

# The Amino Terminus of the Human Multidrug Resistance Transporter ABCC1 Has a U-shaped Folding with a Gating Function\*

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Multidrug resistance is a serious problem in successful cancer chemotherapy. Studies using model cell lines have demonstrated that overexpression of some members of the ATP-binding cassette (ABC) transporter superfamily, such as ABCC1, causes enhanced efflux and, thus, decreased accumulation of multiple anticancer drugs, which leads to increased cell survival. Unlike most other ABC transporters, ABCC1 has an additional membrane-spanning domain (MSD0) with a putative extracellular amino terminus of 32 amino acids. However, the function of MSD0 and the role of the extracellular amino terminus are largely unknown. In this study, we examined the structural folding and the function of the amino terminus. We found that it has a U-shaped folding with the bottom of the U-structure facing cytoplasm and both ends in extracellular space. We also found that this U-shaped amino terminus probably functions as a gate to regulate the drug transport activity of human ABCC1.

The ATP-binding cassette (ABC)<sup>3</sup> membrane transporter superfamily comprises over 1000 members from bacteria to humans, with a wide variety of substrate specificity and function (1, 2). The activity of ABC transporters appear to be dependent on ATP binding and hydrolysis. In humans, 49 ABC transporters are known (for a complete list, see, on the World Wide Web, [nutrigene.4t.com/humanabc.htm](http://nutrigene.4t.com/humanabc.htm)), and some of them (e.g. ABCB1 or Pgp, ABCC1 or MRP1, ABCG2 or BCRP) have been associated with multidrug resistance in cancer chemotherapy (3–7). Overexpression of ABCB1, ABCC1, or ABCG2 alone in cancer cells causes increased drug efflux, which results in decreased cellular accumulation of anticancer drugs, and thus the cells can survive drug treatment.

Unlike most of the known ABC transporters, ABCC1 has an additional membrane-spanning domain (MSD) at its amino terminus with a domain arrangement of MSD0-MSD1-NBD1-MSD2-NBD2 (8, 9). The additional MSD0 consists of five putative transmembrane segments with a predicted extracellular amino terminus (Fig. 1). The functional relevance of the MSD0 to the transporter remains elusive. Although it has been shown that deletion of MSD0 (as little as 66 amino acids from the amino terminus, including the first transmembrane segment) reduced 90% of the LTC<sub>4</sub> transport activity of human ABCC1 (10) and mutation of Cys<sup>7</sup> reduced LTC<sub>4</sub> transport and drug resistance (11), another study showed that the carboxyl core domain (MSD1-NBD1-MSD2-NBD2) of human ABCC1 lacking the entire MSD0 is still functional in transporting substrates, such as LTC<sub>4</sub> (12). It was thought that the loop (L0) linking MSD0 and MSD1 is important for ABCC1 function (10, 12, 13), and it may be involved in drug binding (14). It has also been reported that the MSD0 may play roles in processing and trafficking of human ABCC1 (13, 15).

The predicted extracellular location of the amino terminus of ABCC1 has been confirmed by determining glycosylation status (16, 17) by epitope insertion (18, 19) and by cell-free expression in microsomal membranes (20). However, this prevailing topological orientation of the amino terminus has recently been challenged by a study using a monoclonal antibody (IU2H10) against the amino terminus of human ABCC1, which suggested that part of the amino terminus was exposed intracellularly (21).

To further investigate the exact topological orientation and function of the amino terminus of human ABCC1, we created another monoclonal antibody, IU5C1, and several constructs with HA and FLAG epitope tags at the amino terminus. We found surprisingly that the amino terminus of human ABCC1 has a U-shaped folding with the bottom of the U structure exposed to intracellular space and that this U-shaped structure plays a gating role for the drug transport function of human ABCC1 by plugging into a putative channel.

## EXPERIMENTAL PROCEDURES

**Materials**—Adriamycin, vinblastine, colchicine, VP-16, anti-FLAG monoclonal antibody, and peroxidase- and fluorescein isothiocyanate-conjugated goat anti-mouse IgG were purchased from Sigma. Lipofectamine and G418 were purchased from Invitrogen. ECL reagent and polyvinylidene difluoride

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<sup>3</sup> The abbreviations used are: ABC, ATP-binding cassette; MSD, membrane-spanning domain; HA, hemagglutinin; FACS, fluorescence-activated cell sorting; WT, wild type.

membranes were purchased from Amersham Biosciences and Bio-Rad, respectively. Monoclonal antibodies QCRL-1 and MRPr1 were obtained from Kamiya, whereas the monoclonal anti-HA antibody was from Covance. Synthetic peptides were commercially synthesized by Genemed Synthesis, Inc. All other chemicals were purchased from either Sigma or Fisher.

**Engineering of Tagged ABCC1 Constructs**—To engineer ABCC1<sup>1FLAG2</sup>, amplification of the template construct Yep-FLAG-MRP1-His (22) was performed with primers 5'-GGC-TAGCCACCGGCATGGACTACAAGGACG-3' (forward) and 5'-GGAAGTCTCTTTTCGGCTG-3' (reverse). The product was cloned into pCR®-Blunt (Invitrogen), and the fragment encoding the amino terminus was released by digestion with NheI and BamHI and used to replace the wild type sequence in the ABCC1<sup>WT</sup> construct (21).

For insertion of HA tag (YPYDVPDYAS) at different positions of the amino terminus of human ABCC1, two sequential PCRs were conducted. First, a cDNA cassette (−8 to +867) encoding the amino-terminal 281 amino acids of human ABCC1 was amplified using Pfu polymerase with primers 5'-GGCTAGCCACCGGCATGGCGCTC and 5'-GGAAGTCTCTTTTCGGCTG, and the product was cloned into pCR®-Blunt (Invitrogen). This fragment contains a NheI site at the 5'-end and a BamHI site at the 3'-end. Next, site-directed mutagenesis with the QuikChange XL kit (Stratagene) was used to insert the HA tag with the following primers: for ABCC1<sup>4HA5</sup>, 5'-GGCATGGCGCTCCGGTAC-CCCTACGACGTGCCCCGATTATGCCTCAGGCTTCTGCAGCGCC-3' (forward) and 5'-GGCGCTGCAGAAAGCC-TGAGGCATAATCGGGCACGTCTAGGGGTACCGGAGCGCCATGCC-3' (reverse); for ABCC1<sup>15HA16</sup>, 5'-GGC-TCCGACCCGCTCTACCCCTACGACGTGCCCCGATTATGCCTCATGGGACTGGAATGTC-3' (forward) and 5'-GACATTCCAGTCCCATGAGGCATAATCGGGCACGTCTAGGGGTAGAGCGGGTTCGGAGCC-3' (reverse); for ABCC1<sup>30HA31</sup>, 5'-CAACCCCGACTTCACCTACCCCTACGACGTGCCCCGATTATGCCTCAAAGTGCTTTTCAGAAC (forward) and 5'-GTTCTGAAAGCACTTTGAGGCATAATCGGGCACGTCTAGGGGTAGGTGAAGTCGGGGTTG-3' (reverse). These constructs carrying the mutations were then digested with NheI and BamHI, and the released fragments with inserted tags were used to replace the wild type sequence in the full-length ABCC1 cDNA in pcDNA3.1(+). The final plasmids carrying tagged ABCC1 were named ABCC1<sup>4HA5</sup>, ABCC1<sup>15HA16</sup>, and ABCC1<sup>30HA31</sup>.

To mutate the two N-linked glycosylation sites (Asn<sup>19</sup> → Gln and Asn<sup>23</sup> → Gln) at the amino terminus, the QuikChange XL site-directed mutagenesis kit (Stratagene) was used with primers 5'-CCGCTCTGGGACTGGCAAGT-CACGTGGCAAACCAGCAACCCCGAC-3' (forward) and 5'-GTCGGGGTTGCTGGTTTGGCACGTGATTGCCAG-TCCCAGAGCGG-3' (reverse). The final mutant ABCC1 construct was named ABCC1<sup>QQ</sup>.

ABCC1<sup>ΔN32</sup> (with deletion of the first 32 amino acids) was engineered by amplifying a 1209-bp fragment of ABCC1 using a forward primer carrying a NheI site and a Kozak translation initiation sequence 5'-CTAGCTAGCGCCGATGTTTCA-GAACACGGTC-3' and a reverse primer 5'-CACAGACATG-

AGGTTGAC-3'. The PCR products were then digested with NheI and BamHI to generate a 762-bp fragment, which was subsequently subcloned into ABCC1<sup>WT</sup> digested with NheI and BamHI, resulting in ABCC1<sup>ΔN32</sup>. All of the above mutant constructs were confirmed by double-stranded DNA sequencing.

**Generation of Monoclonal Antibody IU5C1**—Generation of hybridoma-producing anti-ABCC1 antibodies was performed as described previously (21). The hybridoma-producing monoclonal antibody IU5C1 was collected and further characterized in the same way as described previously for IU2H10 (21).

