Mutational Analysis of the Predicted First Transmembrane Segment of Each Homologous Half of Human P-glycoprotein Suggests That They Are Symmetrically Arranged in the Membrane*

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A recent study of P-glycoprotein membrane topology using phoA gene fusions provided evidence that the orientation of the first transmembrane segment of each homologous half of P-glycoprotein was different (Bejá, O., and Bibi, E. (1995) J. Biol. Chem. 270, 12351-12354). To test this hypothesis, we compared the functional consequences of mutations to residues in transmembrane segments (TM)s TM1 and TM7. Mutations to 3 residues occupying homologous positions in TM1 and TM7 resulted in mutant P-glycoproteins that were inactive. These mutations were found to be misprocessed. By contrast, mutations to other residues in TM1 resulted in functional P-glycoproteins. When putative TM1 was replaced with TM7 or TM7 with TM1 to yield TM7/TM7 or TM1/TM1 chimeras, both constructs yielded P-glycoproteins that were capable of conferring drug resistance in transfected cells. The purified P-glycoproteins from mutants TM7/TM7 and TM1/TM1 retained 59 and 28% of verapamil-stimulated ATPase activity, respectively. By contrast, interchanging TM6 and TM12 to yield TM6/TM12, or TM12/TM6 constructs resulted in P-glycoproteins that did not have any detectable ATPase activity and did not confer drug resistance in transfected cells. These results suggest that TM1 and TM7 likely have similar structural and functional roles in P-glycoprotein and that they have identical topologies.

P-glycoprotein is a 170-kDa plasma membrane glycoprotein. It may function as one of several mechanisms responsible for the phenomenon of MDR1 in mammalian cells (2-4). The protein confers resistance to a broad range of cytotoxic agents that do not have a common structure or intracellular target.

Human P-glycoprotein, encoded by the MDR1 gene, consists of 1280 amino acids organized in tandem repeats of 610 amino acids, joined by a linker region of 60 amino acids (5). Each repeat consists of an NH2-terminal hydrophobic domain containing six potential transmembrane sequences followed by a hydrophilic domain containing a nucleotide binding site. The amino acid sequence and domain organization of the protein are typical of the ATP-binding cassette superfamily of transporters (6).

Several lines of evidence suggest that P-glycoprotein interacts with substrates that have partitioned into the lipid bilayer (7, 8). Therefore, initial substrate interactions with P-glycoprotein likely occur at the hydrophobic domains. Accordingly, knowledge of the topology is essential for understanding the mechanism of drug-protein interactions.

Hydropathy analysis of the amino acid sequence of P-glycoprotein suggests a model containing twelve transmembrane segments (5, 9, 10). The NH2- and COOH-terminal halves of the molecule were predicted to be symmetrically arranged in the membrane. Bibi and Bejá (1, 11) have expressed fusion proteins of P-glycoprotein and alkaline phosphatase (phoA fusions) in Escherichia coli in an effort to determine the topology of P-glycoprotein. Their results suggested that the first predicted transmembrane segment of the COOH-terminal half of P-glycoprotein (TM7) was actually two transmembrane segments. By contrast, the first transmembrane segment of the NH2-terminal half of P-glycoprotein was shown to be as predicted by the model. Therefore, the results suggested that the two homologous halves of P-glycoprotein are asymmetrically arranged in the membrane.

In this study, we compared the effects of mutations to residues occupying homologous positions in predicted transmembrane segments TM1 and TM7. We also tested the effects of interchanging TM1 and TM7. We show that mutations to residues occupying homologous positions in predicted TM1 and TM7 had similar effects and that TM1 and TM7 were interchangeable. These results suggest that TM1 and TM7 are symmetrically arranged in the membrane.

