The Glycosylation and Orientation in the Membrane of the Third Cytoplasmic Loop of Human P-Glycoprotein Is Affected by Mutations and Substrates[†]

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Received October 22, 1998; Revised Manuscript Received January 29, 1999

ABSTRACT: Multiple topologies have been detected for the COOH-terminal half of the human multidrug resistance P-glycoprotein (P-gp). In one topology, the predicted third cytoplasmic loop (CL3) is on the cytoplasmic side (P-gp-CL3-cyt) of the membrane. In an alternate topology, CL3 is on the extracellular side of the membrane (P-gp-CL3-ext). It is not known if both forms of P-gp are active because it is difficult to distinguish either topology in the full-length molecule. When the halves of P-gp are expressed as separate polypeptides, the two topologies of the C-Half are readily distinguished on SDS−PAGE, because only the C-Half (CL3-ext) is glycosylated. To test whether both topologies can fold into an active enzyme, we assayed for interaction between the N- and C-Halves of P-gp since functional P-gp requires interaction between both halves. In a mutant P-gp (E875C) that gave about equal amounts of both topologies, only the C-Half (CL3-cyt) could be recovered by nickel chromatography after coexpression with the histidine-tagged N-Half P-gp. The isolated N-Half and E875C C-Half (CL3-cyt) polypeptides, when expressed together, exhibited verapamil- and vinblastine-stimulated ATPase activities that were similar to the wild-type enzyme. We also found that biosynthesis of mutant E875C C-Half in the presence of the N-Half P-gp resulted in enhanced expression of C-Half (CL3-cyt). By contrast, interaction of C-Half (CL3-ext) with N-Half P-gp was not detected. These results show that the topology of the C-Half portion of P-gp greatly influences its interactions with the amino-terminal half of the molecule.

P-glycoprotein (P-gp)[1] is a 170 kDa plasma membrane glycoprotein that confers multidrug resistance when overexpressed in mammalian cells (reviewed in refs *1*−*3*). The protein confers resistance to a broad range of cytotoxic agents that do not have a common structure or intracellular target; examples include anticancer drugs such as vinblastine and doxorubicin; antimicrotubule drugs such as colchicine and podophyllotoxin; and toxic peptides such as valinomycin and gramicidin D. The protein is clinically important because it confers multidrug resistance in cancer (*1*), and interferes in the treatment of AIDS by inhibiting oral absorption and brain entry of HIV-1 protease inhibitors (*2*).

Human P-gp, encoded by the MDR1 gene, consists of 1280 amino acids organized in two tandem repeats of 610 amino acids, joined by a linker region of 60 amino acids. Each repeat consists of an NH_2-terminal hydrophobic domain containing six potential transmembrane (TM) sequences followed by a hydrophilic domain containing a nucleotide-binding site (*3*). The amino acid sequence and domain organization of the protein is typical of the ABC (ATP-binding cassette) superfamily of transporters (*4*).

P-gp has been a particularly useful model protein for studying folding and trafficking (protein kinesis) of polytopic plasma membrane transport proteins because these processes can be manipulated by point mutations (*5*) or substrates (*6*), and the transporter can adopt more than one topology (*7*−*10*). Synthesis of plasma membrane proteins is a complex process involving synthesis of a core-glycosylated intermediate in the endoplasmic reticulum, processing of the sugars into complex carbohydrates in the Golgi apparatus and then transport to the cell surface. The presence of certain point mutations in P-gp, however, causes the protein to be trapped in the endoplasmic reticulum as a core-glycosylated intermediate in association with molecular chaperones such as calnexin and Hsc 70 where it is rapidly degraded (*11*, *12*). The effects of these processing mutations can be overcome by carrying out synthesis in the presence of drug substrates (*6*). Recent results suggest that processing mutants are structurally similar to the core-glycosylated form of the wild-type enzyme but have become trapped in this early step of the folding pathway (*13*). The presence of drug substrates induces superfolding of the processing mutants so that they can overcome this folding barrier to yield functional enzyme.

