (*20*). Should human MRP1 have similar structures, the cytoplasmic opening is likely to be big enough to accommodate a monoclonal antibody. Indeed, structural studies at 22 Å resolution revealed a pore in the center of human MRP1 (*37*).

If the model in Figure 11 is correct, the question that remains is how we can reconcile the findings of this study with previous ones which suggested the extracellular location of the amino terminus of human MRP1 (*8*−*11*). First, this study only indicates the cytoplasmic exposure of the IU2H10 epitope within the amino terminus of human MRP1. The other parts of the amino terminus may still be exposed extracellularly. Second, the conclusion from previous studies mostly relied on the glycosylation status that indicates an extracellular location of the amino terminus. It is possible that the oligosaccharide chains of the amino terminus are located within the channel as depicted in the model (Figure 11). Alternatively, the oligosaccharide chains may retract to the cytoplasmic side with the peptide backbone as demonstrated previously for the E2 glycoprotein (*32*). Currently, it is not known how big the sugar chains at the amino terminus are and whether the channel is big enough to accommodate them without high-resolution structural information. However, it has been shown that inhibition of glycosylation by tunicamycin did not inhibit human MRP1 function (*8*). It is interesting to note that the amino terminus of human MRP7 does not have potential N-linked glycosylation sites at the amino terminus (*27*) (Figure 10A). It has also been observed that mutation of glycosylation sites did not affect the function of human MDR1 (*38*). Thus, it appears that the relative membrane positioning of sugar chains at the amino terminus may not be important for human MRP1 function. Finally, because the amino-terminal end of human MRP1 is projecting toward the extracellular space in our new model, it can thus also accommodate the finding that a nine-amino acid HA epitope (YPYDVPDYA) inserted between Arg^4 and Gly^5 can be stained by the anti-HA antibody outside of cells without permeabilization (*10*). However, it may also be possible that the amino-terminal end of human MRP1 is located inside the cytoplasm, different from the model in Figure 11. Insertion of the nine-amino acid HA epitope may have disrupted the biogenesis of human MRP1 and, thus, helped retain the amino-terminal end extracellularly.

The model in Figure 11, if correct, indicates that the amino terminus may play a role as a gate and be important for MRP1 function. Previously, it has also been shown that deletion of the amino terminus, including the first TM segment, generated an inactive molecule (*12*), although human MRP1 with a deletion of the entire MSD1 remains functional (*13*). We are currently investigating the functional significance of the amino terminus of human MRP1 by engineering various mutations at the amino terminus of human MRP1.

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