Inhibition of Oxidative Cross-linking between Engineered Cysteine Residues at Positions 332 in Predicted Transmembrane Segments (TM) 6 and 975 in Predicted TM12 of Human P-glycoprotein by Drug Substrates*

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Each homologous half of P-glycoprotein consists of a transmembrane domain with six potential transmembrane segments and an ATP-binding domain. Labeling studies with photoactive drug analogs show that labeling occurs within or close to predicted transmembrane segments (TM) 6 (residues 331–351) and TM12 (residues 974–994). To test if these segments are in near-proximity we generated 42 different P-glycoprotein mutants in which we re-introduced a pair of cysteine residues into a Cys-less P-glycoprotein, one within TM6 (residues 332–338) and one within TM12 (residues 975–980) and assayed for cross-linking between the cysteines. All the mutants retained verapamil-stimulated ATPase activity. We found that only the mutant containing Cys-332 and Cys-975 was cross-linked in the presence of oxidant as judged by its decreased mobility on SDS gels. Similar results were obtained when the same mutations were introduced into Cys-less NH2-terminal and COOH-terminal half-molecules of P-glycoprotein followed by coexpression and treatment with oxidant. Cross-linking between Cys-332 and Cys-975, however, was inhibited by verapamil or vinblastine but not by colchicine. These results suggest that residues Cys-332 and Cys-975, which occupy equivalent positions when TM6 and TM12 are aligned, are close to each other in the tertiary structure of P-glycoprotein.

P-glycoprotein, also known as the multidrug resistance protein (product of the human MDR1 gene), is a plasma membrane glycoprotein that is involved in transporting a broad range of cytotoxic drugs from cells (1–3). It may be one of the mechanisms responsible for failure of cancer chemotherapy (4). Studies with knock-out mice show that the protein plays a major role in protecting the animal against various xenotoxins of natural origin (5, 6).

The amino acid sequence deduced from the nucleotide sequence of human MDR1 (7) indicates that each homologous half of P-glycoprotein is composed of six putative transmembrane sequences and a nucleotide-binding site. This arrangement is common to all members of the ABC (ATP-binding cassette) superfamily of transport proteins (8, 9).

The predicted transmembrane regions of P-glycoprotein appear to be particularly important for function since the enzyme appears to interact with substrates that are embedded in the lipid bilayer (10, 11). Labeling studies using photolabeled analogs of drug substrates (12–17), such as [3H]azidopine (12, 14, 16), [125I]iodoarylazidoprazosin (15, 16), and 6-O-[(2-3(4-azido-3-)] iodophenyl)propionamido[ethylforskolin (16), suggested that the labeling sites are closely associated with TM6 and TM12. These photolabeling studies suggest that there may be two distinct drug-labeling sites or that the two labeled segments are part of a single drug-labeling site. Therefore, knowledge about the three-dimensional arrangement of the transmembrane segments is crucial for understanding how P-glycoprotein functions. Such information has not been available due to technical difficulties in crystallizing the protein for use in x-ray diffraction studies. Several elegant approaches to studying helix packing such as site-directed excimer fluorescence (18) or designed metal ion-binding sites and site-directed spin labeling (19) have been developed for the lactose permease of Escherichia coli. These approaches have provided important insight about the protein. Unfortunately, these approaches require relatively large amounts of highly purified protein that is difficult to achieve with most eukaryotic polytopic membrane proteins.

In this study, we have attempted to determine the proximity of residues in predicted TM6 and TM12 of P-glycoprotein by cross-linking cysteine residues that have been placed in different positions of the molecule. We re-introduced pairs of cysteine residues into a Cys-less mutant of P-glycoprotein, one in predicted TM6 and one in predicted TM12. The mutants were expressed in HEK 293 cells, treated with oxidant, and the cross-linked protein detected by changes in electrophoretic mobility on SDS gels. We found that Cys-332 and Cys-975 were cross-linked in the presence of oxidant, and cross-linking was inhibited by some drug substrates. These results suggest that these residues are close to each other in the three-dimensional structure of P-glycoprotein.