**Transfection and Selection of HEK293 Stable Clones**—HEK293 cells were plated and grown to subconfluence. 5 μg of each construct encoding the wild type and mutant human ABCC1 was transfected into HEK293 cells using Lipofectamine Plus as previously described (21). 2 days after transfection, cells were split, and 1% of the cells were used for selection with G418 (800 μg/ml) for 2 weeks. The G418-resistant clones were picked and then expanded for further analysis of ABCC1 expression using Western blot and FACS. These clones were maintained in medium containing G418 at a concentration of 200 μg/ml for further studies.

**Enzyme-linked Immunosorbent Assay, Western Blot, Confocal Imaging, and FACS Analyses**—Enzyme-linked immunosorbent assay, Western blot, confocal imaging, and FACS analyses were performed exactly as previously described (21). For peptide inhibition, different concentrations of various peptides were mixed with the primary antibody and incubated for 1 h at room temperature prior to probing the blot or saponin-permeabilized cells. For FACS analysis in the presence of vanadate, cells were aliquoted into a microtiter plate at a density of 0.5 × 10<sup>6</sup> cells/well. The cells were then incubated with Adriamycin in the presence of sodium vanadate at 37 °C for 1 h. The incubation medium was then removed by centrifugation, and the cells were stained with the standard protocol as described above.

**Statistical Analyses**—All protein activity data (log-transformed EC<sub>50</sub>) were fitted into a linear mixed model, in which the correlation among paired data were modeled. All *p* values were calculated based on this mixed model, and *p* values less than 5% were considered significant. The advantage of this approach is to allow different experiments to share the same variance information. Model diagnosis was also performed, and it appeared that equal variance and normal assumptions worked well in our data set after the EC<sub>50</sub> values were log-transformed. The analysis was conducted in SAS, PROC MIXED (available on the World Wide Web at [www.sas.com/](http://www.sas.com/)).

## RESULTS

**Characterization and Epitope Mapping of the Monoclonal Antibodies IU5C1 and IU2H10**—The monoclonal antibody IU2H10 has been characterized, and its epitope has been mapped to 10 amino acids (<sup>8</sup>SADGSDPLWD<sup>17</sup>) in the amino terminus of human ABCC1 (21). During our screening of monoclonal antibodies raised against the amino terminus of human ABCC1, we found another clone, IU5C1 of the IgG1 subtype. As shown by both Western blot analysis of membranes isolated from human ABCC1-transfected HEK293 or drug-se-

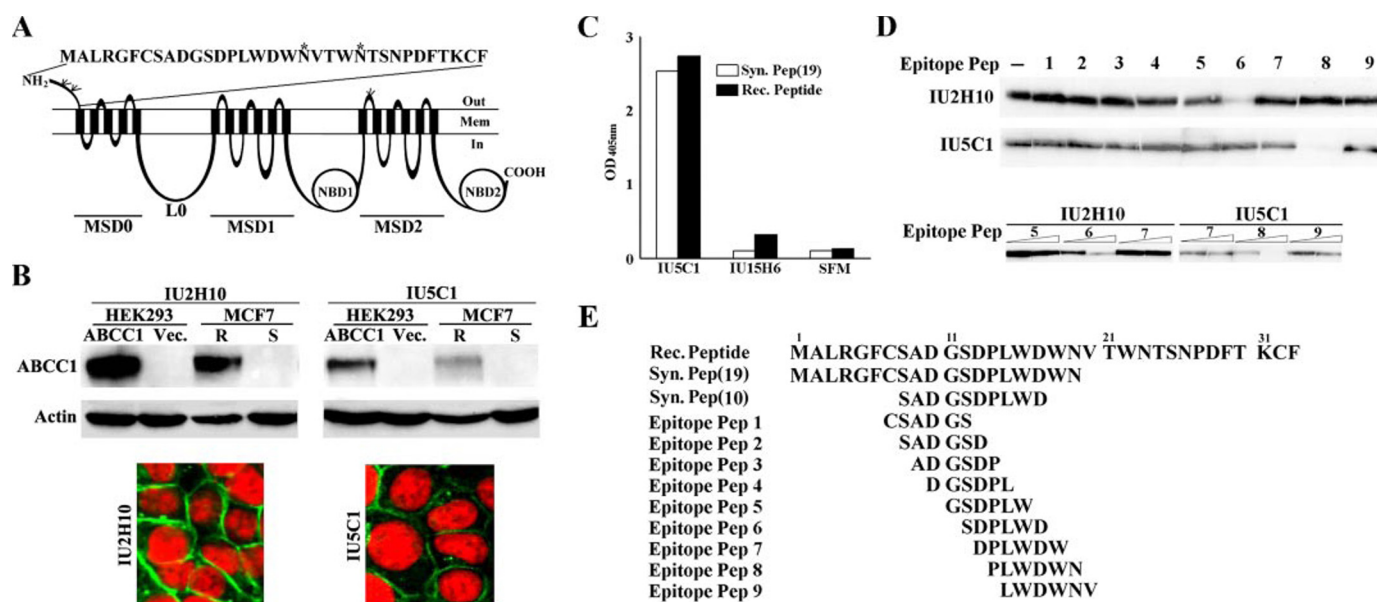


FIGURE 1. **Characterization of monoclonal antibody IU5C1 and IU2H10.** A, schematic folding of human ABCC1 with sequence of the putative extracellular amino terminus. The glycosylation sites are indicated by branched symbols and asterisks. B, Western blot and immunofluorescence staining. Isolated membranes were used for Western blot analysis using IU2H10 and IU5C1.  $\beta$ -Actin was used as a loading control. R, resistant; S, sensitive. Human ABCC1<sup>WT</sup>-transfected HEK293 cells were stained by IU2H10 and IU5C1 for immunofluorescence analysis. The nuclei were counterstained with propidium iodide. C, enzyme-linked immunosorbent assay. The recombinant peptide of 33 amino acids and the synthetic peptide of 19 amino acids were used to test IU5C1. D, epitope mapping. IU2H10 and IU5C1 were first incubated with synthetic epitope peptides at 25 µg/ml (top panel) or 5 and 10 µg/ml (bottom panel) prior to probing the blots. E, sequence of recombinant and synthetic peptides used for epitope mapping.

lected MCF7 cells and confocal immunofluorescence analysis of ABCC1-transfected HEK293 cells (Fig. 1B), IU5C1 reacted specifically with human ABCC1 similarly as IU2H10 with the exception that IU5C1 had a lower titer than IU2H10.

To determine whether IU5C1 has a different epitope from IU2H10 in the amino terminus of human ABCC1, we first employed the enzyme-linked immunosorbent assay to determine if the epitope of IU5C1 is located in the amino-terminal 19 amino acids. As shown in Fig. 1C, IU5C1 reacted with the synthetic peptides of 19 amino acids as well as with the recombinant peptide immunogen of 33 amino acids representing the amino terminus of human ABCC1. It also reacted with the synthetic peptides of 10 amino acids (<sup>8</sup>SADGSDPLWD<sup>17</sup>) (data not shown). Thus, the epitope of IU5C1 is probably located within these 10 amino acids of human ABCC1, the same as IU2H10 (21). To further map the IU5C1 and IU2H10 epitope, we performed a peptide walking experiment using a series of synthetic peptides of six amino acids each (Fig. 1E). These peptides were then tested for their ability to block the reaction of IU5C1 and IU2H10 to human ABCC1 on Western blot. As shown in Fig. 1D, IU2H10 activity was blocked by peptide 6 (<sup>12</sup>SDPLWD<sup>17</sup>), whereas IU5C1 activity was blocked by peptide 8 (<sup>14</sup>PLWDWN<sup>19</sup>) in a concentration-dependent manner. Thus, probably the epitopes for IU2H10 and IU5C1 are <sup>12</sup>SDPLWD<sup>17</sup> (peptide 6) and <sup>14</sup>PLWDWN<sup>19</sup> (peptide 8), respectively, and their epitopes overlap with a shift of 2 amino acids.

**Membrane Orientation of the IU5C1 Epitope**—Previously, we have shown that the monoclonal antibody IU2H10 can stain ABCC1 only in permeabilized cells, suggesting that its epitope in the amino terminus is located intracellularly (21), creating a controversial issue regarding the membrane orientation of the

amino terminus of human ABCC1. To determine if the results were not due to the use of the specific antibody IU2H10, we performed a similar study using IU5C1. As shown in Fig. 2A, IU5C1 stained ABCC1-expressing cells only when the cells were permeabilized by saponin or by Triton X-100, similar to that stained by IU2H10 shown previously (21). Live cells and cells transfected with vector alone were not stained by IU5C1. In addition, the IU5C1 staining of ABCC1-expressing cells under permeabilized conditions was completely blocked by the peptide 8 but not by peptide 7 (Fig. 2B), suggesting that the IU5C1 staining was specific to human ABCC1. Thus, the IU5C1 epitope in the amino terminus of human ABCC1 is probably also located intracellularly.