There is considerable evidence that the NH_2-terminal half of P-gp contains 6 TM segments (*14*−*16*). Alternative topologies, however, have been detected for the COOH-terminal half of P-gp. It appears that the segment of P-gp that is responsible for these alternate topologies is the

[†] This research was supported by a grant to D.M.C. as part of a group grant from the Medical Research Council of Canada and a grant from the Canadian Cystic Fibrosis Foundation of Canada. D.M.C. is a Scholar of the Medical Research Council of Canada.

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[1] Abbreviations: C-Half, COOH-terminal half molecule of P-gp (residues 681 to 1280); CL, cytoplasmic loop; Cyt, cytoplasmic; Ext, extracellular; N-Half, NH_2-terminal half molecule of P-gp (residues 1−682); P-gp, P-glycoprotein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TM, transmembrane segment.

10.1021/bi982525y CCC: $18.00
Published on Web 03/31/1999

predicted third intracellular loop (CL3). P-gp molecules have been identified in which CL3 is on the cytoplasmic side of the membrane (CL3-cyt) (*14*, *16*) or on the extracellular side (CL3-ext) of the membrane (*7*, *10*). Both CL3-cyt (*14*, *16*) and CL3-ext (*9*) forms of P-gp have been detected at the cell surface. It is not known, however, whether both forms of P-gp function as drug pumps because of the difficulties in separating these very similar polypeptides.

To test whether P-gp (CL3-cyt) or P-gp (CL3-ext) were both active, we characterized the interactions between the two halves of P-gp (N-Half and C-Half) that were expressed as separate polypeptides. Both halves of P-gp can be expressed as separate polypeptides, but drug-stimulated ATPase activity is only observed when the two halves are expressed contemporaneously (*17*), suggesting that direct interaction between the two halves of P-gp is essential for activity (*18*). Accordingly, we tested whether the C-Half molecules with either CL3-cyt or CL3-ext topology could interact with the N-Half molecule. Our results suggest that only the CL3-cyt C-Half molecule is able to interact with the N-Half molecule and that this interaction is promoted by the presence of drug substrates.

EXPERIMENTAL PROCEDURES

Generation of Constructs. Full-length MDR1 cDNA and the cDNAs coding for the NH_2- and COOH-terminal half-molecules, modified to encode the epitope for monoclonal antibody A52 at the COOH-terminal ends of the proteins, were inserted into the mammalian expression vector pMT21 as described previously (*18*). Oligonucleotide-directed mutagenesis was carried out as described previously (*19*). For purification purposes, a full-length MDR1 cDNA and the cDNAs coding for the half-molecules were modified to encode for 10 histidine residues at the COOH-ends of the proteins (*18*, *20*). The sequence at the COOH-terminus of the full-length P-gp that would normally end as TKRQ now became TKRA$(His)_{10}$LDPRQ. The sequence at the COOH-terminus of the NH_2-terminal half-molecule was RKLA$(His)_{10}$-LDPRQ.

Purification of P-gp Mutants and Measurement of ATPase Activity. For purification of P-gp mutants, HEK 293 cells transfected with cDNAs coding for the histidine- and/or A52-tagged P-gps were solubilized with 1% (w/v) *n*-dodecyl-β-D-maltoside and the mutant P-gps purified by nickel-chelate chromatography. Drug-stimulated ATPase activity was determined as previously described (*20*).

To test for association between the A52-tagged COOH-half molecule (C-Half-A52) and the histidine-tagged NH_2-half molecule (N-Half-His), a copurification method was used (*18*). Both cDNAs were coexpressed in HEK 293 cells. The cells were solubilized with 1% (w/v) dodecyl-β-D-maltoside and the insoluble material was removed by centrifugation at 16000*g* for 15 min. The solubilized extracts were subjected to nickel-chelate chromatography and the eluted fraction subjected to immunoblot analysis with monoclonal antibody A52 (*12*).

Immunological Procedures. The cells were lysed in SDS sample buffer (63 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, and 2% (v/v) β-mercaptoethanol) containing 50 mM EDTA and protease inhibitors (10 μM E-64, 12 μg/mL leupeptin, 100 units/mL aprotinin, 50 μg/mL 4-(2-aminoethyl)benzenesulfonyl fluoride, and 25 μg/mL benzamidine). The samples were then subjected to SDS−PAGE, electroblotted onto nitrocellulose and developed with monoclonal antibody A52 (*19*) or with a rabbit polyclonal antibody against P-gp followed by enhanced chemiluminescence (Amersham) as described previously (*12*).

