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Review

Determining the structure and mechanism of the human multidrug resistance P-glycoprotein using cysteine-scanning mutagenesis and thiol-modification techniques

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Abstract

The multidrug resistance P-glycoprotein is an ATP-dependent drug pump that extrudes a broad range of hydrophobic compounds out of cells. Its physiological role is likely to protect us from exogenous and endogenous toxins. The protein is important because it contributes to the phenomenon of multidrug resistance during AIDS and cancer chemotherapy. We have used cysteine-scanning mutagenesis and thiol-modification techniques to map the topology of the protein, show that both nucleotide-binding domains are essential for activity, examine packing of the transmembrane segments, map the drug-binding site, and show that there is cross-talk between the ATP-binding sites and the transmembrane segments.

Keywords: P-glycoprotein; ABC transporter; Cysteine-scanning mutagenesis; Disulfide crosslinking; Drug transport; Dibromobimane

Contents

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1. Introduction

P-glycoprotein (P-gp) was discovered by Juliano and Ling [1]. It was overexpressed in cell lines that were initially selected for resistance to a particular cytotoxic agent. These cell lines were subsequently shown to be resistant to many other cytotoxic agents that had diverse chemical structures and intracellular targets (multidrug resistance). It is generally accepted that P-gp confers multidrug resistance by acting as an ATP-dependent drug pump at the cell surface to remove hydrophobic compounds out of the lipid bilayer (Fig. 1) (see comprehensive reviews in [2–4]).

Expression of P-gp in various mammalian tissues is quite variable [5–11]. The relatively high levels of P-gp expression in the epithelial cells of the intestine, blood-brain barrier, blood-testis barrier, and adrenal glands suggests that P-gp plays a very important role in protecting the organism from the onslaught of numerous toxins in our diet and environment [12].

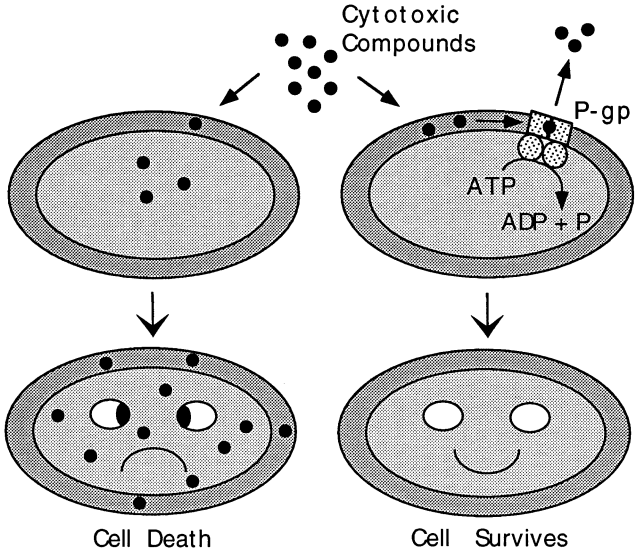

Fig. 1. Expression of P-gp confers multidrug resistance. Cytotoxic compounds enter the cell by diffusing through the lipid bilayer before binding to their intracellular target. Cells overexpressing P-gp (right) extract the cytotoxic compound from the lipid bilayer and use the energy from ATP hydrolysis to extrude them from the cell. These cells are viable even with the continued presence of the toxic compounds. The cells that do not express P-gp (left) accumulate these compounds and die very shortly.

The exact physiological role of P-gp, however, is not known.

Studies on P-gp 'knock-out' mice show that the protein is not essential. The mice remain fertile and do not display any obvious phenotypic abnormalities. They are, however, hypersensitive to accumulation of cytotoxic drugs, and this is most acute in the brain [13,14].