**The U-shaped Folding of the Amino Terminus of Human ABCC1**—Previously, it has been found that an HA tag engineered following the Arg<sup>4</sup> residue (18) and a FLAG tag engineered following Asn<sup>23</sup> with mutations of glycosylation sites (19) at the amino terminus were both found outside of cells. These observations, together with our findings on the relative membrane orientation of IU2H10 and IU5C1 epitopes, raised a possibility that the amino terminus of human ABCC1 may have a U-shaped folding with the bottom of the U (IU2H10 and IU5C1 epitopes) located intracellularly and the two ends (tags) located extracellularly.

To test this possibility, we first engineered a series of constructs with HA (YPYDVPDYAS) or FLAG tags (DYKDDDDK) inserted at different positions in the amino terminus of human ABCC1 (ABCC1<sup>1</sup>FLAG<sup>2</sup>, ABCC1<sup>15</sup>HA<sup>16</sup>, and ABCC1<sup>30</sup>HA<sup>31</sup>) (Fig. 3A) in addition to the HA- and FLAG-tagged constructs (ABCC1<sup>4</sup>HA<sup>5</sup> and ABCC1<sup>FL</sup>FLAG<sup>-Q</sup>) published elsewhere (18, 19). These constructs were transfected into HEK293 cells, and stable clones with similar expression levels of ABCC1 were selected for further characterization. All newly tagged ABCC1



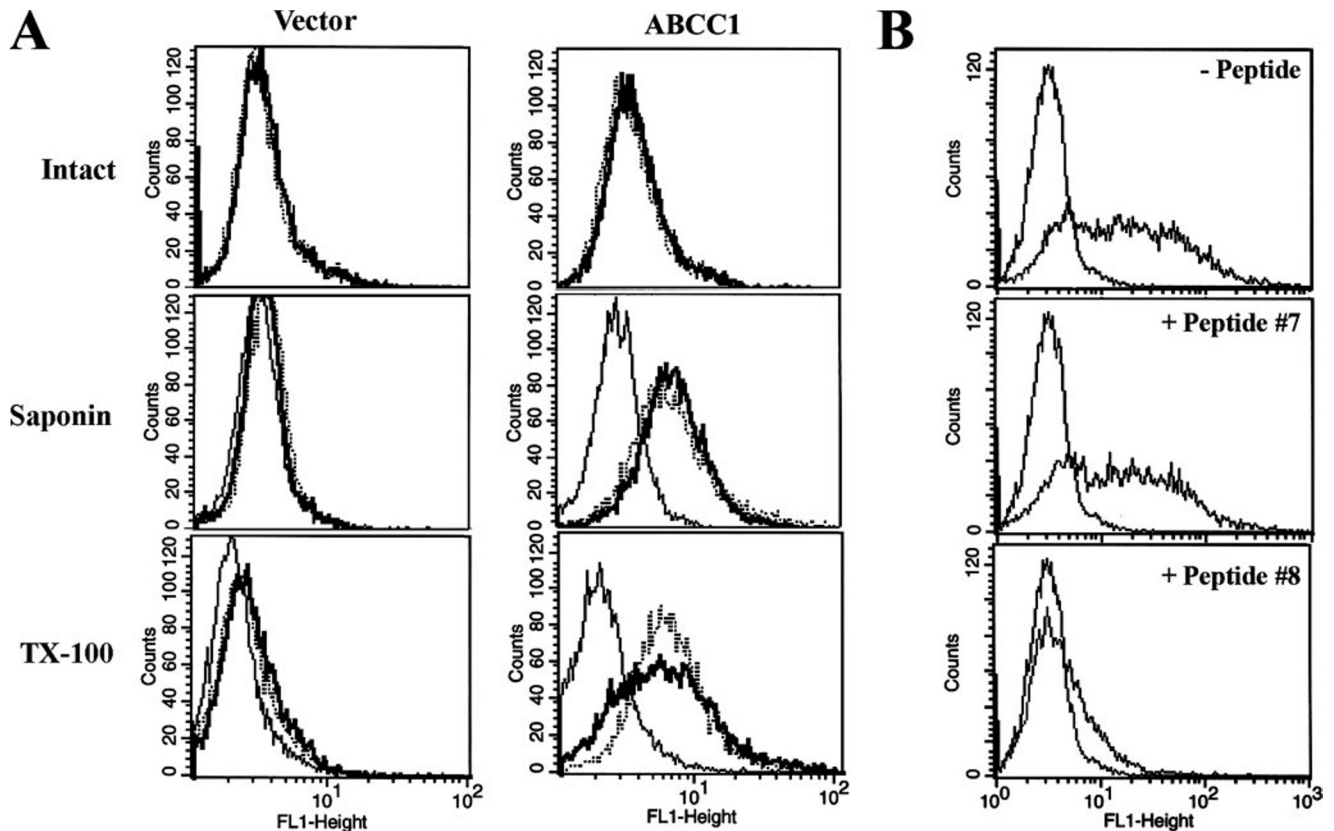


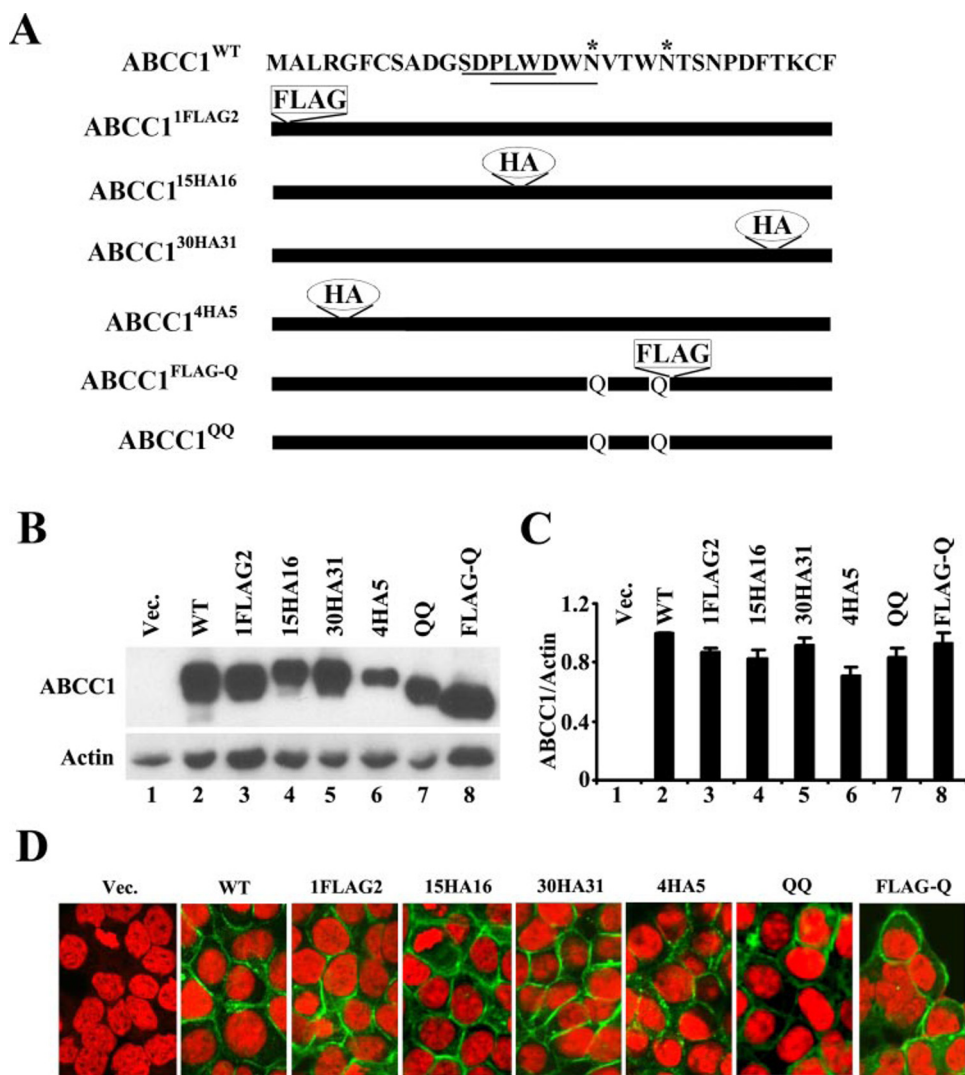
FIGURE 2. **FACS analysis of human ABCC1<sup>WT</sup>-transfected HEK293 cells with IU5C1.** *A*, IU5C1 staining. Human ABCC1<sup>WT</sup>- and vector-transfected HEK293 cells were probed with IU5C1 (thick line), IU15H6 (thin line; nonspecific negative control), and QCRL-1 (dotted line; positive control) in the absence or presence of saponin or Triton X-100 followed by secondary antibody conjugated with fluorescein isothiocyanate for FACS analysis. *B*, epitope peptide inhibition. IU5C1 and IU15H6 were first incubated without or with synthetic peptide 7 or 8 (see Fig. 1) at 25  $\mu$ g/ml prior to probing saponin-permeabilized HEK293 cells with ABCC1<sup>WT</sup>.

constructs were well expressed, as shown by Western blot analyses of isolated membranes (Fig. 3, *B* and *C*), and all ABCC1 appeared to traffic normally onto plasma membranes as determined by immunofluorescence analysis of permeabilized cells (Fig. 3*D*). It is noteworthy that both ABCC1<sup>FLAG-Q</sup> and ABCC1<sup>Q<sub>Q</sub></sup> have a smaller size than others on the Western blot (Fig. 3*B*) due to a lack of glycosylations in the amino terminus.