RESULTS

Point Mutations can Influence the Topology of P-gp. Figure 1 shows the two topologies of P-gp. The CL3-cyt topology shown in Figure 1A is that predicted from hydropathy plots of the human MDR1 gene (*3*). In this model, the consensus glycosylation site (residues N809, T810, and T811) in the COOH-half is located in the cytoplasmic loop between predicted TM 8 and TM 9, and is not glycosylated (Figure 1A). This is because glycosylation of membrane glycoproteins occurs only on the lumenal side of the endoplasmic reticulum and Golgi apparatus (*21*). Figure 1A is the predominant form of P-gp that has been detected at the cell surface (*14*−*16*). Other studies, however, reported that a portion of P-gp had the CL3-ext topology (Figure 1B) (*7*, *10*). Skach et al. (*10*) also showed that TM8 and TM9 were exposed to the extracellular surface in the P-gp (CL3-ext) molecules, while predicted TM7 and TM10 were shown to be membrane bound. Similarly, both predicted TM11 and TM12 have also been shown to be membrane bound (*22*). In the P-gp (CL3-ext) topology, glycosylation occurs at residue N809 (see below) of the consensus site in the COOH-half (Figure 1B). While the P-gp (CL3-ext) topology (Figure 1B) has also been detected at the cell surface (*7*), it has been difficult to compare its properties to that of P-gp (CL-3-cyt). This is because the P-gp (CL3-ext) form of P-gp appears to be present in only minor amounts relative to the P-gp (CL3-cyt) form. It has, however, been demonstrated that alternate topologies are present in both full-length and truncated forms of P-gp (*9*). It has not been possible to isolate the two forms of full-length P-gp, and it is difficult to distinguish the two forms on SDS−PAGE because of their small size difference (about 2−3 kDa). The inability to detect a shift in the size of P-gp on SDS−PAGE suggested that either the carbohydrate moiety at this site was not extensively modified in the Golgi apparatus or that the glycosylated variant does not leave the endoplasmic reticulum. To overcome these problems, we studied the two variants of P-gp using half-molecule forms of the enzyme. We previously showed that contemporaneous expression of both NH_2-(N-Half) and COOH-half (C-Half) molecules of P-gp results in the formation of a functional complex (*17*). Coupling of drug binding to stimulation of ATPase activity requires interactions between both halves of P-gp and is not observed when either half-molecule is expressed alone. The half-molecules appear to interact in a manner that resembles the full-length P-gp since they exhibit similar drug-stimulated ATPase activities as the intact P-gp molecule (*18*).

Expression of each half-molecule as an individual polypeptide allowed us to detect the alternative topologies of the C-Half by SDS−PAGE, because of the different glycosylation patterns of the P-gp (CL3-ext) and P-gp (CL3-cyt) C-Half molecules (Figure 1, panels C and D). As shown in Figure 2 (lane 2), expression of wild-type C-Half P-gp in HEK 293 cells results in the appearance of two products. The majority (>80%) of the product is unglycosylated (C-