MATERIALS AND METHODS

Construction of Mutants—A full-length MDR1 cDNA, modified to encode the epitope for monoclonal antibody A52 (20) at the COOH-terminal end of the protein, was inserted into the mammalian expression vector pMT21, as described previously (21). The sequence at the COOH terminus of P-glycoprotein that would normally end as TKRQ, now became TKRAISLISNSCSPEFDDDLPAGEQREACHRGRDPQ. Oligonucleotide-directed mutagenesis was carried out as described previously (21). The construction of a Cys-less mutant of P-glycoprotein-A52, in which the codons for cysteine residues were simultaneously mutated to alanine, was described previously (22). For purification purposes the A52 epitope was replaced with a histidine tag as described previously.

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(23). The cDNAs coding for either the NH$_2$-terminal or COOH-terminal half-molecules of P-glycoprotein and containing the epitope for monoclonal antibody A52 were generated as described previously (24). Cys-less NH$_2$- and COOH-terminal half-molecules were created by mutating all the codons for cysteine to alanine. Purification of P-glycoprotein Mutants and Measurement of Verapamil-stimulated ATPase Activity—Purification of P-glycoprotein (His)$_6$ was carried out as described previously (23). Briefly, 20 (10 cm diameter) culture dishes of subconfluent HEK 293 cells were transfected with mutant MDR1 cDNA, and membranes were prepared 40 h later as described previously (23). The membranes were solubilized with 1% (w/v) n-dodecyl-β-D-maltoside (Sigma) and P-glycoprotein (His)$_6$ isolated by nickel-chelate chromatography using nickel spin columns (Ni-NTA, Qiagen).

To measure ATPase activity, purified P-glycoprotein was diluted with an equal volume of 100 mg/ml crude sheep brain phosphatidylethanolamine (Sigma, Type IIIs commercial grade), which had been previously washed with Tris-buffered saline to remove traces of phosphate, and then sonicated. 100 ng of purified P-glycoprotein was incubated with 1 mM verapamil, and ATPase activity was initiated by the addition of an equal volume of buffer containing 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl$_2$, and 10 mM ATP. The samples were incubated at 37 °C for 40 min, and the amount of inorganic phosphate liberated was determined by the method of Chifflet et al. (25).

**RESULTS**

Mutagenesis and ATPase Activity of Mutant P-glycoproteins—Each homologous half of P-glycoprotein consists of a transmembrane domain with six predicted transmembrane segments and an ATP-binding domain (Fig. 1A). Predicted TM6 and TM12 are also directly connected to the nucleotide-binding domains. There is a relatively high degree of homology between TM6 and TM12 as 11 of 21 residues are identical (Fig. 1B). We have previously shown that mutation of either Phe-335 → Ala or Phe-978 → Ala, which occurs at homologous positions when TM6 and TM12 are aligned (Fig. 1B), profoundly altered the drug resistance profiles of the mutant proteins (28) and their ATPase activity (23). Five of the mutants, Cys-335/Cys-975, Cys-335/Cys-977, Cys-334/Cys-978, and Cys-337/Cys-978, had greater than 50% of wild-type ATPase activity (23).

These results suggested that Phe-335 and Phe-978 and/or surrounding residues were of particular importance and may be close enough to each other, such that they could be cross-linked if they were changed to cysteine.