We next studied the staining pattern of the tagged ABCC1 in live and permeabilized cells using FACS analyses of different antibodies (Fig. 4). The wild type untagged ABCC1 (ABCC1<sup>WT</sup>) was stained by IU2H10 and IU5C1 only under permeabilized conditions (Fig. 4, compare *A* with *B*). Neither HA nor FLAG antibody stained the cells expressing ABCC1<sup>WT</sup> (Fig. 4*C*). ABCC1 with the FLAG tag inserted between Met<sup>1</sup> and Ala<sup>2</sup> (ABCC1<sup>1FLAG2</sup>) was stained by FLAG antibody under both live (Fig. 4*D*) and permeabilized conditions (Fig. 4*F*), suggesting that the amino-terminal end of the amino terminus of human ABCC1 is exposed to the extracellular space. ABCC1 with the HA tag inserted between Arg<sup>4</sup> and Gly<sup>5</sup> (ABCC1<sup>4HA5</sup>) was also stained by HA antibody in live cells (Fig. 4*G*), suggesting that the HA tag between Arg<sup>4</sup> and Gly<sup>5</sup> is exposed extracellularly. Similar to that in ABCC1<sup>WT</sup>, the IU2H10 and IU5C1 epitopes in both ABCC1<sup>1FLAG2</sup> and ABCC1<sup>4HA5</sup> were detected only following cellular permeabilization (Fig. 4, *E* and *H*), suggesting that these original epitopes in both ABCC1<sup>1FLAG2</sup> and ABCC1<sup>4HA5</sup> are located intracellularly as ABCC1<sup>WT</sup>.

It is interesting to note, however, that the HA staining of ABCC1<sup>4HA5</sup> in the live cells was less than that in the saponin-permeabilized cells (Fig. 4, compare *G* with *I*). The reduced live staining of HA tag in ABCC1<sup>4HA5</sup> occurs probably because the HA tag is too close to the lipid bilayer and cannot be stained effectively by the HA antibody. Saponin is a plant glycoside consisting of a steroid or a triterpene attached to a carbohydrate chain. It may change the membrane lipid fluidity in addition to its ability to permeabilize cells. Treatment with saponin may have helped expose the epitope by changing the membrane fluidity because of its closeness to the lipid bilayer. If this is the case, staining of ABCC1<sup>4HA5</sup> at 37 °C, which also increases membrane lipid fluidity, may be increased compared with staining at 4 °C. Indeed, the HA staining of ABCC1<sup>4HA5</sup> at 37 °C was increased to a level similar to that in the saponin-treated cells (data not shown). Thus, the membrane fluidity affects the staining of HA tags in ABCC1<sup>4HA5</sup>. On the other hand, the IU2H10 epitope cannot be stained at 37 °C (data not shown), ruling out the possibility that the inaccessibility of the IU2H10 epitope was due to the lipid fluidity.

Insertion of the HA tag between Leu<sup>15</sup> and Trp<sup>16</sup> (ABCC1<sup>15HA16</sup>) disrupts the IU2H10 and IU5C1 epitopes (Fig. 3*A*). As shown in Fig. 4*K*, no IU2H10 or IU5C1 staining of the ABCC1<sup>15HA16</sup>-expressing cells under either live or permeabilized conditions were observed, consistent with the disruption of their epitopes by the tag insertion. Interestingly, the staining



**FIGURE 3. Engineering and expression analysis of tagged ABCC1.** *A*, schematic demonstration of mutant ABCC1 with tags. Glycosylation sites are indicated by asterisks, and the IU2H10 and IU5C1 epitopes are underlined. The positions of tags are indicated. *B*, Western blot analysis. Membranes isolated from stable cell clones expressing wild type (untagged) and tagged or mutant ABCC1 were used for Western blot analysis using antibody MRP1. Actin was used as a loading control. *C*, ABCC1 expression level. The relative intensity of ABCC1 in Western blot was determined using Scion imaging software and normalized using actin as a control. The data were from four experiments. *D*, immunofluorescence staining. Stable cell clones expressing wild type (untagged) and tagged or mutant ABCC1 were fixed and permeabilized before staining with MRP1 (for ABCC1<sup>15HA16</sup>) and IU5C1 (for all others). Vector-transfected cells were used as negative controls.

of ABCC1<sup>15HA16</sup> by HA antibody was observed only under permeabilized conditions (Fig. 4, compare *J* and *L*). This observation confirms that the central portion of the amino terminus of human ABCC1, where the IU2H10 and IU5C1 epitopes are situated, is located intracellularly.

Previously, it has been observed that the FLAG epitope inserted between Asn<sup>23</sup> and Thr<sup>24</sup> with mutations of Asn<sup>19</sup> and Asn<sup>23</sup> in the amino terminus to Gln residues (ABCC1<sup>FLAG-Q</sup>) was detected extracellularly by the FLAG antibody (19), suggesting that the amino terminus of human ABCC1 is exposed to the extracellular space. It is of interest to determine the relative membrane location of the IU2H10 and IU5C1 epitopes in ABCC1<sup>FLAG-Q</sup>. As shown in Fig. 4*M*, both IU2H10 and IU5C1 can stain live cells expressing the ABCC1<sup>FLAG-Q</sup> mutant, suggesting that the IU2H10 and IU5C1 epitopes in the mutant ABCC1<sup>FLAG-Q</sup> are located extracellularly. This finding is

intriguing, because the central portion containing the IU2H10 and IU5C1 epitopes in the amino terminus of human ABCC1<sup>FLAG-Q</sup> was apparently relocated to the extracellular space by the insertion of FLAG tag at the carboxyl side of the IU2H10 and IU5C1 epitopes. To determine whether this relocation was due to the elimination of the two glycosylation sites at the amino terminus of ABCC1<sup>FLAG-Q</sup>, we engineered another construct (ABCC1<sup>QQ</sup>) with mutations of the two glycosylation sites (Asn<sup>19</sup> → Gln and Asn<sup>23</sup> → Gln) at the amino terminus without insertion of any tags. Staining of the cells expressing ABCC1<sup>QQ</sup> by IU2H10 and IU5C1 occurred only when the cells were permeabilized (Fig. 4, compare *S* and *T*). Thus, mutation of the glycosylation sites in the amino terminus did not cause the relocation of the IU2H10 and IU5C1 epitopes in human ABCC1.

It appears that the tag insertion at the carboxyl-terminal side, but not the amino-terminal side, of the IU2H10 and IU5C1 epitopes causes the relocation of these epitopes. This difference may be due to the fact that the carboxyl-terminal end is inflexible by attaching to the first TM segment anchored in the membranes. To further test this possibility, we engineered another construct by inserting an HA tag between Thr<sup>30</sup> and Lys<sup>31</sup> (ABCC1<sup>30HA31</sup>), and a stable cell line expressing this construct was tested. As shown in Fig. 4*P*, the

IU2H10 and IU5C1 epitopes could be detected in live cells expressing ABCC1<sup>30HA31</sup>, suggesting that the IU2H10 and IU5C1 epitopes of ABCC1<sup>30HA31</sup> are located extracellularly and confirming that the tag insertion at the carboxyl side of IU2H10 and IU5C1 epitopes relocates these epitopes. Interestingly, the HA tag in the ABCC1<sup>30HA31</sup> could not be detected by HA antibodies in live cells (Fig. 4*P*). This observation is probably due to the fact that the HA tag inserted following Thr<sup>30</sup> is too close to membranes, which creates steric hindrance. However, a weak staining was observed when the cells were treated with saponin (Fig. 4*R*), suggesting that the staining of the tag may be enhanced by increasing the membrane lipid fluidity as for the HA tag in ABCC1<sup>4HA5</sup>. To support this argument, we performed a staining at 37 °C as described above for ABCC1<sup>4HA5</sup> and also found a weak staining of live cells (data not shown). Thus, the HA tag inserted between Thr<sup>30</sup> and Lys<sup>31</sup> is close to