Chemotherapy is a major form of treatment for many cancers. In tumors that do not express endogenous P-gp, there is initial success with remission of the tumor during chemotherapy. Unfortunately, the tumors recur in many cases because of induction of P-gp expression following chemotherapy. In these cases, the prognosis is poor. Tumors that express endogenous P-gp usually complicate the chemotherapy regimen. In these instances, the chemotherapy regimen usually involves polypharmacy, and use of drugs that have different chemical structures as well as different intracellular targets (e.g., *Vinca* alkaloids, epipodophyllotoxins, taxanes and actinomycin D). Combination chemotherapy is used not only to attack different aspects of the tumor cell cycle, but it also coincidentally inhibits P-gp. An example is the inclusion of cyclosporin A in the chemotherapy regimen for treating retinoblastoma [2]. Treatment of retinoblastomas with vincristine alone is usually not successful because a high percentage of these tumors overexpress P-gp. Addition of a relatively high dose of cyclosporin A during vincristine administration, however, results in remission of the tumor. The combination therapy works because P-gp has a relatively higher affinity for cyclosporin A. Therefore, cyclosporin A likely acts as a competitive inhibitor [15].

P-gp has also been implicated in the poor therapeutic response of AIDS patients to protease inhibitors. Protease inhibitors such as indinavir, nelfinavir and saquinavir are substrates of P-gp [16,17]. The relatively high levels of expression of P-gp in the gastrointestinal tract would account for the poor oral absorption of these drugs. The problem is compounded when P-gp in the blood-brain barrier prevents penetration of these drugs into the central nervous system.

Although combination chemotherapy to circum-

vent the action of P-gp can result in the remission of some tumors, it is not without disadvantages. The use of several drugs during treatment also results in increased side effects for the patient, and this is often an important limiting factor during therapy (i.e. a balance between killing the tumor and 'killing' the patient). In order to develop a second generation of inhibitors that are specific for P-gp, it is important to know about its biosynthesis, structure and mechanism. A particularly useful method for understanding the structure and mechanism of P-gp is to use cysteine-scanning mutagenesis and thiol-modification techniques. In this review, we summarize some of the results from using a functional cysteine-less (Cys-less) P-gp for biochemical analysis.

2. Construction of a functional Cys-less P-gp

P-gp is a typical ABC (ATP-binding cassette) transporter [18]. The 1280 amino acids of human P-gp are arranged in four domains: two transmembrane domains (TMDs) with six predicted transmembrane (TM) segments each and two nucleotide-binding domains (NBDs) [19–22]. The molecule is tandemly duplicated with the two halves showing about 43% amino acid identity. The protein contains seven endogenous cysteine residues at positions 137, 431, 717, 956, 1074, 1125 and 1227 (Fig. 2). All of the cysteines were mutated to alanine [23] resulting in a Cys-less P-gp. Alanine is a relatively small neutral amino acid that should cause minimal structural perturbations.

Initially, it seemed that serine would be a better substitution than alanine because it is structurally similar to alanine. We found, however, that substitution of cysteine with serine in the Walker A consensus sites (residues 431 or 1074) caused structural disruption of P-gp (Loo and Clarke, unpublished). P-gp mutants C431S or C1074S failed to mature to the cell surface and were rapidly degraded.

Two modifications were made to the Cys-less P-gp. The first modification was to add the epitope for monoclonal antibody A52 (derived from SERCA1 Ca^{2+}-ATPase [24]) to the COOH-terminal end of the protein. This allowed us to follow expression of Cys-less P-gp. The modification did not alter expression or activity [25]. SERCA1 is not normally expressed in the mammalian cell lines used for expression of the P-gp mutants. Therefore, the monoclonal antibody A52 used in immunoblots results in very low background. The A52 antibody tag is also useful for quantitating the amount of P-gp-A52 expressed using an ELISA with the purified SERCA1 as a standard. The SERCA1 Ca^{2+}-ATPase is readily purified from rabbit muscle.