EXPERIMENTAL PROCEDURES

Oligonucleotide-directed Mutagenesis—A full-length MDR1 cDNA that was modified to encode the epitope for monoclonal antibody AS2 (12) at the COOH-terminal end of the protein was inserted into the mammalian expression vector pMT21 as described previously (13). The sequence at the COOH terminus of P-glycoprotein that would normally end as TKRQ now became TKRA(His)10LDPR. The MDR1 cDNA was modified to encode 10 histidine residues at the COOH end of the protein. The sequence at the COOH terminus of P-glycoprotein that would normally end as TKRQ now became TKRA(His)10LDPR. Oligonucleotide-directed mutagenesis was carried out as described previously (13). For purification purposes, a full-length MDR1 cDNA was modified to encode for 10 histidine residues at the COOH end of the protein. The sequence at the COOH terminus of P-glycoprotein that would normally end as TKRQ now became TKRA(His)10LDPR.

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The abbreviations used are: MDR, multidrug resistance; TM, transmembrane segment.
CAINGGLQPAAFIE,… with TM1 resulted in the sequence 712-VGTLAAIIHGAGLPLMLAPF732.

For TM6 and TM12 chimeras, BstEI sites at the codons for residues Glu-331 (TM6) and Glu-972 (TM12) and NheI sites at residues Pro-350 (TM6) and Ser-992 (TM12) were created by site-directed mutagenesis. The BstEI to NheI fragments were then replaced with TM6 or TM12. Replacement of the sequence of putative TM6, 331-LTVFSSLIGAFSGQSAP531 with TM12 resulted in the sequence 331-VTLVFSAVVF-GAMAVQVAS351. Replacement of the sequence of putative TM12, 974-VLVFSAVVF-GAMAVQVSS994, with TM6 resulted in the sequence 974-TTVFSSLIGAFSVQGAS994.

Cell Culture—Procedures for transient transfection of human HEK 293 cells or stable transfection of mouse NIH 3T3 cells, followed by selection in the presence of vinblastine (5 nM) or colchicine (45 nM), have been described previously (13). For stable selection in the presence of drug substrates, the cDNA coding for the mutant P-glycoprotein and containing the epitope for monoclonal antibody A52 at the COOH-terminal end of the protein was used.

Purification of P-glycoprotein Mutants and Measurement of Mg2+-ATPase Activity—For purification of P-glycoprotein mutants, membranes were prepared from HEK 293 cells transfected with the cDNA coding for the histidine-tagged P-glycoprotein mutant, and the mutant P-glycoprotein purified by nickel-chelate chromatography (14). The purified P-glycoprotein was diluted with an equal volume of 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl2, and 10 mM ATP. The samples were incubated at 37°C for 30 min and the amount of inorganic phosphate liberated was determined by the method of Chifflet et al. (15).

Endoglycosidase H Digestion—P-glycoprotein—A52 cDNAs were expressed in HEK 293 cells. The transfected cells were then solubilized with buffer containing 50 mM sodium citrate, pH 5.5, 0.5% (w/v) SDS, 10 mM EDTA, and 1% (v/v) 2-mercaptoethanol. The samples were divided into two equal portions, which were then incubated either with (-) or without (+) endoglycosidase H (100 units, New England Biolabs, Inc.) for 15 min at room temperature. The digestion was stopped by addition of an equal volume of buffer containing 0.25 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, and 20% (v/v) glycerol. The reaction mixtures were subjected to SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose, and developed with monoclonal antibody A52 and enhanced chemiluminescence (Amersham Corp.) as described previously (16).

RESULTS

Mutational Analysis of TM1—The residues predicted in TM1 and TM7 are shown in Fig. 1. The topology is based on the hydropathy plots of the primary structure of P-glycoprotein (5, 9, 10) and is consistent with the observation that the extracellular loop between TM1 and TM2 contains the glycosylation sites. Nine of the 21 residues in TM1 are identical to the residues in TM7, and these residues occupy homologous positions in each transmembrane segment as predicted by hydropathy profiles. Recently, Béja and Bibi (1) used phoA gene fusions of P-glycoprotein to show that the 21 residues of TM7 likely consist of two transmembrane segments (TM7b). To distinguish between the two models of TM7, mutational analysis was carried out on the residues predicted in TM1. The rationale was that mutation of residues occupying homologous positions in both transmembrane segments should have similar effects if model TM7a is correct. If the effects are different, then the results would support model TM7b. Accordingly, site-directed mutagenesis was used to change each residue (except for Ala, Ile, and Gly) of TM1 to Ala. Alas, Ile, and Gly were changed to Leu, Ser, and Val, respectively, to be consistent with our previous studies (13, 16–18). The mutant cDNAs were then transfected into NIH 3T3 cells and selected for drug-resistant colonies in the presence of colchicine (45 nM) or vinblastine (5 nM). Mutations were classified as having no effect when drug-resistant colonies were obtained in the presence of either drug.