Accordingly, site-directed mutagenesis was used to change the codon for each residue surrounding Phe-335 and Phe-978 to cysteine (Fig. 1B). A series of mutant P-glycoproteins was then generated in which we re-introduced a pair of cysteine residues, one in predicted TM6 (residues 332–338) and the other in predicted TM12 (residues 975–980) in a functional Cys-less P-glycoprotein (23). It was important to determine whether the mutant proteins retained drug-stimulated ATPase activity since cross-linking of an inactive P-glycoprotein may not provide useful information. Therefore, the cDNAs of these mutant P-glycoproteins were also modified to contain a histidine tag at the COOH end of the molecule to facilitate purification by nickel-chelate chromatography (23). The mutant proteins were expressed in HEK 293 cells and purified. All the mutants yielded the mature 170-kDa form of P-glycoprotein together with varying amounts of 150-kDa immature form of the enzyme (data not shown) The varying amounts of immature P-glycoprotein is a consistent feature of transient expression in either COS-1 or HEK 293 cells. We have previously shown that the Cys-less mutant matures more slowly than wild-type enzyme (22) and that a significant amount of the product isolated by nickel-chelate chromatography consists of the immature form (150 kDa) of the enzyme (23). The immature form of the enzyme is sensitive to digestion with endoglycosidase H, suggesting that it is the core-glycosylated form of the enzyme and likely resides in the endoplasmic reticulum (23). Therefore, none of the mutations prevented maturation of the enzyme. The purified mutants were then reconstituted with phosphatidylethanolamine and assayed for ATPase activity in the presence of 1 mM verapamil. At this saturating concentration of verapamil, Cys-less P-glycoprotein exhibited an ATPase activity of 0.74 μmol/min/mg enzyme. The relative activities of the 42 mutants are shown in Table I. The majority of mutants, except for mutants Cys-332/Cys-976, Cys-333/Cys-978, Cys-334/Cys-978, and Cys-337/Cys-978, had greater than 50% of the ATPase activity of Cys-less P-glycoprotein. Mutants Cys-332/Cys-976, Cys-333/Cys-978, Cys-334/Cys-978, and Cys-337/Cys-978 had 47, 29, 24, and 30% of Cys-less P-glycoprotein ATPase activity, respectively. The three mutants with the lowest activities all included a change of Phe-978 → Cys. This is consistent with the previous observation that mutant Phe-978 → Ala had significantly reduced verapamil-stimulated ATPase activity (23).

Five of the mutants, Cys-335/Cys-975, Cys-335/Cys-976, Cys-335/Cys-978, Cys-335/Cys-977, and Cys-335/Cys-980, had 2–3-fold higher ATPase activities in the presence of verapamil compared with Cys-less P-glycoprotein. All of these

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; TM, transmembrane segment(s); PBS, phosphate-buffered saline.

2 T. W. Loo and D. M. Clarke, unpublished observations.
Verapamil-stimulated ATPase activities of double Cys mutants relative to Cys-less P-glycoprotein

| Mutation in TM6 | Mutation in TM12 | Relative ATPase activitya |
|-----------------|------------------|--------------------------|
| Leu-332 → Cys   | Leu-975 → Cys    | 103                      |
|                 | Leu-976 → Cys    | 47                       |
|                 | Val-977 → Cys    | 78                       |
|                 | Phe-978 → Cys    | 88                       |
|                 | Ser-979 → Cys    | 105                      |
|                 | Ala-980 → Cys    | 95                       |
| Thr-333 → Cys   | Leu-975 → Cys    | 74                       |
|                 | Leu-976 → Cys    | 52                       |
|                 | Val-977 → Cys    | 60                       |
|                 | Phe-978 → Cys    | 29                       |
|                 | Ser-979 → Cys    | 109                      |
|                 | Ala-980 → Cys    | 95                       |
| Val-334 → Cys   | Leu-975 → Cys    | 100                      |
|                 | Leu-976 → Cys    | 87                       |
|                 | Val-977 → Cys    | 85                       |
|                 | Phe-978 → Cys    | 24                       |
|                 | Ser-979 → Cys    | 88                       |
|                 | Ala-980 → Cys    | 97                       |
| Phe-335 → Cys   | Leu-975 → Cys    | 292                      |
|                 | Leu-976 → Cys    | 241                      |
|                 | Val-977 → Cys    | 218                      |
|                 | Phe-978 → Cys    | 115                      |
|                 | Ser-979 → Cys    | 246                      |
|                 | Ala-980 → Cys    | 229                      |
| Phe-336 → Cys   | Leu-975 → Cys    | 90                       |
|                 | Leu-976 → Cys    | 83                       |
|                 | Val-977 → Cys    | 74                       |
|                 | Phe-978 → Cys    | 73                       |
|                 | Ser-979 → Cys    | 86                       |
|                 | Ala-980 → Cys    | 107                      |
| Ser-337 → Cys   | Leu-975 → Cys    | 76                       |
|                 | Leu-976 → Cys    | 87                       |
|                 | Val-977 → Cys    | 71                       |
|                 | Phe-978 → Cys    | 30                       |
|                 | Ser-979 → Cys    | 114                      |
|                 | Ala-980 → Cys    | 79                       |
| Val-338 → Cys   | Leu-975 → Cys    | 89                       |
|                 | Leu-976 → Cys    | 112                      |
|                 | Phe-978 → Cys    | 76                       |
|                 | Ser-979 → Cys    | 100                      |
|                 | Ala-980 → Cys    | 92                       |