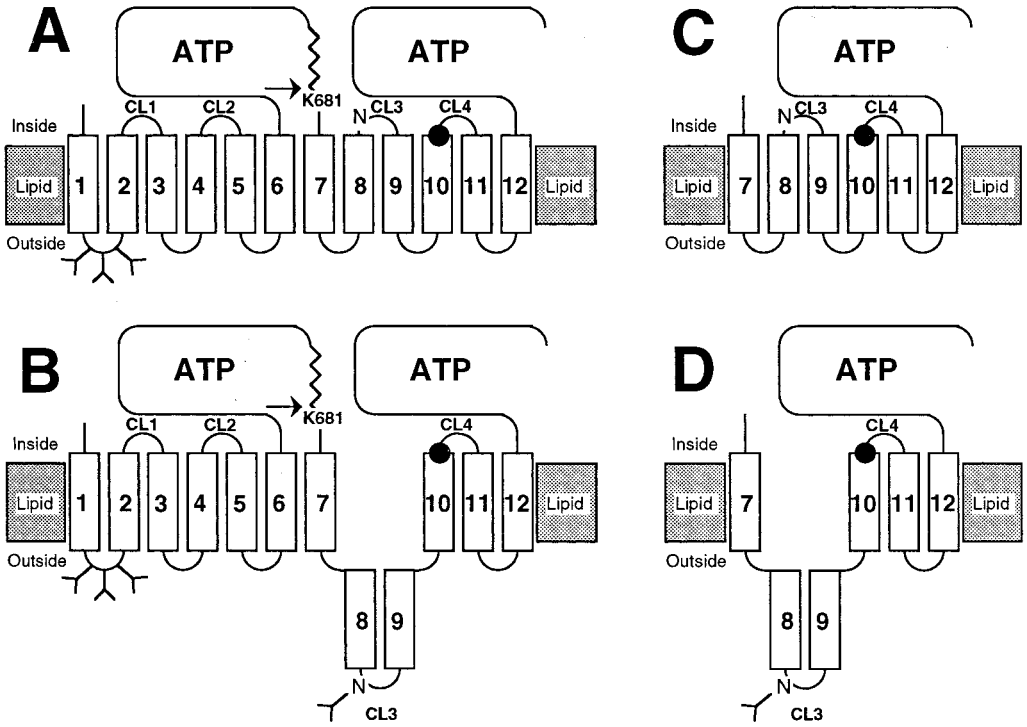

FIGURE 1: Topologies of P-gp. (A) The predicted CL-3(cyt) topology of human MDR1 P-gp. The 12 transmembrane segments are represented as rectangles. The two ATP-binding domains are located on the cytoplasmic side of the membrane whereas the glycosylated sites (Y) are on the extracellular side of the membrane. The location of the only consensus glycosylation in the COOH-terminal half of the molecule (N) is indicated. (B) P-gp with a CL-3(ext) alternative topology. The main structural differences are that the (N-T-T at position N809) consensus glycosylation site would now be accessible for glycosylation and TM8 and TM9 are not in the membrane. This model is based on the results of Skach et al. (*10*). The zig-zag lines represent the linker regions, and the arrow shows where the molecule was divided in half for expression of half-molecule polypeptides. Mutation E875C is shown as a shaded circle in TM 10. (C) C-Half polypeptide with CL-3(cyt) topology. (D) C-Half polypeptide with CL-3(ext) topology. The C-Half polypeptide contains residues 681−1280 of P-gp. An initiating methionine preceded residue 681.

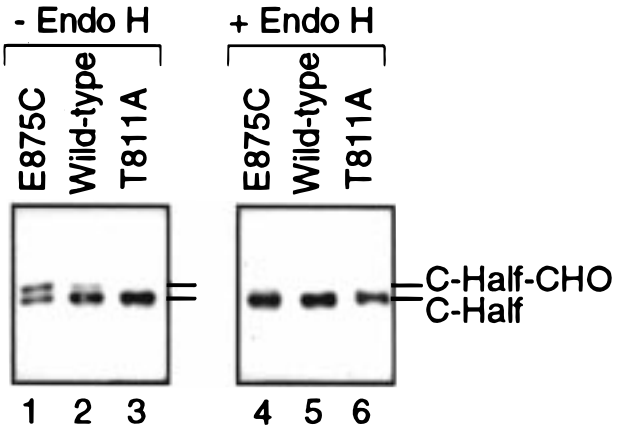

FIGURE 2: Detection of two orientations of the C-Half polypeptide. Whole cell extracts of cells expressing the A52-tagged wild-type C-Half, mutant E875C C-Half or mutant T811A C-Half P-gps were treated with (+Endo H) or without (−Endo H) endoglycosidase H and subjected to SDS−PAGE and immunoblot analysis with monoclonal antibody A52. C-Half and C-Half-CHO represent unglycosylated and glycosylated forms, respectively, of the C-Half molecule.