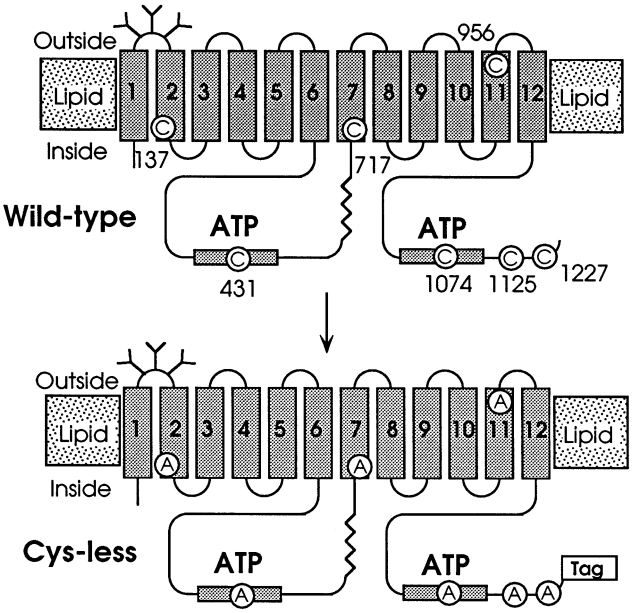

Fig. 2. Construction of Cys-less P-glycoprotein. Wild-type P-gp contains seven cysteine residues that were mutated to alanine resulting in the Cys-less P-gp. A tag was added to the COOH-end to facilitate detection by immunoblot analysis (epitope for monoclonal antibody A52) or for purification (a 10-histidine) by nickel-chelate chromatography. The numbered rectangles represent the TM segments, and the horizontal rectangles represent the 'Walker A' consensus ATP-binding sites.

Instead of the A52 tag, 10 histidine residues were added at the COOH-end of P-gp [26]. Histidine-tagged P-gp mutants can be expressed, purified by nickel-chelate chromatography and assayed for activity in 3–4 days. The fortuitous availability of nickel-NTA spin columns (Qiagen, Inc.) made it relatively easy to do 12–24 purifications at one time. We have constructed histidine-tagged P-gps containing either six or 10 histidines at the COOH-end. Although both types of P-gp can be recovered by nickel-chelate chromatography, using P-gp with a $(His)_{10}$ tag results in purer enzyme. This is because higher concentrations of imidazole (80 mM versus 20 mM for $(His)_{10}$ and $(His)_6$, respectively) can be used during

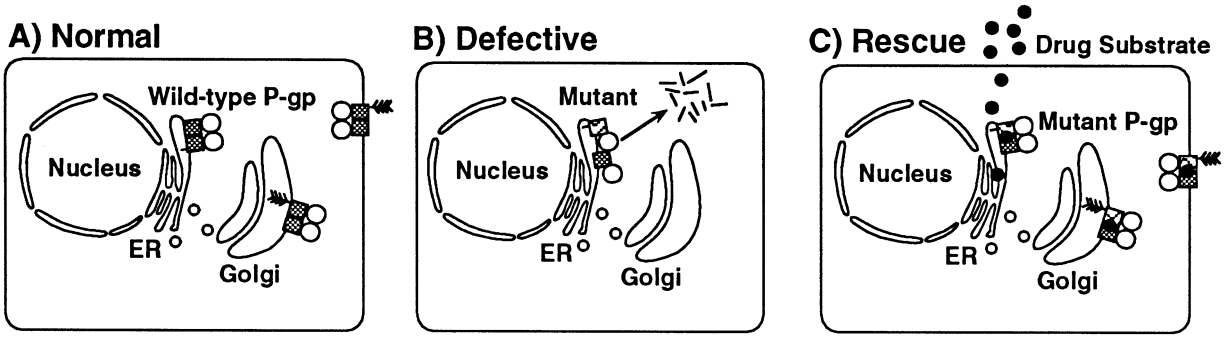

Fig. 3. Schematic representation of rescue of processing mutants by drug substrates. (A) Wild-type P-gp is synthesized in the endoplasmic reticulum (ER) as a core-glycosylated protein that is then processed in the Golgi apparatus and subsequently trafficked to the plasma membrane. (B) P-gp processing mutants are retained in the ER as core-glycosylated intermediates and are rapidly degraded. (C) The hydrophobic drug substrates diffuse to the ER, where they stabilize the processing mutant so that it adopts a 'near-native' structure and leaves the ER for processing (in the Golgi) and transport to the plasma membrane.

the column washes to remove more impurities with P-gp-(His)$_{10}$. Large-scale expression and purification of histidine-tagged P-gp can be achieved with baculovirus vectors and Sf9 insect cells (Loo and Clarke, unpublished; [27]). The disadvantage with the insect cell system is that it is tedious and time consuming (takes weeks to months).