*a* The P-glycoprotein(His)_{10} mutants were purified by nickel-chelate chromatography, reconstituted with lipid, and ATPase activity measured in the presence of saturating levels of verapamil (1 mM). Each mutant was expressed, purified, and assayed for ATPase activity twice.

mutants included a change of Phe-335 → Cys. This is also consistent with our previous finding that mutant Phe-335 → Ala exhibits increased ATPase activity in the presence of verapamil compared with wild-type enzyme (23).

**Oxidative Cross-linking of Full-length Molecules**—If a pair of cysteine residues is in near-proximity then oxidation would result in the formation of a disulfide bond. A cross-link between a residue within predicted TM6 and a residue in predicted TM12 could result in a protein with altered mobility on SDS gels compared with that of the uncross-linked species. This would serve as a rapid and simple assay to detect for the presence of cross-linking. Accordingly, we expressed all the mutants in HEK 293 cells and then subjected whole cell samples to oxidation with Cu^{2+}(phenanthroline)_{3}. The samples were analyzed by SDS-PAGE without reducing agent, and P-glycoprotein was detected by immunoblotting with a rabbit polyclonal antibody to P-glycoprotein (27). As shown in Fig. 2A, only mutant Cys-332/Cys-975 yielded an immunoreactive product that migrated more slowly on SDS gels after treatment with oxidant. By contrast, when a mutant P-glycoprotein containing only Cys-332 or Cys-975 was treated with oxidant, there was no shift in electrophoretic mobility (Fig. 2B). Simi-}

![Fig. 2. Electrophoretic mobility of full-length P-glycoprotein mutants after oxidative cross-linking.](image-url)
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A52 epitope tag. These results confirm that restoration of drug-stimulatable ATPase activity is due to physical association between the two half-molecules. Therefore, the two half-molecules appear to associate in a manner similar to the full-length molecule and are therefore ideally suited for cross-linking studies.

Accordingly, we first constructed Cys-less versions of the half-molecules and attached A52 epitope tags at the COOH end of each molecule. We have previously found that the presence of the epitope tags did not interfere with the interactions between the two halves (24). We were unable to purify enough of the histidine-tagged half-molecules by nickel-chelate chromatography for measurement of drug-stimulated ATPase activity. We could, however, detect expression of the half-molecules in HEK 293 cells by immunoblot analysis. A series of Cys-less NH2-terminal half-molecules was then constructed that contained a single cysteine at positions 332–338 of predicted TM6. A similar series of Cys-less COOH-terminal half-molecules was also constructed in which a single cysteine residue was introduced at one of positions 975–980 of predicted TM12. The cDNAs coding for a Cys-less NH2-terminal half-molecule and a Cys-less COOH-terminal half-molecule were co-expressed in HEK 293 cells and, as shown in Fig. 3A, treatment of membranes containing the mutation Leu-332 → Cys in the Cys-less NH2-terminal half-molecule and the mutation Leu-975 → Cys in the Cys-less COOH-terminal half-molecule with oxidant at 37 °C resulted in the time-dependent appearance of an immunoreactive product of apparent mass 160 kDa and the comcomitant disappearance of the half-molecule products. After 16 min, the majority of the immunoreactive material appeared as the 160-kDa product. By contrast, oxidations of membranes containing other combinations of cysteine residues in the half-molecules did not result in the appearance of the 160-kDa product and were similar to those shown for Cys-335 in Cys-less NH2-terminal half-molecule and Cys-975 in Cys-less COOH-terminal half-molecule as shown in Fig. 3A. Similarly, no cross-linking was detected when Cys-332 in Cys-less NH2-terminal half-molecule was coexpressed with the Cys-less COOH-terminal half-molecule or when Cys-975 in Cys-less COOH-terminal half-molecule was coexpressed with the Cys-less NH2-terminal half-molecule (Fig. 3B). Therefore, these results indicate that Cys-332 and Cys-975 can form a disulfide cross-link, even when expressed on separate polypeptides of P-glycoprotein.