Half) while a minor amount contains carbohydrate that is sensitive to digestion by endoglycosidase H (lane 5; C-Half-CHO). The C-Half-CHO appears to be quite sensitive to proteolytic digestion. When cells expressing the C-Half molecule were solubilized with SDS sample buffer containing no protease inhibitors, the C-Half-CHO form of the molecule was not detected. In the presence of protease inhibitors, however, both C-Half and C-Half-CHO forms were detected. In a previous study (*17*), we could not detect the presence of C-Half-CHO because protease inhibitors were not included in the SDS sample buffer. Similarly, we have also observed that the biosynthetic intermediates of the cystic fibrosis transmembrane conductance regulator (CFTR) were also quite sensitive to proteolytic digestion after solubilization with SDS sample buffer containing no protease inhibitors (*23*). Addition of carbohydrate in the C-Half of P-gp occurs at the single consensus (N-T-T) glycosylation site (at position N809) between TM8 and TM9. Mutation of T811 within the consensus glycosylation sequence to alanine prevented glycosylation of the C-Half molecule (Figure 2, lane 3). Since N809 is within CL-3, this result is interpreted to mean that the wild-type C-Half-CHO represents the P-gp (CL3-ext) topology, while the unglycosylated C-Half has the P-gp (CL3-cyt) topology. The relatively low amount of the P-gp (CL3-ext) present makes it difficult to compare the properties of C-Half (CL3-cyt) and C-Half (CL3-ext). Therefore, it would be convenient if the amount of C-Half (CL3-ext) could be increased. During structure−function analysis studies (unpublished observations), we found a single mutant, E875C, that gave increased amounts of glycosylated C-Half polypeptide. This mutant is a useful tool for studying the interaction between the N-Half and the alternative topologies of the C-Half molecules. As shown in Figure 2 (lane 1), expression of the COOH-half of mutant E875C in HEK 293 cells resulted in the appearance of about equivalent amounts of the glycosylated (C-Half-CHO) and unglycosylated (C-Half) forms of the protein.

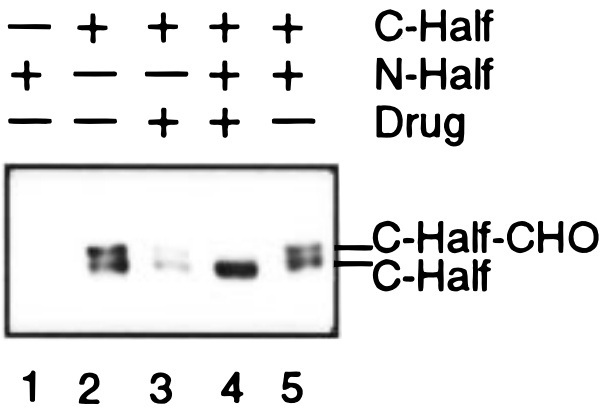

FIGURE 3: Effect of drug substrates or N-Half P-gp on Expression of C-Half. HEK 293 cells were transfected with A52-tagged mutant E875C C-Half cDNA with or without (+ or −) N-Half P-gp cDNA. The transfected cells were incubated for 24 h with (+drug) or without (−drug) 10 mM cyclosporin A. Whole cell extracts were then subjected to SDS−PAGE followed by immunoblot analysis with monoclonal antibody A52. A smaller amount of sample was loaded in lane 4 because of the higher expression levels of C-Half-A52 under these conditions.

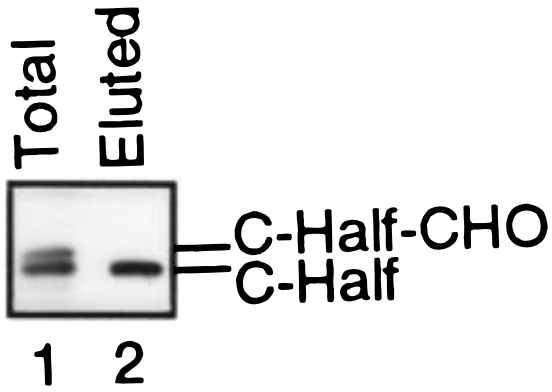

FIGURE 4: Copurification of C-Half-A52 with N-Half-His P-gp. HEK 293 cells were cotransfected with histidine-tagged N-Half and A52-tagged mutant E875C C-Half cDNAs, and solubilized with *n*-dodecyl-β-D-maltoside and the extract subjected to nickel-chelate chromatography using 10 mM imidazole in the washing steps. Samples of the solubilized extract (Total) and that eluted from the nickel column with 300 mM imidazole (Eluted) were subjected to SDS−PAGE analysis and immunoblot analysis with monoclonal antibody A52. The positions of the glycosylated (C-Half-CHO) or unglycosylated (C-Half) molecules are shown.