Although expression of Cys-less P-gp in insect cells does not appear to be a problem [28], the same cannot be said for expression in mammalian cells. An initial problem with expression of the Cys-less P-gp in mammalian cells was that biosynthesis was inefficient relative to the wild-type enzyme. Introduction of other cysteine residues at different places in the Cys-less P-gp further inhibited maturation of the protein. This appears to be a common problem when generating a functional Cys-less protein. For example, replacement of individual cysteine residues is well tolerated by the PMA1 H^+-ATPase, but multiple replacements adversely affected the structure of the transporter such that it was unstable and did not retain much activity [29].

A method to 'boost' expression of mature Cys-less P-gp in mammalian cells was discovered during work on processing mutants of P-gp. During structure-function studies, we found that about 12% of the point mutations in P-gp affected processing of the protein [25,30,31]. These mutations are found in the TM segments, the intracellular or extracellular loops, the linker region and in either nucleotide-binding domain. The major product in P-gp-processing mutants expressed in HEK 293 cells is a protein of 150 kDa, while that in wild-type P-gp is a protein of 170 kDa. The processing mutants are retained in the endoplasmic reticulum as core-glycosylated biosynthetic intermediates in association with chaperones such as calnexin and Hsc70 and are rapidly degraded [32,33]. The presence of these point mutations must cause P-gp to adopt an unfavorable conformation that is recognized by the cell's quality control mechanism. It was a pleasant surprise to discover that the presence of drug substrates during biosynthesis of these processing mutants 'rescued' all of these processing mutants such that the major product was now a protein of 170 kDa. These rescued mutant proteins are transported to the plasma membrane in a functional form and are as stable as the wild-type protein [34]. These results suggested that the drug/substrate-binding site(s) must exist transiently in the misprocessed mutants during biosynthesis (as in the wild-type enzyme), and that the folding pathway for P-gp must involve numerous transitional conformational states that are in equilibrium. The presence of drug/substrate at a critical point must stabilize a particular structural conformation of the misprocessed mutants long enough to allow proper folding and subsequent trafficking to the cell surface. If the drug/substrate is not present to act as a 'scaffold' at a critical point, then these transient structures can proceed to a malfolded state, and are subsequently degraded (degradation pathway). Preliminary evidence suggests that this may indeed be the case. As P-gp travels through the folding pathway, its structure changes from a trypsin-