To determine if cross-linking between the transmembrane segments was specific, we tested for possible interactions between TM6 and TM11. Each residue at equivalent positions in predicted TM6 (residues Leu-332–Val-338) and TM11 (residues Met-949–Cys-956) was systematically changed to cysteine for measurement of drug-stimulated ATPase activity (30–33). This suggests that drug binding leads to a conformational change in the protein. To test whether drug substrates affect cross-linking between TM6 and TM12, the 42 different double Cys mutants were incubated with verapamil, vinblastine, or colchicine at room temperature for 10 min and then treated with oxidant. None of the 56 double Cys mutants yielded detectable cross-linked product using either full-length molecules or half-molecule forms of the enzyme (data not shown). These results suggested that cross-linking between Cys-332 and Cys-975 was specific.

Effect of Drug Substrates on Cross-linking—Drug substrates such as verapamil, vinblastine, or colchicine stimulate ATPase activity (30–33). This suggests that drug binding leads to a conformational change in the protein. To test whether drug substrates affect cross-linking between TM6 and TM12, the 42 different double Cys mutants were incubated with verapamil, vinblastine, or colchicine before treatment with oxidant. Except for mutant Cys-332/Cys-975, the other cysteine mutants did not show any change in the pattern of cross-linking in the presence of verapamil, vinblastine, or colchicine (data not shown). The presence of verapamil or vinblastine affected cross-linking between Cys-332 and Cys-975 (Fig. 4). The amount of cross-linked species decreased with increasing levels of either verapamil or vinblastine. Inhibition of cross-linking was maximal in the presence of 1 μM verapamil or 100 μM vinblastine. These concentrations of drug substrates correspond to the levels required for maximal drug-stimulated ATPase activity. There was, however, little inhibition of cross-linking in the presence of 5 mM colchicine, the concentration at which maximal colchicine-stimulated ATPase activity occurs. It was possible that the mutant did not interact with colchicine. To test for this possibility, the cDNAs coding for Cys-less P-glycoprotein and mutant L332C + L975C were transfected into NIH 3T3 cells, and drug-resistant colonies were selected in the presence of 45 nM colchicine or 5 nM vinblastine. We found that both mutants yielded a similar number of vinblastine- and colchicine-resistant colonies (data not shown), suggesting that both mutants were still functional. We have found that determination of the drug resistance profile of a mutant may not be as sensitive a measure of drug-stimulated ATPase activity for detecting subtle changes in the mutant protein (43). This may be due to the fact that the drug resistance profile is an indirect measure of P-glycoprotein function, whereas drug-
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Fig. 5. Drug-stimulated ATPase activities. Wild-type, Cys-less, and mutant L332C + L975C forms of P-glycoprotein (His)_{10} were purified by nickel-chelate chromatography and ATPase activity measured in the presence of phospholipid and 1 mM verapamil, 5 mM colchicine, or 100 μM vinblastine. The fold stimulation is the ratio of the ATPase activity in the presence of drug substrate to that found in the absence of drug substrate.

stimulated ATPase activity is a direct measure of function. Accordingly, we purified both histidine-tagged mutant proteins and assayed for drug-stimulated ATPase activity in the presence of phospholipid. As shown in Fig. 5, the ATPase activities of both mutants (Cys-less and L332C + L975C) were similar in the presence of 5 mM colchicine, 1 mM verapamil, and 100 μM vinblastine. Compared with the wild-type enzyme, both Cys-less and L332C + L975C mutants had lower drug-stimulated ATPase activities.