Interaction of the CL3-ext and CL3-cyt C−Half Molecules with Substrates and the N−Half Polypeptide. A question we wished to address was whether C-Half molecules with either topology could also interact with drug substrates. Since mutant E875C gave relatively equal amounts of C-Half molecules with both topologies, it was used for further analysis.

To test whether C-Half with either topology could interact with drug substrates, we used an in vivo drug binding assay. In this assay, the presence of drug substrates during biosynthesis promotes interactions between the N- and C-Half molecules of P-gp (*13*). This association can be easily monitored by adding a histidine tag to the N-Half molecule (N-Half-His) and assaying for association with an epitope tagged (A52) C-Half molecule (C-Half-A52), and nickel-chelate chromatography. Increased amounts of the C-Half-A52 are recovered by the nickel column when C-Half-A52 is coexpressed with N-Half-His in the presence of drug substrate (cyclosporin A). Cyclosporin A was used during biosynthesis because it has a relatively high affinity for P-gp (*6*).

We first tested the effect of the N-Half P-gp and drug substrates on the expression of the C-Half (CL3-cyt) and C-Half (CL3-ext). To monitor the expression of the C-Half polypeptide, an A52 epitope tag was attached to mutant E875C C-Half molecule, while the N-Half was not tagged. The untagged N-Half molecule of P-gp was coexpressed with the A52-tagged C-Half of mutant E875C in the presence or absence of drug substrate. Figure 3 shows the relative expression levels of glycosylated (C-Half-CHO) or unglycosylated (C-Half) forms of mutant E875C C-Half when expressed in the presence or absence of untagged N-Half molecule and drug substrate (cyclosporin A). When E875C C-Half was expressed alone, the CL3(cyt) and CL3(ext) topologies were present in about equivalent amounts (Figure 3, lane 2). Similar results were obtained when the C-Half of mutant E875C was expressed alone in the presence of drug substrate (lane 3) or when it was coexpressed with the N-Half in the absence of substrate (lane 5). By contrast, the unglycosylated C-Half polypeptide CL-3(cyt) was the major product when the C-Half was expressed in the presence of both drug and N-Half. The glycosylated C-Half (C-Half-CHO) was not detected (lane 4). These results show that accumulation of CL-3(cyt) was enhanced relative to CL-3(ext), when expression was carried out in the presence of cyclosporin A and the N-Half polypeptide. This suggests that the C-Half with the CL-3(cyt) topology is much more capable of interacting with the N-Half to form the drug-binding site(s).

We then tested whether the C-Half with the CL-3(cyt) topology preferentially interacts with the N-Half polypeptide. In this assay, a histidine-tagged N-Half molecule was coexpressed with an A52 epitope-tagged E875C C-Half. This enabled us to monitor the association between the two halves after nickel-chelate chromatography. If the interaction between the two halves is relatively strong, then the C-Half-A52 molecules should be retained by the nickel column in association with the N-Half-His, even in the presence of relatively high concentrations of salt (0.5 M NaCl) and detergent (1% dodecyl-β-D-maltoside) during nickel chelate chromatography. Histidine-tagged N-Half P-gp (N-Half-His) was coexpressed with the A52-tagged C-Half of mutant E875C (C-Half-A52) in HEK 293 cells, solubilized with n-dodecyl-β-D-maltoside, and the solubilized material was subjected to nickel chromatography (*18*). Figure 4 (lane 1) shows that both glycosylated (C-Half-CHO) and unglycosylated (C-Half) molecules were expressed and solubilized with detergent. Following nickel-chelate chromatography, however, only the unglycosylated form (C-Half) was eluted from the column. The glycosylated C-Half (C-Half-CHO) was not retained by the nickel column and was detected in the flow-through fractions (data not shown). These results suggest that only C-Half (CL3-cyt) forms strong associations with the N-Half polypeptide.