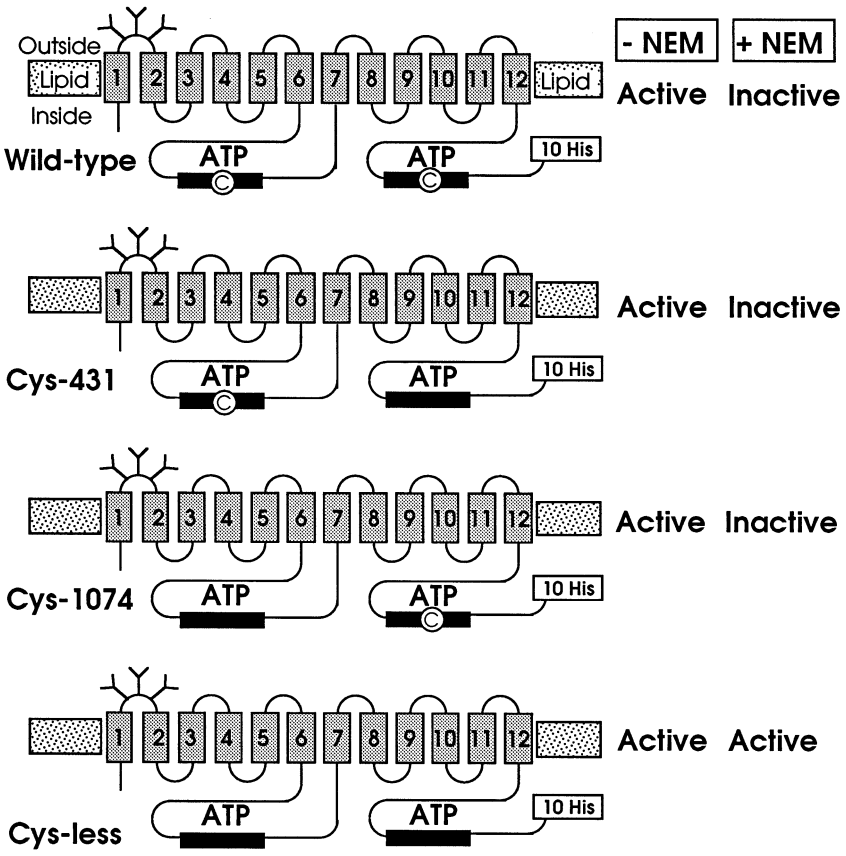

Fig. 4. Role of the nucleotide-binding domains. A cysteine residue was introduced into either Walker A consensus site (Cys431 or Cys1074) of Cys-less P-gp. The ATPase activities of the mutants in the presence or absence of NEM (− or +NEM) were determined. The ATPase activity of Cys-less P-gp was not inhibited by NEM.

sensitive conformation to a relatively trypsin-resistant one [35]. This 'drug rescue' of the processing mutants is schematically represented in Fig. 3.

Cysteine-scanning mutagenesis and thiol chemistry were then used to answer the following questions:

Are both nucleotide-binding domains essential for activity and are they equivalent?
How is the protein folded in the membrane?
Where are the drug-binding site(s)?
How is ATP hydrolysis coupled to drug transport?
How does drug binding or ATP hydrolysis affect P-gp conformation?

3. Importance of the two nucleotide-binding domains

A fundamental question about P-gp was the role of the nucleotide-binding domains (NBDs). Shimabuku et al. [36] fused the NH_2-terminal half or the COOH-terminal half of P-gp with *lacZ* and showed that only the NH_2-terminal half could hydrolyze ATP with a specific activity of 180 nmol Pi/min/mg enzyme. The COOH-terminal half-*lacZ* fusion was not expressed in sufficient amounts in NIH 3T3 or yeast cells for measuring activity. When we expressed the NH_2- and COOH-terminal halves as separate polypeptides in Sf9 insect cells, however, both halves had low levels of basal ATPase activity [28]. These results suggested that both nucleotide-binding domains could hydrolyze ATP. A similar conclusion was reported for the hamster P-gp [37]. It was interesting to note that substrate-stimulated ATPase activity was only observed when both halves were co-expressed. The absence of drug-stimulated ATPase activity in each half-molecule suggested that the ATPase activities were not coupled to drug/substrate

binding. These results showed that coupling of ATPase activity to drug binding requires interaction between both halves of P-gp. Al-Shawi et al. [38] showed that the ATPase activity of hamster P-gp was inhibited by *N*-ethylmaleimide (NEM). Maximal inactivation of ATPase activity was coincident with incorporation of two NEMs per P-gp. Labeling by NEM occurred at two sites, with equal distribution of label between the NH_2 and COOH halves of the molecule. Labeling by NEM was inhibited by the presence of ATP. Therefore, it was predicted that the cysteine residues in the Walker A consensus sequences of either NBD (GNSGCGKS and GSSGCGKS, respectively) likely reacted with NEM.

To test the contribution of either NBD to P-gp function, the Cys-less P-gp was modified so that a single cysteine residue was introduced into the Walker A consensus sequence (C431 or C1074) in either NBD (Fig. 4). Covalent modification of either C431 or C1074 with NEM inactivated the basal or drug-stimulated ATPase activities [39]. In both mutants, inactivation of ATPase activity was inhibited by the presence of ATP. The NBDs did not appear to be equivalent since the concentrations of NEM required for inhibiting ATPase activity were different (half-maximal inhibition of 7 μM for C431 and 35 μM for C1074). These results suggested that both nucleotide-binding domains are essential for activity, and that both NBDs must function in a regulated manner. The Senior group have used vanadate trapping of nucleotides to show that only one NBD is in a catalytic transition state at a time, and have proposed an alternating site catalysis mechanism [40].