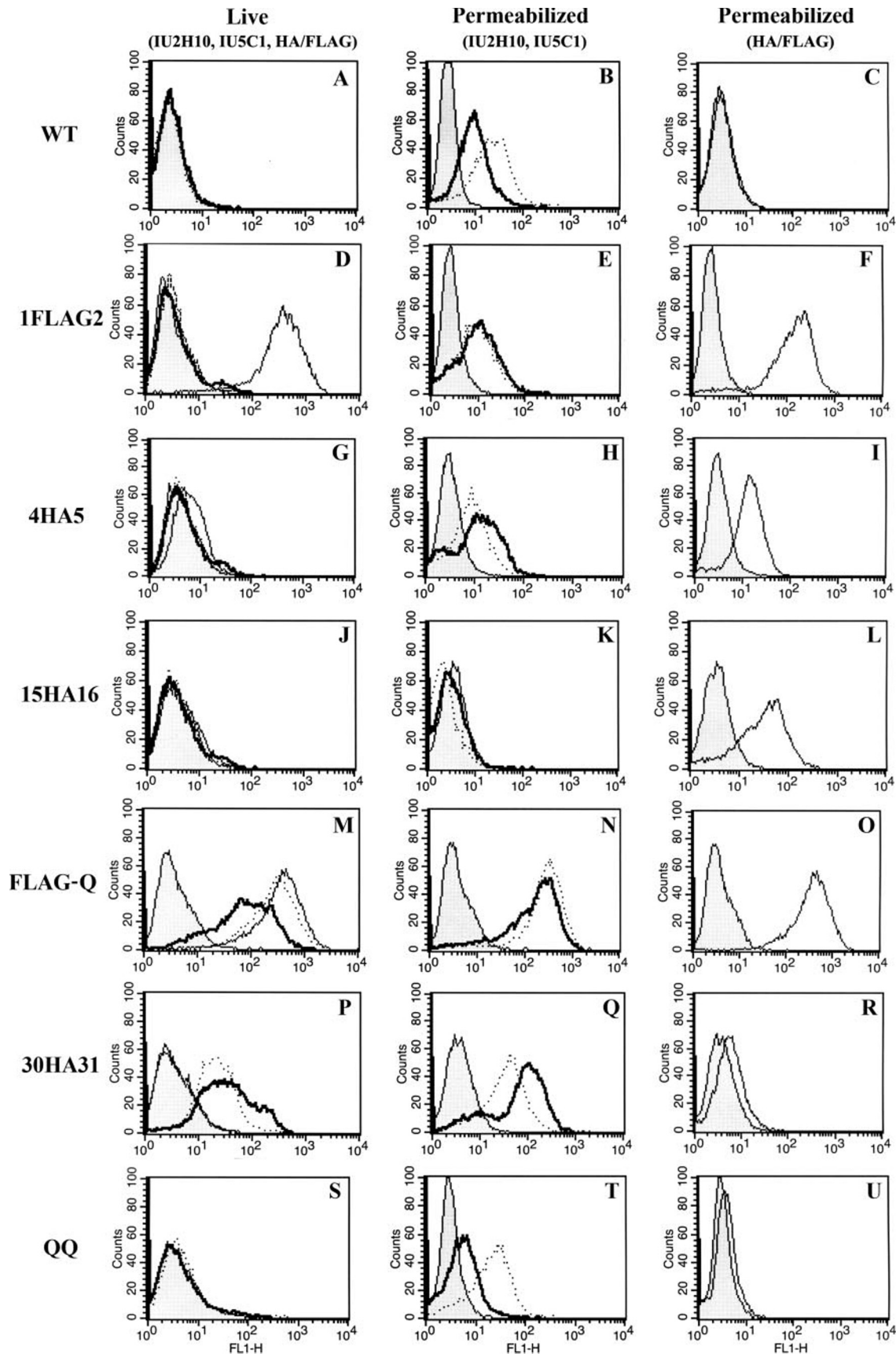
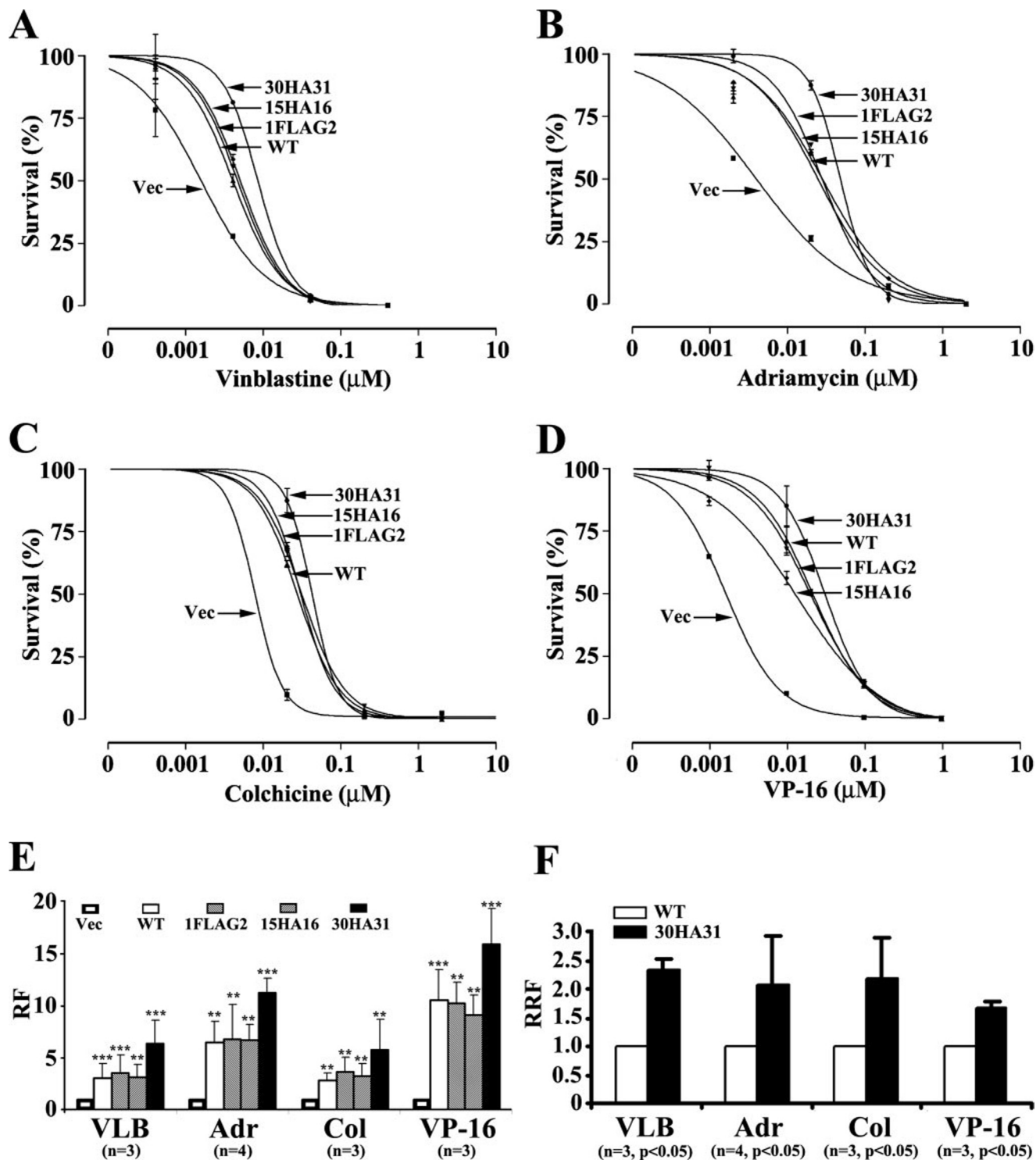


FIGURE 4. Membrane orientation of the amino terminus of human ABCC1 in HEK293 cells. Stable HEK293 cells expressing wild type and various tagged or mutant ABCC1 were subjected to staining under live (left column) or saponin-permeabilized (middle and right columns) conditions. The antibodies used were IU5C1 (thick line), IU2H10 (dotted line), HA or FLAG (thin line), or nonspecific control antibody IU15H6 (thin line with gray area under curve).





**FIGURE 5. Drug resistance function of human ABCC1.** Stable HEK293 cells expressing ABCC1<sup>WT</sup>, ABCC1<sup>4HA5</sup>, ABCC1<sup>15HA16</sup>, and ABCC1<sup>30HA31</sup> were subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay using anticancer drugs vinblastine (A), Adriamycin (B), colchicine (C), and VP-16 (D). Vector-transfected cells were used as a negative control. E, a summary of resistance factor (RF) of ABCC1 compared with vector-transfected cells for different anticancer drugs. \*\* and \*\*\*, statistically significant differences with  $p < 0.05$  and  $p < 0.01$ , respectively. F, relative resistance factor (RRF) between ABCC1<sup>30HA31</sup> and ABCC1<sup>WT</sup> for different anticancer drugs after normalization to ABCC1 expression level. VLB, vinblastine; Adr, Adriamycin; Col, colchicine.

the extracellular surface of the membrane lipid bilayer, consistent with the predicted topological structure.