Drug-Stimulated ATPase Activity. To test whether the C-Half (CL3-cyt) that associated with N-Half-His formed an active complex, we assayed for drug stimulated ATPase activity. It has been demonstrated that there is a good correlation in the turnover numbers between transport of

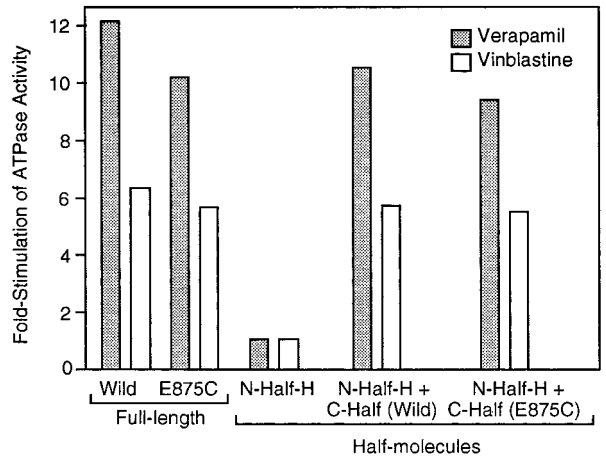

FIGURE 5: Drug-stimulated ATPase activities of wild-type and mutant E875C P-gps. P-gp cDNAs were expressed in HEK 293 cells in the presence of 10 μM cyclosporin A. Equivalent amounts of histidine-tagged full-length wild-type or mutant E875C P-gps isolated by nickel-chelate chromatography, were added to lipid and assayed for ATPase in the absence or presence verapamil (1 mM) or vinblastine (0.05 mM). Histidine-tagged N-Half (N-Half-H) that was expressed alone or coexpressed with A52 epitope-tagged wild-type or mutant E875C C-Half were isolated by nickel-chelate chromatography. Equivalent amounts of the histidine-tagged N-Half were also assayed for drug-stimulated ATPase activity after addition of lipid. *Fold-stimulation* indicates the ratio of activity with drug substrate to that found without substrate.

vinblastine and vinblastine stimulation of ATPase activity (*24*). Therefore, we tested for vinblastine-stimulated ATPase activity. Verapamil was also chosen as it is the most potent stimulator of human P-gp ATPase activity. Full-length P-gp showed 12.1- and 6.3-fold stimulation of ATPase activity in the presence of 1 mM verapamil and 0.05 mM vinblastine, respectively. The full-length mutant E875C P-gp also exhibited drug-stimulated ATPase activities that were about 80−90% of the wild-type enzyme (Figure 5). A similar pattern of drug-stimulated ATPase activity was observed with the half-molecules. The wild-type half-molecules showed 10.5- and 5.8-fold drug-stimulated ATPase activities in the presence of verapamil and vinblastine, respectively. Coexpression of wild-type N-Half-His with mutant E875C C-Half-A52 in the presence of cyclosporin A resulted in the formation of an active complex that had 85−95% of the activity of the wild-type enzyme (Figure 5). Isolated N-Half-His showed no drug-stimulated ATPase activity when expressed alone. These results confirm that the C-Half (CL3-cyt) forms a functional complex with the N-Half polypeptide.