4. Topology of P-gp

The Cys-less P-gp was a useful tool for determining the topology of P-gp at the cell surface. Single cysteine residues were introduced at different locations in P-gp. Mutants that still conferred drug resistance in transfected cells were used for topology studies. Study of the inactive molecules may not be informative because they may be misfolded. The mutant P-gps were transiently expressed in HEK 293 cells and reacted with thiol-specific membrane-permeant (biotin maleimide; 3-(*N*-maleimidylpropionyl) biocytin; Molecular Probes, Inc.) or membrane-impermeant (stilbenedisulfonate maleimide; 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid) compounds. The rationale was that treatment with biotin maleimide would biotinylate any Cys residues, and that pretreatment with membrane-impermeant stilbenedisulfonate maleimide would block the exposed extracellular cysteine residues. The biotinylated proteins were immunoprecipitated and detected by Western blot analysis with streptavidin conjugated to horseradish peroxidase [23]. The topology obtained was consistent with the model predicting six TM segments in each homologous half of P-gp. Kast et al. [41,42] inserted a small antigenic peptide epitope (YPYDVPDYAIEGR) containing part of the hemagglutinin (HA) of influenza virus at various positions in the mouse P-gp, and showed that the topology was consistent with the predicted model (‘6+6’ TM model). The topology of P-gp (Fig. 2) is still controversial. Other topologies (for example, six TM segments in the NH_2 half and four TM segments in the COOH half; ‘6+4’ TM model) have also been reported [43–47]. A possible explanation for these alternative topologies is that they do exist at the cell surface, but at such low levels that it may not be possible to isolate enough material for biochemical studies. Another explanation is that the different topologies could be inherent to the folding properties of the truncated forms of P-gp used in these studies. Point mutations in P-gp can indeed alter the levels of

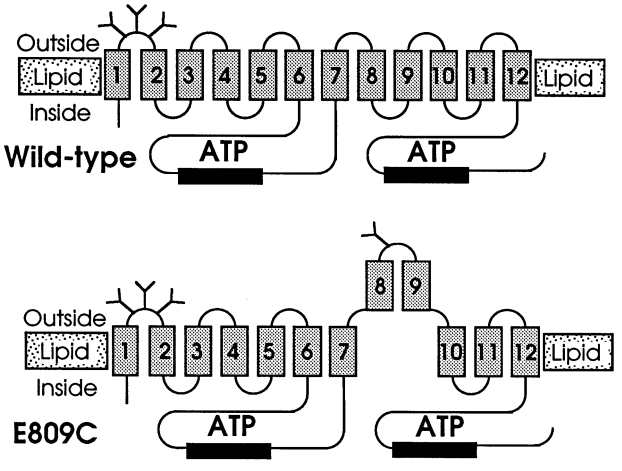

Fig. 5. Topologies of P-gp. Wild-type P-gp showing the predicted ‘6+6’ TM arrangement. Some mutations such as E875C cause about 50% of P-gp to adopt a ‘6+4’ TM arrangement. In this conformation, N809 in the loop connecting TMs 8 and 9 is glycosylated.

the different topologies present in the cell. For example, the mutation E875C promotes the formation of a topology where the predicted cytoplasmic loop 3 is on the opposite side of the membrane and where Q809 is now glycosylated (Fig. 5). It appears, however, that only P-gp with the '6+6' TM arrangement interacts with drug substrates [48].

Determining the topology of polytopic membranes using cysteine-scanning mutagenesis and thiol-specific compounds has become increasingly popular. For example, this approach has been successfully used for the serotonin transporter [49], the F_1F_0 ATP synthase [50,51], AE1 protein [52], the yeast vacuolar proton ATPase [53] and the bradykinin β2 receptor [54]. A variety of other thiol-reactive compounds have also been used. For example, *N*-biotinylaminoethylmethane thiosulfonate (MTSEA-biotin) was used to label the serotonin reporter [49].

5. Identification of residues in the drug-binding domains

In order to understand how P-gp is able to transport such a variety of hydrophobic compounds, it is important to identify the residues that participate in drug binding. One method for identifying the domains that bind drugs is to use photoactive radioactive analogs of drug substrates to label P-gp, followed by proteolytic or chemical cleavage to identify the labeled fragment. The results from such studies suggest that the photolabeled residue(s) are in TM3 [55], TM4 [55,56], TM5 [55,57], TM6 [55,57,58], TM7 [59], TM8 [59], TM11 [60] and TM12 [58–61].