**Gating Function of the Amino Terminus of Human ABCC1—**Previously, ABCC1<sup>4HA5</sup> and ABCC1<sup>FLAG-Q</sup> have been shown to be functional to transport drug substrates (18, 19). We next

determined if the tagged ABCC1 newly engineered in this study is also functional for drug resistance. For this purpose, we performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays of the stable clones expressing ABCC1<sup>WT</sup>, ABCC1<sup>1FLAG2</sup>, ABCC1<sup>15HA16</sup>, and ABCC1<sup>30HA31</sup> using four

anticancer drugs. As shown in Fig. 5, *A–D*, all of these clones are resistant to vinblastine, Adriamycin, colchicine, and VP-16 compared with cells transfected with vector alone. Although the resistance factors to all drugs are similar between ABCC1<sup>WT</sup>, ABCC1<sup>1FLAG2</sup>, and ABCC1<sup>15HA16</sup>, they all appear to be lower than that of ABCC1<sup>30HA31</sup> (Fig. 5*E*). Further analysis showed that the resistance factors of ABCC1<sup>30HA31</sup> to all drugs are significantly higher than that of ABCC1<sup>WT</sup> after normalizing to their expression levels (Fig. 5*F*), suggesting that ABCC1<sup>30HA31</sup> is more active in effluxing anticancer drug substrates than ABCC1<sup>WT</sup>. Because ABCC1<sup>30HA31</sup> has an extracellularly located amino terminus compared with ABCC1<sup>WT</sup>, ABCC1<sup>1FLAG2</sup>, and ABCC1<sup>15HA16</sup>, we hypothesized that the amino terminus of human ABCC1 may function as a gate with a U-shaped folding by plugging into a putative channel in ABCC1<sup>WT</sup>. The amino-terminal gate may experience transition between two different conformations, open and closed, as shown in Fig. 7*C*. Insertion of HA epitope at the carboxyl end of the amino terminus (ABCC1<sup>30HA31</sup>) may have forced the gate to open, which allows the protein to transport anticancer drugs more efficiently.

To test the above hypothesis, we generated another construct (ABCC1<sup>ΔN32</sup>) with deletion of the entire putative amino-terminal gate (32 amino acids). We proposed that deletion of the amino terminus would remove the putative gate, which should have an effect on ABCC1 activity similar to or better than that of ABCC1<sup>30HA31</sup> to allow a more efficient transport of substrates. A HEK293 clone with stable expression of ABCC1<sup>ΔN32</sup> with correct membrane localization (Fig. 6*E*) was generated and used to determine drug resistance. As shown in Fig. 6, *A–D* and *F*, ABCC1<sup>ΔN32</sup> appeared to be much more active than ABCC1<sup>WT</sup>, with significantly higher resistance factors.

To further test the above hypothesis, we analyzed whether the central portion of the amino terminus relocates to extracellular space (conformational change) to open the channel during the transport process and thus expose the amino terminus extracellularly. For this purpose, we used vanadate as an inhibitor to trap the intermediate stage of a putative conformational change to detect the HA epitope in ABCC1<sup>15HA16</sup>, which may be transiently exposed extracellularly. As shown in Fig. 7*A*, the staining of HA epitope was observed in live cells in the presence of vanadate, suggesting that HA epitope in the central portion of the amino terminus is relocated from the inside to the outside of cells during the transport process and caught outside of cells by using vanadate. This change was not observed in the absence of vanadate or in vector-transfected cells. Similar observations have also been made with the ABCC1<sup>WT</sup> using IU2H10 as a probe (data not shown). Thus, it is likely that the central portion of the amino terminus of human ABCC1 experiences a conformational change (gate opening) by relocating from extracellularly inaccessible to accessible during the transport process.

If the amino-terminal sequence functions as a gate and plugs into a putative channel, it is possible that the addition of a synthetic peptide representing the amino-terminal gate may block the function of ABCC1 by irreversibly plugging into the channel. We tested this possibility by preincubating

isolated membrane vesicles for 30 min with or without a synthetic 19-amino acid peptide (for sequence, see Syn.Pept (19) in Fig. 1*E*), followed by analysis of ATP-dependent LTC<sub>4</sub> uptake as previously described (11). As shown in Fig. 7*B*, the ATP-dependent LTC<sub>4</sub> uptake was completely inhibited by the peptide, suggesting that the peptide may directly interact with and inhibit the function of ABCC1.

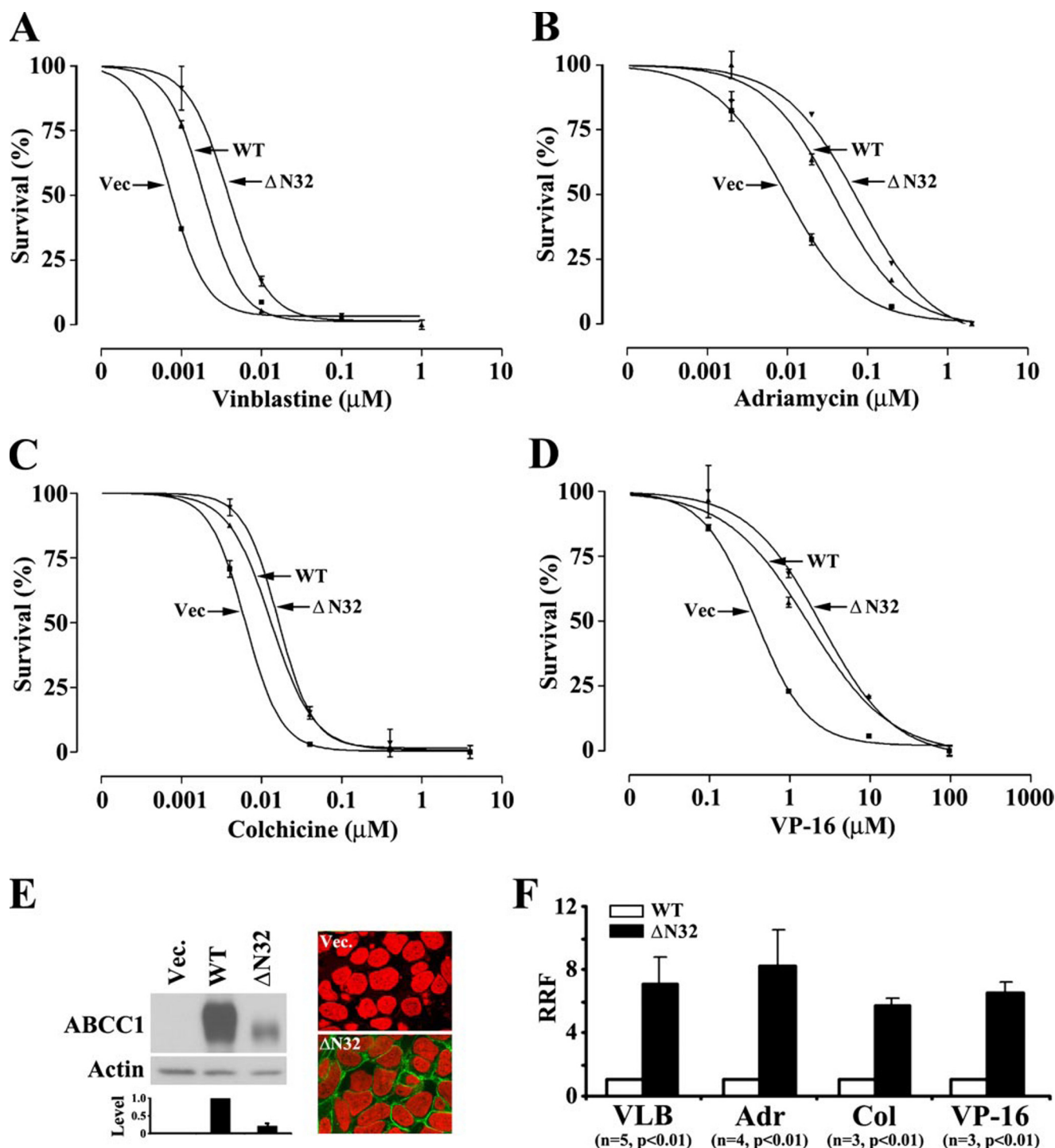
## DISCUSSION

In this study, we further investigated the membrane orientation of the amino terminus of human ABCC1 and its potential function as a gate. Two monoclonal antibodies, IU2H10 and IU5C1, directed against the central portion of the amino terminus of human ABCC1 were generated and used for determining the membrane orientation of their epitopes. We also engineered HA or FLAG tags into different positions at the amino terminus for determination of the folding of the amino terminus. We found that the amino terminus of human ABCC1 probably had a U-shaped folding with the bottom of the U in cytoplasm and the two ends in extracellular space. This U-shaped folding of the amino terminus may serve as a gate for the function of human ABCC1 by plugging into a putative channel formed by the membrane-spanning domains (Fig. 7*C*).