DISCUSSION

The results in this study confirm that the COOH-terminal half of P-gp can exist in at least two different topologies when it is first synthesized in the endoplasmic reticulum (*10*, *14*, *16*, *22*). In wild-type P-gp, the C-Half with the CL-3(cyt) topology (unglycosylated form) is present in significantly higher levels than the CL-3(ext) topology (glycosylated form) when expression is carried out in HEK 293 cells. It is possible that the CL-3(cyt) topology of the C-Half is more energetically favored during the initial folding events or that the CL-3(ext) topology is more susceptible to proteolytic degradation. We found that increased levels of Cl-3(ext) were present when solubilization of the cells was carried out in the presence of protease inhibitors. In addition, we have noted that increased levels of CL-3(ext) were also present when expression was carried out in the presence of proteasome inhibitors such as lactacystin or MG-132 (unpublished observations). Therefore, the low level of expression would make it difficult for the CL-3(ext) topology to be detected even if this form of the protein was correctly transported to the cell surface of HEK 293 cells. It is also quite possible that the relative levels of the different topologies may be influenced by cell type. This may explain why Zhang et al. (*9*) could detect P-gps with altered topologies on the cell surface of Chinese hamster ovary cells or human SKOV cells while others could only detect P-gp with the conventional CL-3(cyt) topology at the cell surface (*14*). While it is clear that P-gps with the CL-3(cyt) and CL-3(ext) TM topologies exist, they appear to interact differently with the N-Half P-gp. This is supported by the finding that the C-Half with CL-3(ext) topology did not interact with the N-Half molecule when assayed by nickel-chelate chromatography (Figure 4). When the C-Half of mutant E875C was synthesized to the presence of the N-Half polypeptide and cyclosporin A, only the amount of unglycosylated C-Half was increased. This suggests that expression of C-Half(cyt) was enhanced by the presence of cyclosporin A and N-Half P-gp or that glycosylation of C-Half(ext) was suppressed. This latter explanation would be possible if the N-Half polypeptide interacted with the C-Half polypeptide before synthesis was complete. Similar results were obtained with other drug substrates such as capsaicin and verapamil (data not shown). There is convincing evidence to suggest that drug binding requires the participation of residues from both homologous halves of P-gp. For example, we showed that either half-molecule expressed alone will exhibit low levels of ATPase activity, but both half-molecules must be present to couple drug binding to stimulation of ATPase activity (*17*). We recently showed that P-gp "quarter-molecules" (transmembrane domains only) can mediate drug−protein interactions (*13*). The nucleotide binding domains are not required for drug binding, and each individual transmembrane domain does not bind drug substrate. When both transmembrane domains were coexpressed in the same cell however, the presence of drug substrates induces the two proteins to interact and adopt a protease-resistant conformation. Therefore, transmembrane domain interactions are critical for interactions between the two halves of P-gp as well as for drug binding. The results (Figure 4) suggest that CL-3(cyt) may be more efficient in forming these types of interactions than CL-3(ext).

It is surprising that a single mutation, E875C, promotes an alternative topology in P-gp. This change appears to affect folding rather than the activity of the enzyme. When expressed in the presence of drug substrates, the mutant E875C showed vinblastine- and verapamil-stimulated ATPase activity that was similar to that of wild-type enzyme (Figure 5). In the absence of drug substrate, maturation of mutant E875C was inefficient, such that the majority of the product was a core-glycosylated 150 kDa protein (data not shown). It has been proposed that the topology of a eukaryotic polytopic membrane-spanning protein is determined by the amino acid charge difference across the first TM segment (*25*). This Charge Difference Hypothesis was suggested from extensive studies done by von Heijne's group on the structural determinants for the membrane topology of proteins expressed in *Escherichia coli* (*26*, *27*). The

remaining TM segments were proposed to fold in the membrane in a passive and processive manner. Recent experiments (*28*), however, suggest that the topological determinants of eukaryotic membrane proteins are quite complex. It appears that sequences downstream of the first TM segment are also important in determining the local topology of the protein. Sato et al. (*28*) showed that the topology of the first TM segment of the Glut1 glucose transporter could be altered by changing the charge difference across the membrane while the topology of the rest of the molecule remained unchanged. This is consistent with the observation that there are many important factors that determine the insertion and orientation of a TM segment. These include factors such as the local net charge, hydrophobicity of a TM segment, and the folding of surrounding regions (*29*−*32*). In P-gp, it appears that the E875C mutation influences topological folding of the C-terminal domain such that increased amounts of CL-3(ext) are the major product. Skach et al. (*10*) showed that TM8 and TM9 in P-gp-CL-3(ext) reside outside the membrane (ER lumen). It appears that the ability of P-gp to adopt this alternative topology in hamster P-gp may due in part to the unique topogenic properties of TM8 (*33*). It is therefore possible that the E875C mutation promotes the formation of P-gp with this topology. The main difference between the two topologies is in the strength of the interactions between the two halves of the molecule. It is possible that TM8 and/or TM9 are important for the interaction between the two halves. In both P-gp (*13*) and the cystic fibrosis transmembrane conductance regulator (CFTR) (*34*), the data suggests that the two transmembrane domains mediate association between the halves of these proteins.

ACKNOWLEDGMENT

We are grateful to Dr. David H. MacLennan for the cDNA coding for the A52 epitope and monoclonal antibody and to Dr. Randal Kaufman (Boston) for pMT21.

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BI982525Y