Another approach has been to use mutational analysis to identify residues that when changed, alter activity or substrate specificity of the transporter [25,30,31,62–65]. The results from such studies suggest that residues on one face of TM segments 5, 6, 11 and 12 are particularly sensitive to mutations. A potential problem, however, is that the mutations in most domains, including the nucleotide-binding domains [66,67] can alter the substrate specificity of P-gp. It is also difficult to determine whether mutations affecting substrate specificity are due to functional perturbations or due to perturbations in the global structure of P-gp. An example is the spontaneous mutation G185V that conferred increased resistance to colchicine and decreased resistance to vinblastine [68,69]. This mutation was subsequently found to have pleiotropic effects on P-gp as a result of structural alterations [70].

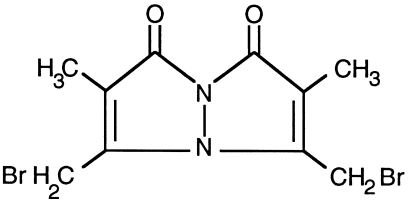

Fig. 6. Structure of dibromobimane. Dibromobimane is a thiol-specific compound that stimulated the ATPase activity of Cys-less P-gp. It can be used as a crosslinking agent due to the presence of a bifunctional group.

A quite different method for identifying residues that line the drug-binding sites would be to use cysteine-scanning mutagenesis with a thiol-reactive substrate. Once a reactive residue is identified then protection experiments can be used to identify whether that particular residue contributes to the binding of other substrates.

Dibromobimane (dBBn) is a thiol-reactive compound and a substrate of P-gp. It is a hydrophobic compound (Fig. 6) containing two thiol-reactive groups, and can also be used as a thiol crosslinking reagent. It stimulates the ATPase activity of Cys-less P-gp about 8-fold at a concentration of 1 mM [71]. Another useful property of dBBn is that it can be converted to a non-substrate (i.e. does not stimulate ATPase activity of P-gp) by quenching with cysteine. Initial studies were done on residues in TM6 and TM12. The ATPase activities of mutants L339C and A342C (TM6), and mutants L975C, V982C and A985C (TM12) were inhibited by dBBn. Protection from inactivation was observed with verapamil, vinblastine and colchicine, suggesting that these residues were important for interaction of these substrates with P-gp. While this approach has many advantages, one drawback is that all residues that participate in binding dBBn may not react with it.

6. Disulfide crosslinking to assess TM packing in the membrane

Most evidence suggests that the TM segments interact with substrates. TM6 and TM12 appear to be

particularly important. In the linear model of P-gp, TM6 and TM12 are relatively far apart. If TM6 and TM12 contribute to the same drug-binding site, then they would be expected to be close to each other in the three-dimensional structure of the protein. Such high-resolution structures have not been available due to technical difficulties in crystallizing P-gp for X-ray diffraction studies. A relatively low resolution structure of P-gp (25 Å), however, was recently reported by Rosenberg et al. [72].

A method that does not require large amounts of protein for studying the TM packing of P-gp in the membrane is disulfide crosslinking analysis. Such an approach was initially used to characterize the oligomeric nature of the aspartate and Tar receptors in bacteria [73–76]. These investigators took advantage of the fact that these proteins formed stable homodimers and that the crosslinked products were readily detected by shifts in their electrophoretic mobilities in SDS-PAGE gels.

Changes in the electrophoretic mobility of P-gp in SDS-PAGE gels were used to assay for disulfide crosslinking between residues in TM6 and TM12. Crosslinking was detected in mutants F343C/M986C, G346C/G989C and P350/S993C [77,78]. Crosslinking experiments with the half molecules of P-gp confirmed that the changes in electrophoretic mobilities of these mutants were indeed due to disulfide crosslinking [77]. The NH_2 and COOH halves of P-gp interact because they show drug-stimulated ATPase activity when coexpressed in the same cell.

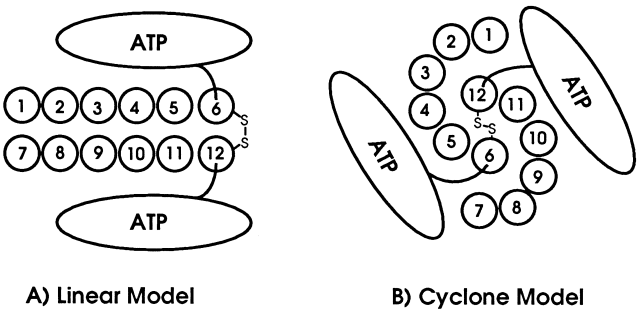

Fig. 7. Models of TM packing in the membrane. Disulfide crosslinking studies show that TMs 6 and 12 are close together. On this basis, aerial views of two models are presented. (A) The 'Parallel' model in which TMs 1–7 are parallel to TMs 7–12. (B) The 'Cyclone' model in which TMs 1–6 are anti-parallel to TMs 7–12. The TMs are represented as numbered circles.