The epitopes for both IU2H10 and IU5C1 are located in the central portion of the amino-terminal 32 amino acids. Using peptide walking, we mapped their epitopes within six amino acids each. The epitope for IU2H10 has a sequence of <sup>12</sup>SDPLWD<sup>17</sup>, and the epitope for IU5C1 has a sequence of <sup>14</sup>PLWDWN<sup>19</sup>. The two epitopes overlap with a two-amino acid shift. Generation of two different monoclonal antibodies to this region suggests that this portion of the amino terminus is highly immunogenic and further supports the conclusion that the epitopes are located intracellularly at resting state of the protein. It is also noteworthy that the mutants ABCC1<sup>FLAG-Q</sup> and ABCC1<sup>QQ</sup>, with the mutation of Asn<sup>19</sup> to Gln, which is the last residue in the IU5C1 epitope, are still reactive to IU5C1 (Fig. 4, *M* and *T*). This observation suggests that the last residue Asn<sup>19</sup> in the IU5C1 epitope can be changed to a homologous residue Gln. On the other hand, deleting the residue Asn<sup>19</sup> would abolish its reactivity to IU5C1 as demonstrated by peptide walking (Fig. 1).

Within the ABC superfamily of transporters, only a few members (*e.g.* ABCC1, ABCC2, ABCC3, ABCC6, ABCC8, ABCC9, and ABCC10) have the MSD0 with a predicted extracellular amino terminus. Among these members, the length of the predicted extracellular amino terminus varies, and the sequence is not well conserved (11). Currently, the functional role of the amino terminus and the MSD0 is not well known. For ABCC1, the core domain lacking the entire MSD0 appears to function normally (12), suggesting that MSD0 is dispensable. On the other hand, removal of the first 66 amino acids (10) or mutation of a single Cys (Cys<sup>7</sup>) residue (11) essentially knocked out ABCC1 function. Despite these findings, the existence of such a domain in several ABC transporters of different functions suggests that it is important for either structure, function, or both, which we currently do not understand. Indeed, it has been found that MSD0 may contribute to the binding of the substrate LTC<sub>4</sub>





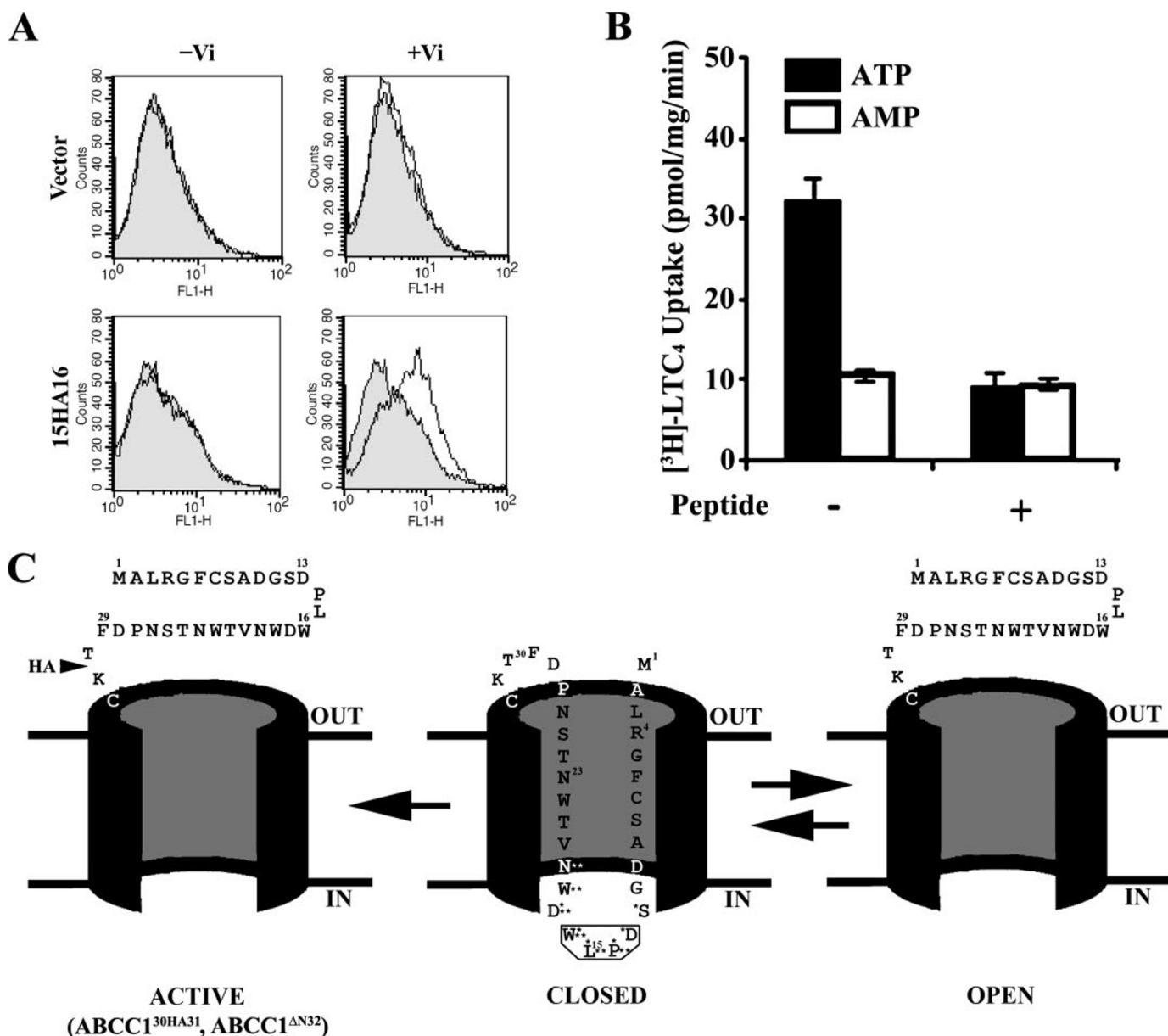
**FIGURE 6. Expression and drug resistance function of human ABCC1 $\Delta\text{N32}$ .** Stable HEK293 cells expressing ABCC1<sup>WT</sup> and ABCC1 $\Delta\text{N32}$  were subjected to a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay using anticancer drugs vinblastine (A), Adriamycin (B), colchicine (C), and VP-16 (D). Vector-transfected cells were used as a negative control. E, Western blot and immunofluorescence analyses of ABCC1 $\Delta\text{N32}$  expression. The relative intensity of ABCC1 in Western blot was determined using a Scion imaging software and normalized using actin as a control. The data were from four experiments. F, relative resistance factor (RRF) between ABCC1 $\Delta\text{N32}$  and ABCC1<sup>WT</sup> for different anticancer drugs after normalization to ABCC1 expression level. VLB, vinblastine; Adr, Adriamycin; Col, colchicine.

and glutathione (14), cellular processing and trafficking of ABCC1 (15), and dimerization of ABCC1.<sup>4</sup>

In a previous study, we unexpectedly found that the IU2H10

epitope in the central portion of the amino terminus of human ABCC1 can be stained only in permeabilized cells (21), suggesting that it is located intracellularly. This finding contradicts the prevailing model in which the amino terminus is thought to be extracellular inferred from several previous studies (16–20). The results from the current study suggest that the putative

<sup>4</sup>Y. Yang, Y. Liu, Z. Dong, J. Xu, Z. Liu, and J.T. Zhang, unpublished observations.



**FIGURE 7. Conformational changes of the amino terminus and its gating role.** *A*, membrane reorientation. HEK293 cells harboring vector control or ABCC1<sup>15HA16</sup> were first treated with or without vanadate before they were stained by HA antibody. *B*, effect of amino-terminal peptide on ATP-dependent [<sup>3</sup>H]LTC<sub>4</sub> uptake in isolated membrane vesicles. Membrane vesicles from HEK293 cells expressing human ABCC1<sup>WT</sup> were pretreated with or without 40 mM synthetic peptide Syn.Pep (19) (see Fig. 1). The vesicles were then pelleted and washed before being used for [<sup>3</sup>H]LTC<sub>4</sub> uptake analysis in the presence of ATP or AMP. Results shown are means  $\pm$  S.D. of triplicate determinations from a representative experiment. *C*, schematic model of the gating role of the amino terminus of human ABCC1. Binding of substrate at the cytoplasmic side may trigger the gate to open. ABCC1 with its amino terminus truncated (ABCC1<sup>ΔN32</sup>) or relocated (ABCC1<sup>30HA31</sup>) is constitutively active.

extracellular amino terminus of human ABCC1 probably has a U-shaped structure. Although the bottom of the structure (central portion containing IU2H10 and IU5C1 epitopes) is exposed to intracellular space, both ends of the U-structure are located extracellularly. The intracellular opening of the putative channel may be big enough for antibodies to access their epitopes in the bottom of the U-structure.