We were hindered in our attempts to determine whether the cross-linked P-glycoprotein was active. It appears that the presence of copper during the cross-linking step interfered with subsequent purification of P-glycoprotein by nickel-chelate chromatography.

DISCUSSION

Cysteine-scanning mutagenesis combined with oxidative cross-linking was first used to characterize the transmembrane segments of receptor proteins that mediate chemotaxis in E. coli such as the aspartate (26, 34, 35), Tar (36), and Trg (29) bacterial chemotactic receptors. These investigators were able to take advantage of the fact that these proteins form stable homodimers so that cross-linked homodimers were readily detected by shifts in electrophoretic mobility in SDS gels. In this study, we found that this approach may also be amenable to monomeric proteins. Cross-linking of Cys-332 and Cys-975 of P-glycoprotein caused the protein to migrate with altered electrophoretic mobility in SDS gels. It was interesting that introduction of an intramolecular cross-link into SERCA1 Ca^{2+}-ATPase with glutaraldehyde also slows its electrophoretic mobility on SDS gels (37). These observations suggest that alterations in electrophoretic mobility due to intramolecular cross-linking may be a general phenomenon. Therefore, the approach of cysteine mutagenesis combined with oxidative cross-linking may be applicable for mapping the three-dimensional arrangement of the transmembrane segments of a polytopic membrane protein even if it exists as a monomer.

Interpretation of such data, however, should be undertaken with considerable caution as discussed by Falko and Koschland (34) and Yu et al. (38). A major concern is that cross-linking may result from the random trapping of a conformational fluctuation in the protein. Another potential problem is that introduction of cysteine residues may cause the protein to adopt a non-native conformation so that cross-linking may not pertain to the native structure. For P-glycoprotein, the introduction of cysteines at positions 332 and 975 did not appear to perturb the native structure as the protein exhibited verapamil-stimulated ATPase activity at levels similar to the Cys-less parent molecule. In addition, cross-linking between Cys-332 and Cys-975 was observed using two different approaches. One approach used the full-length molecule, and the second approach involved the use of half-molecule forms of P-glycoprotein.

In this study, cross-linking of the cysteine residues appeared to be quite specific. Only one (Cys-332) of the seven cysteine residues in TM6 (residues 332–338) and one (Cys-975) of six in TM12 (residues 975–980) was cross-linked, whereas no cross-linking was observed between the cysteine residues in TM6 and TM11 (residues 949–956). It is unlikely that there would have been a large number of cross-linked residues detected in this study since there are several requirements for cross-linking to occur. It has been estimated that the a-carbons of the cross-linked cysteine residues can only be a maximum distance of 7 Å from each other, with the average being 5–6 Å (39). In addition, the cysteine residues must be on facing sides of the two interfacing helices for disulfide bonds to form (26). The environment surrounding the cysteine residues also appears to be important. Some cysteine residues in predicted transmembrane helices do not appear to be reactive with compounds such as N-ethylmaleimide (26, 40). Therefore, these cysteine residues would unlikely form disulfide bonds even if their a-carbons were within 7 Å of each other.