Mutants that did not yield any drug-resistant colonies were considered to be inactive. We found that changes to 3 residues in TM1 (Gly-54 → Val, Ala-58 → Leu and Gly-62 → Val) resulted in mutant P-glycoproteins that were inactive (Fig. 1). To test whether these mutations interfered with processing of P-glycoprotein, their cDNAs were transiently expressed in HEK 293 cells and whole cell extracts were subjected to immunoblot analysis with monoclonal antibody A52. Expression in HEK 293 cells allows for rapid expression and assessment of the structural integrity of the mutant proteins. As shown in Fig. 2, the major product of these inactive mutants was a protein with an apparent mass of 150 kDa. By contrast, expression of the cDNAs coding for wild-type or the remaining mutants yielded a major product with an apparent mass of 170 kDa. The 150-kDa proteins were sensitive to digestion with endoglycosidase H (Fig. 2), suggesting that these products are core-glycosylated intermediates that are in the endoplasmic reticulum.
Each clone were analyzed by immunoblotting with monoclonal antibody A52 to identify clones expressing mutant P-glycoprotein-A52. As shown in Fig. 3, stable cell lines expressing wild-type enzyme contain the 170-kDa protein as the major product, whereas cells expressing the mutants (Gly-54 → Val, Ala-58 → Leu and Gly-62 → Val) contained the 150-kDa (core-glycosylated) protein as the major product. These stable cell lines were then incubated at 26 °C for 48 h, and whole cell extracts were subjected to immunoblot analysis. As shown in Fig. 3, all three mutants were temperature sensitive. At a lower growth temperature, each mutant yielded mature P-glycoprotein (170 kDa) as the major product.

These results are similar to the mutational analysis of TM7 (19). Mutation of residues occupying homologous positions in TM7 (Gly-714, Ala-718, and Gly-722) also resulted in mutant P-glycoproteins that were misprocessed and temperature sensitive. These results suggest that residues in TM1 and TM7 play similar roles and support model TM7α. TM7, however, is sensitive to change at this position in TM7. This mutation seems to have only a moderate effect on folding, however, because processing to the fully mature form of the enzyme could be restored when the cells were incubated at 26 °C (19).

All of the deleterious mutations in TM1 and TM7 caused misprocessing of P-glycoprotein. It is not known, however, if the misprocessed mutants are functional. One approach to address this question is to purify the mutant P-glycoproteins for measurement of ATPase activity. Accordingly, we attached 10 tandem histidine residues at the COOH end of P-glycoprotein (14) to facilitate purification of the mutant P-glycoprotein by nickel-chelate chromatography. Although this approach worked well for wild-type enzyme, we were unable to purify any P-glycoprotein products for mutants Gly-54 → Val, Ala-58 → Leu or Gly-62 → Val (data not shown). When the different fractions of the nickel-chelate chromatography were analyzed by immunoblot analysis, however, it became apparent that the mutant proteins were not retained by the resin. Analysis of the nickel chromatography fractions of wild-type and mutant Gly-54 → Val are shown in Fig. 4. The majority of the wild-type P-glycoprotein-(His)10 is retained by the column and is recovered by elution with 300 mM imidazole. By contrast, the P-glycoprotein of mutant Gly-54 → Val is not retained by the resin and is detected in the flow-through fraction. Similar results were obtained with mutants Ala-54 → Leu and Gly-62 → Val (data not shown). These results suggest that mutations to Gly-54, Ala-58, and Gly-62 result in misfolding of the enzyme such that the histidine tag is inaccessible for binding to the nickel column. Therefore, the presence of a histidine tag at the COOH terminus of P-glycoprotein provides a sensitive assay for detecting whether a mutation causes misfolding of the protein and supports the observation that misprocessed P-glycoproteins are likely to be misfolded.