Alternatively, the amino terminus of human ABCC1 may sit on the putative channel extracellularly rather than traverse it in such a way that the IU2H10 and IU5C1 epitopes are inaccessible on the outside of cells. However, we think that this possibility is less likely. We clearly showed that the access of IU2H10

and IU5C1 epitopes was membrane permeabilization-dependent using several permeabilization agents (Fig. 2) and that temperature (data not shown) and urea (21) treatment, which both could disrupt the extracellular folding of the amino terminus and membrane fluidity but not membrane permeability, did not increase the staining of these epitopes. Furthermore, similar results were observed using an isolated Fab fragment of IU2H10,<sup>5</sup> suggesting that the size of the antibody was not a cause of its inability to stain its epitopes in live cells.

<sup>5</sup> Q. Chen and J.-T. Zhang, unpublished observations.

To form a U-shaped folding, a  $\beta$ -turn structure may exist at the bottom of the U, where the IU2H10 and IU5C1 epitopes are located. Examination of the IU2H10 and IU5C1 epitope sequences showed four residues that are highly likely to occupy the four positions of a  $\beta$ -turn according to the studies by Chou and Fasman (23, 24). These residues are Asp<sup>13</sup>, Pro<sup>14</sup>, Leu<sup>15</sup>, and Trp<sup>16</sup>, which probably occupy the  $f_i$ ,  $f_{i+1}$ ,  $f_{i+2}$ , and  $f_{i+3}$  positions of the  $\beta$ -turn, respectively, and they are the center of the IU2H10 epitope. Empirically, Pro and Trp residues are located predominantly in the second and fourth positions of a  $\beta$ -turn, respectively, whereas Asp residues, which have the highest bend potential, can occupy any of the four positions (23, 24). Because the amino terminus has only 32 residues, it is unlikely to form two antiparallel  $\alpha$ -helices to cross the membrane. Previously, examination of the sequence using an online program (available on the World Wide Web at [www.expasy.ch/tools/#secondary](http://www.expasy.ch/tools/#secondary)) has shown that the amino terminus contains at least a stretch of sequence (<sup>16</sup>WDWNVTW<sup>22</sup>) that has high potential to form a  $\beta$ -strand (21). We thus speculate that the U-shaped structure of the amino terminus of human ABCC1 is formed by an antiparallel  $\beta$ -sheet with a  $\beta$ -turn at <sup>13</sup>DPLW<sup>16</sup>. This  $\beta$ -turn makes up the IU2H10 and IU5C1 epitopes and is exposed to the cytoplasmic space.

If the epitopes of IU2H10 and IU5C1 consist of the  $\beta$ -turn, insertion of HA epitope into this position would have disrupted this structure in the ABCC1<sup>15HA16</sup>. However, it appeared that the amino terminus of ABCC1<sup>15HA16</sup> had normal folding, and the protein functioned normally. We further examined the sequence of the HA epitope (YPYDVPDYAS) and found that it too comprises a sequence (VPDY) that qualifies for a  $\beta$ -turn structure. Clearly, further studies are needed to investigate whether these  $\beta$ -turn structures exist and if they are important for the folding of the amino terminus of human ABCC1.

The amino terminus of human ABCC1 contains two *N*-linked glycosylation sites (Asn<sup>19</sup> and Asn<sup>23</sup>) and it appears that both sites are glycosylated. Mutation of these two sites significantly reduced the apparent size of ABCC1 due to reduced glycosylation (Fig. 3B). These *N*-linked sugar chains did not appear to affect the reactivity of the IU2H10 to the amino terminus (21). In this study, we further confirmed this conclusion by the finding that the removal of the two glycosylation sites by mutating the two Asn residues to Gln did not enhance the reactivity of IU2H10 and IU5C1 to ABCC1. Mutation of the two glycosylation sites also did not affect the membrane orientation of the IU2H10 and IU5C1 epitopes. Thus, it is likely that these sugar chains do not play any role in the U-shaped folding of the amino terminus. Furthermore, we found that the mutation of the two glycosylation sites was not responsible for the relocation of the amino terminus to extracellular space as found with the ABCC1<sup>FLAG-Q</sup> (19).

In the current study, we also found that the HA tags inserted between Arg<sup>4</sup> and Gly<sup>5</sup> and between Thr<sup>30</sup> and Lys<sup>31</sup> are not well stained in live cells by HA antibody. However, increasing the membrane lipid fluidity by increasing temperature enhanced the HA antibody reactivity to these tags. These observations suggest that the HA tags inserted at these positions are very close to the extracellular surface of the membrane lipid bilayer, which will sterically hinder the antibody reaction, and

increasing the membrane lipid fluidity helps the exposure of these tags. These observations also support the conclusion that both ends of the amino-terminal U-structure are located on the extracellular side of the membrane.

Interestingly, we found that insertion of HA (ABCC1<sup>30HA31</sup>) or FLAG (ABCC1<sup>FLAG-Q</sup>) tags into the second half of the amino terminus (second strand of the  $\beta$ -sheet structure) relocated the  $\beta$ -turn-containing IU2H10 and IU5C1 epitopes into extracellular space, whereas insertions at the first half (ABCC1<sup>1FLAG2</sup> and ABCC1<sup>4HA5</sup>) of the amino terminus did not. This is probably due to the fact that the second half is anchored into membranes by attaching to the first TM segment. Insertion into the first half (between Met<sup>1</sup> and Ala<sup>2</sup> and between Arg<sup>4</sup> and Gly<sup>5</sup>) would force an extension of the amino terminus into the extracellular space without any significant effect on the  $\beta$ -sheet structure. On the other hand, insertion into the second half (between Asn<sup>23</sup> and Thr<sup>24</sup> and between Thr<sup>30</sup> and Lys<sup>31</sup>) would disrupt the structure due to the rigidity of the second half with an anchor in membranes, which may force an impaired movement of the second strand of the  $\beta$ -sheet. This impaired relaxation may induce the relocation of the amino terminus from the putative channel to the extracellular space.

The amino terminus of human ABCC1 consists of largely hydrophilic residues, and thus, the U-shaped folding is unlikely to traverse directly the hydrophobic lipid bilayer. In a previous study (21), we found that the IU2H10 epitope in the amino terminus was relocated to extracellular space with the deletion of the carboxyl core domain (MSD1-NBD1-MSD2-NBD2), suggesting that the core domain is required for the membrane traversal of the U-shaped amino terminus. Thus, we proposed that the U-shaped amino terminus probably traverses the membrane through a putative channel formed by the transmembrane segments in the carboxyl core domain (Fig. 7C). By plugging into the putative channel, the U-shaped amino terminus may play a gating role for ABCC1 function. As the gate opens to allow substrate transport, the amino terminus will be transiently relocated to the extracellular space, and the IU2H10 and IU5C1 epitopes would be accessible extracellularly. We indeed detected these epitopes extracellularly in the presence of the ATPase inhibitor vanadate (Fig. 7A). We also found that removal of the amino terminus (ABCC1 <sup>$\Delta$ N32</sup>) or permanently relocating the amino terminus to extracellular space (ABCC1<sup>30HA31</sup>) enhanced the activity of ABCC1, possibly by generating a constitutively active molecule with constant opening of the gate (Figs. 5 and 6). Careful examination of the previous study (19) showed that the resistance factors of ABCC1<sup>FLAG-Q</sup>-transfected cells to VP-16 and daunorubicin are similar to that of ABCC1<sup>WT</sup>-transfected cells. However, the expression level of ABCC1<sup>FLAG-Q</sup> is only about half of ABCC1<sup>WT</sup> in that study. Thus, ABCC1<sup>FLAG-Q</sup> is probably twice as active as ABCC1<sup>WT</sup>, consistent with our findings of ABCC1<sup>30HA31</sup> (Fig. 5F).

Currently, it is unclear why human ABCC1 would need such a gating for its function and what triggers the gate to open. It is tempting to speculate that the gate of ABCC1 may function as a sensor of intracellular drugs and that as the intracellular drug concentration reaches a critical level and binds to ABCC1, it may then cause the gate to open and cause an efflux of drugs.



Thus, the gating mechanism can be another level of regulation. It has been shown previously that the amino-terminal domain of ABCC1 contains a substrate-binding site (14). The binding of substrates to this site may trigger the gate to open. Currently, we are testing this hypothesis.

In summary, we have demonstrated in this study that the amino terminus of human ABCC1 has a U-shaped folding with the central bottom portion facing the cytoplasm and the two ends in extracellular space. The U-structure probably consists of an antiparallel  $\beta$ -sheet. Four amino acid residues located in the overlapping IU2H10 and IU5C1 epitopes (six amino acid residues each) in the bottom of the U-structure probably form a  $\beta$  turn. This U-shaped structure may function as a gate by plugging into a putative channel formed by the membrane-spanning domain in the carboxyl core of human ABCC1.

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