An interesting observation in this study is that verapamil and vinblastine but not colchicine could inhibit cross-linking between residues Cys-332 and Cys-975. These results appear to mimic the ability of these substrates to inhibit labeling of P-glycoprotein by photoactive drug analogs. Both verapamil and vinblastine are also relatively good inhibitors of labeling of P-glycoprotein by photoactive analogs such as [3H]azidopine, whereas colchicine is a relatively poor inhibitor of labeling of P-glycoprotein by these drug analogs (12, 41, 42). It is possible that vinblastine and verapamil could induce a conformational change upon drug-protein interaction and/or that these compounds may physically prevent cross-linking between Cys-332 and Cys-975 or labeling by photoactive drug analogs. Physical inhibition by drug substrates could occur if Cys-332 and Cys-975 are close to or within the verapamil or vinblastine drug-binding site(s). Similarly, little inhibition of cross-linking of Cys-332 and Cys-975 or poor inhibition of photoactive labeling of P-glycoprotein by colchicine suggests that either the colchicine-binding site(s) is different from that of verapamil or vinblastine or that binding of colchicine to P-glycoprotein does not result in a conformational change in the protein.

It should be noted that the approach used in this study does have some limitations. One problem is that it was not possible to determine conclusively whether the immature form of the enzyme was also cross-linked. Evidence suggesting that the immature form of P-glycoprotein may also be cross-linked is the observation that the half-molecule forms P-glycoprotein can be efficiently cross-linked (Fig. 3). The NH2-terminal half-molecule is sensitive to endoglycosidase H digestion, suggesting that it is likely to be in the endoplasmic reticulum. Therefore, the conditions during cross-linking should also have allowed the immature form of P-glycoprotein to be cross-linked. Fig. 4 also shows that cross-linking in the presence of increasing concentrations of verapamil or vinblastine resulted in an increase in the amount of both the 170- and 150-kDa form of P-glycoprotein. This effect was most pronounced in the presence of 1000 μM verapamil or 100 μM vinblastine. Another potential limitation is the low efficiency of disulfide bond formation between cysteine residues buried in the membrane. This may be due to their relatively low reactivity. For example, we have found that pretreatment of cells expressing any of the mutants

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with lipid-soluble thiol-specific reagents such as the biotin derivatives, 3-(N-maleimidomPropionyl)-biotin (Molecular Probes) or N-iodoacetyl-N-biotinylhexylenediamine (Pierce), did not affect ATPase activity or label the mutant P-glycoproteins.\(^2\)

Cysteine-scanning mutagenesis and cross-linking in other parts of P-glycoprotein should identify other residues that are in near-proximity. These results will provide insight into the three-dimensional arrangement of the transmembrane domains of the molecule, and the effect of drug substrates on disulfide cross-linking may provide an important tool for studying drug-protein interactions.