Construction and Expression of Internal Chimeras of P-glycoprotein—The results of mutational analyses of residues of TM1 and TM7 suggest that both transmembrane segments likely play similar structural roles in the homologous halves of P-glycoprotein and may be interchangeable. To test this possibility, mutant P-glycoproteins were constructed in which the predicted TM1 and TM7 segments were changed to yield TM1/TM1, TM7/TM7, or TM7/TM1 chimeric P-glycoproteins. For comparison, we also interchanged the predicted transmembrane segments TM6 and TM12 to yield the chimeras TM6/TM6, TM12/TM12, or TM12/TM6. Genetic (16, 17) and biochemical (20, 21) analyses have provided evidence that TM6 and TM12 play important roles in P-glycoprotein-substrate interactions.
The cDNAs coding for the chimeric P-glycoproteins were transfected into NIH 3T3 cells and selected for vinblastine-resistant colonies. Vinblastine-resistant colonies were obtained for wild-type P-glycoprotein (TM1/TM7) and mutant (TM7/TM7). It is possible that direct selection may not have allowed the detection of residual activity that might have been detected under less stringent conditions. Therefore, we used an indirect selection method by cotransfecting NIH 3T3 cells with the cDNAs coding for the mutant chimeras together with pWL-neo (Statagene) and selected for G418-resistant clones. Whole cell extracts of individual clones were subjected to immunoblot analysis with monoclonal antibody A52 as described under “Experimental Procedures.”

Chimera (TM7/TM7) had 59, 58, and 70% of the maximal wild-type P-glycoprotein ATPase activity in the presence of verapamil, vinblastine, and colchicine, respectively. Similarly, mutant (TM1/TM1) had 28, 36, and 37% of the maximal wild-type activity, whereas mutants (TM6/TM6), (TM12/TM12), or (TM12/TM6) had little detectable drug-stimulated ATPase activity (<10% of wild type). These results are consistent with the ability of the mutants to confer drug resistance in transfected cells. Measurement of drug-stimulated ATPase activity appears to be a very sensitive assay for detecting subtle changes in the function of a mutant P-glycoprotein. This is seen in mutant TM7/TM7 that exhibited only 58% of wild-type vinblastine-stimulated ATPase activity, but both mutant TM7/TM7 and wild-type P-glycoprotein yielded a similar number of vinblastine-resistant colonies. Drug-resistant colonies could be obtained after transfection of NIH 3T3 cells with mutants (TM7/TM7) and (TM1/TM1) but not with mutants (TM7/TM1), (TM6/TM6), (TM12/TM12), or (TM12/TM6). These results suggest that, to some extent, TM7 and TM1 are interchangeable. Replacement of TM1 with TM7 results in a mutant P-glycoprotein that retains more than 50% of the wild-type functional activity but appears to mature faster than mutant TM7/TM7 that exhibited only 58% of wild-type ATPase activity (Fig. 7).

DISCUSSION

Knowledge of the topology of P-glycoprotein is essential for understanding the mechanism of drug transport because the
predicted transmembrane domains appear to be involved in drug-protein interactions. In the predicted secondary structure of P-glycoprotein, each half of the molecule contains six transmembrane segments that are symmetrically arranged in the membrane. Several different approaches have been used to test this hypothesis. Béja and Bibi (1) have expressed mouse P-glycoprotein-alkaline phosphatase fusion proteins in E. coli to show that TM1 and TM7 are asymmetrically arranged in the membrane. These authors (11) have also shown that putative TM4 may be located on the extracellular surface. Another approach that favored the asymmetric topology for each homologous half of P-glycoprotein has been the expression of P-glycoprotein prolactin chimeras in Xenopus oocytes. Using this approach, Skach and Lingappa (23) reported that the topologies of TM1-TM2 and TM7-TM8 were asymmetric. They also reported that the COOH half of the molecule spans the membrane only four rather than the predicted six times (24). Finally, multiple topologies for P-glycoprotein have been reported in studies using cell-free translation systems (25–27).

In contrast to the above findings, the results of other studies support the predicted model for P-glycoprotein. In a previous study, we constructed a Cys-less human P-glycoprotein to study its topology (28). Cysteine residues were then introduced into predicted extracellular or cytoplasmic loops of Cys-less P-glycoprotein, and the topology of the protein was determined by using membrane-permeant and impermeant thiol-specific reagents. The results obtained were consistent with the predicted structural model. Kast et al. (29) also studied the topology of the NH₂-terminal half of murine mdr3 by epitope insertion. Their results were also consistent with the predicted model. In addition, two monoclonal antibodies that react with the external domain of P-glycoprotein (MRK-16 (30) and MM4.17 (31)) map to a stretch of amino acids between TM7 and TM8. These results support the symmetrical model based on hydrophathy analysis.

Comparison of the effects of mutations in each half of P-glycoprotein also supports a symmetric organization. Changes in substrate specificity were observed when mutations were made to residues occupying homologous positions in each half of P-glycoprotein. Examples include Pro-233 in TM4 and Pro-866 in TM10 (17), Phe-335 in TM6 and Phe-978 in TM12 (17). In the present study, mutations to residues occupying homologous positions in predicted TM1 and TM7 also affected processing of P-glycoprotein. In addition, we found that a fully mature and functional P-glycoprotein could be synthesized even when the two transmembrane segments were interchanged to yield TM7/TM7 and TM1/TM1 chimeras.

It is possible that the reason for the differences in the topology in the region of TM7 is that Béja and Bibi (1) used mouse P-glycoprotein, whereas the present study is with human P-glycoprotein. As shown in Fig. 8, there is only 67% amino acid identity (28 of the 42 residues) in the region of TM7 between mouse and human P-glycoprotein.

All of the studies that have provided evidence in support of a symmetric arrangement of the two halves of P-glycoprotein have involved the use of functional molecules that have been expressed in intact cells. By contrast, the studies that support an asymmetric arrangement of P-glycoprotein have used truncated molecules of P-glycoprotein that, in some cases, have been fused to large reporter molecules. Addition of large passenger molecules and the use of truncated P-glycoprotein could lead to misfolding of P-glycoprotein. Folding of P-glycoprotein...
appears to be very sensitive to even small changes in the molecule. We have observed that biosynthesis and maturation of P-glycoprotein can be particularly sensitive to even conserved single amino acid changes in the transmembrane segments (this study) or in the cytoplasmic loops (18). These mutant P-glycoproteins are likely misfolded (this study) and are retained in the endoplasmic reticulum by chaperones such as calnexin (19). Recently, it has been shown that some integral membrane proteins, such as the CH1P 28 water channel (32), are synthesized with a topology that is different from the final mature form of the protein (“topological maturation”). A similar process may occur in P-glycoprotein.

In this study, we found that mutants such as Gly-54 → Val, Ala-58 → Leu and Gly-62 → Val that are only core glycosylated (150 kDa) are indeed misfolded, because they could not be purified by nickel-chelate chromatography. This result suggests that the histidine tag at the COOH-end of the molecule was masked as a result of the protein being misfolded. By contrast, the core-glycosylated (150-kDa) form of histidine-tagged P-glycoprotein of mutants (TM1/TM1) (this study) and Cys-less P-glycoprotein (14) that are eventually processed to the fully mature (170-kDa) form are readily purified by nickel chromatography. These results suggest that the use of a histidine-tagged P-glycoprotein provides another sensitive method for detecting proteins that are indeed misfolded and can be used to differentiate core-glycosylated proteins that are misfolded from those that are processed more slowly to the mature form.

We observed that the various chimeras exhibited less activity than did wild-type enzyme. This was not surprising in the cases in which TM6 and TM12 were interchanged. Evidence from mutational studies indicates that both of these transmembrane segments may play a role in drug transport, but transmembrane segments TM5-TM6 and TM11-TM12 may be absolutely essential for modulating substrate specificity. It is important for overall activity, whereas TM5-TM6 and TM11-TM12 are critical for ion selectivity (34).

In summary, the results of this study further support the predicted model of P-glycoprotein, in which the two halves are symmetrically arranged in the membrane. Similar conclusions have been reported for cystic fibrosis transmembrane conductance regulator in which its topology was studied by insertion of consensus glycosylation sites at various positions of the molecule (35).

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