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REFERENCES

1. Endicott, J. A., and Ling, V. A. (1989) Annu. Rev. Biochem. 58, 137–171
2. Gottesman, M. M., and Pastan, I. (1993) Annu. Rev. Biochem. 62, 385–427
3. Gottesman, M. M., Currier, S., Bruggemann, E., Lelong, I., Stein, W., and Pastan, I. (1994) Curr. Top. Membr. 41, 3–17
4. Bradley, G., and Ling, V. (1994) Cancer Metastasis Rev. 13, 223–233
5. Schinkel, A. H., Smit, J. J. M., van Tellingen, O., van Deemter, L., and Pastan, I. (1994) Curr. Top. Membr. 41, 3–17
6. Schinkel, A. H., Mol, C. A. M., van Deemter, L., and Pastan, I. (1995) Eur. J. Cancer 31, 1295–1298
7. Chen, C., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1986) Cell 47, 381–389
8. Higgins, C. F. (1992) Annu. Rev. Cell Biol. 8, 67–113
9. Higgins, C. F. (1995) Cell 82, 693–696
10. Raviv, Y., Pollard, H. B., Bruggemann, E. P., Pastan, I., and Gottessman, M. M. (1990) J. Biol. Chem. 265, 3975–3980
11. Homolya, L., Holle, Z., Germann, U. A., Pastan, I., Gottesman, M. M., and Sarkadi, B. (1993) J. Biol. Chem. 268, 21493–21496
12. Greenberger, L. M., Yang, C.-P. H., Gindin, E., and Horwitz, S. B. (1990) J. Biol. Chem. 265, 4394–4401
13. Bruggemann, E. P., Germann, U. A., Gottesman, M. M., and Pastan, I. (1989) J. Biol. Chem. 264, 15483–15488
14. Bruggemann, E. P., Currier, S. J., Gottesman, M. M., and Pastan, I. (1992) J. Biol. Chem. 267, 21020–21026
15. Greenberger, L. M. (1993) J. Biol. Chem. 268, 11417–11425
16. Morris, D. I., Greenberger, L. M., Bruggeman, E. P., Cardarelli, C., Gottesman, M. M., Pastan, I., and Seamon, K. B. (1995) Mol. Pharmacol. 46, 329–337
17. Zhang, X., Collins, K. L., and Greenberger, L. M. (1995) J. Biol. Chem. 270, 5441–5448
18. Wu, J., Perrin, D. M., Sigman, D. S., and Kaback, H. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9186–9190
19. Voss, J., Hubbel, W. L., and Kaback, H. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12390–12393
20. Zobrycka-Gaarn, K., MacDonald, G., Phillips, L., Jorgensen, A. O., and MacLennan, D. H. (1984) J. Bioenerg. Biomembr. 16, 441–446
21. Loo, T. W., and Clarke, D. M. (1993) J. Biol. Chem. 268, 3143–3149
22. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 843–840
23. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 21449–21452
24. Loo, T. W., and Clarke, D. M. (1994) J. Biol. Chem. 269, 7750–7756
25. Chifflet, S., Torriglia, A., Chiesa, E., and Tolos, S. (1998) Anal. Biochem. 168, 1–4
26. Lynch, B. A., and Kosibald, D. E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10402–10406
27. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 21839–21844
28. Loo, T. W., and Clarke, D. M. (1993) J. Biol. Chem. 268, 19965–19972
29. Lee, G. F., Burrows, G. G., Lebert, M. R., Dutton, D. P., and Hazelbauer, G. L. (1994) J. Biol. Chem. 269, 29920–29927
30. Ambudkar, S. V., Leong, I. H., Zhang, J., Cardarelli, C. O., Gottesman, M. M., and Pastan, I. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8472–8476
31. Sarkadi, B., Price, E. M., Bouzere, R. C., Germann, U. A., and Scarborough, G. A. (1992) J. Biol. Chem. 267, 4554–4558
32. Sharom, F. J., Yu, X., and Doige, C. A. (1993) J. Biol. Chem. 268, 24197–24202
33. Al-Shawi, M. K., and Senior, A. E. (1993) J. Biol. Chem. 268, 4197–4206
34. Falke, J. J., and Koshland, D. E. (1987) Science 237, 1596–1600
35. Chervitz, S. A., and Falke, J. J. (1995) J. Biol. Chem. 270, 24043–24053
36. Fukula, A. A., and Simon, M. I. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4144–4148
37. MacLennan, D. H., Clarke, D. M., Loo, T. W., and Skejicz, I. S. (1992) Acta Physiol. Scand. Suppl. 67, 141–150
38. Yu, H., Kono, M., McKee, T. D., and Oprimi, D. D. (1995) Biochemistry 34, 14963–14969
39. Katz, B. A., and Kossiakoff, A. (1986) J. Biol. Chem. 261, 15480–15485
40. Altenbach, C., Flitsch, S. L., Khorana, H. G., and Hubbel, W. L. (1989) Biochemistry 28, 7806–7812
41. Germann, U. A., Willingham, M. C., Pastan, I., and Gottesman, M. M. (1990) Biochemistry 29, 2295–2303
42. Morris, D. I., Speicher, L. A., Ruoho, A. E., Tew, K. D., and Seamon, K. B. (1991) Biochemistry 30, 8371–8379
43. Loo, T. W., and Clarke, D. M. (1996) J. Biol. Chem. 271, 15414–15419