Cysteine residues were introduced into the Cys-less forms of either half-molecule. They were coexpressed and assayed for the presence of crosslinked products. The results showed that F343C (TM6)+M986C (TM12), G346C (TM6)+G989C (TM12), and P350C (TM6)+S993C (TM12) (Loo and Clarke, unpublished observations) could be crosslinked. These results suggest that TM6 and TM12 close together in the three-dimensional structure of P-gp.

A considerable amount of crosslinking data will be required for understanding the packing of TM segments in the membrane. To this end, development of a feasible working model might provide a clearer picture for designing future crosslinking experiments. Data from the TM6/TM12 crosslinking experiments

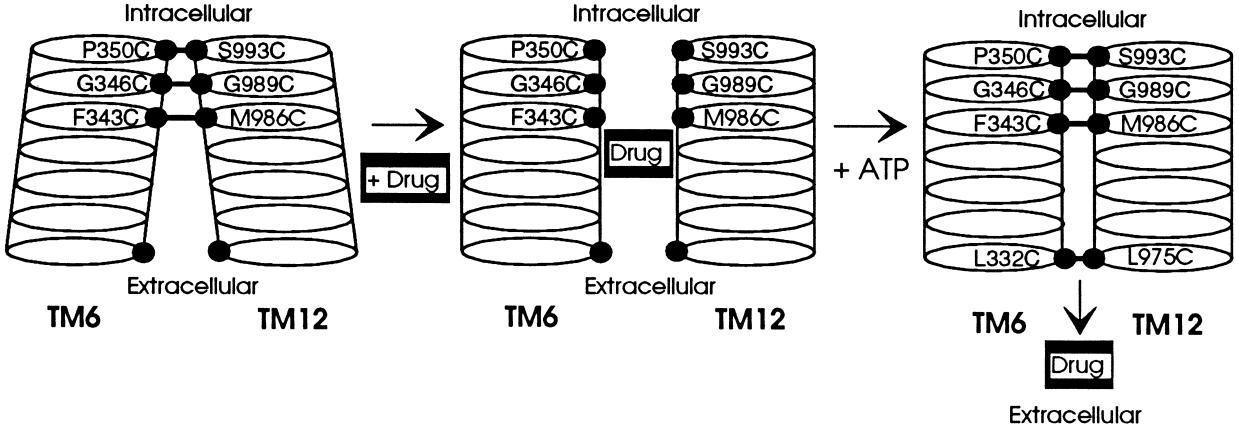

Fig. 8. Model of conformational changes between TM6 and TM12 during drug transport. (A) In the absence of drug substrates or ATP, crosslinking is observed between Cys residues in TM6 and TM12 that are close to the cytoplasmic side of the lipid bilayer. (B) Binding of drug substrate prevents crosslinking between TM6 and TM12. (C) ATP hydrolysis causes movement between TM6 and TM12 such that crosslinking is also detected between residues that are close to the extracellular side of the bilayer.

can be used to design two practical models. In the 'Parallel' model, TMs 1–6 are parallel to TMs 7–12 (Fig. 7A). The model predicts that crosslinking would be possible between TM1/TM7, TM2/TM8, TM3/TM9 and so on. Another arrangement of the TMs is the 'Cyclone' model (Fig. 7B). In this model, TMs 1–6 are anti-parallel to TMs 7–12. This model predicts a different pattern of crosslinking, with crosslinking expected between TMs 4–6 and TMs 10–12, and not between TMs 1 and 2 and TMs 7 and 8. More recent results support the 'Cyclone' model (Loo and Clarke, manuscript in preparation).

Crosslinking studies are quite informative, since they can provide insight into the dynamic nature of the TM segments. The presence of substrates and ATP during crosslinking changes the pattern of crosslinking and can be used to detect the range of movement of the TM segments. These results provide insight into the conformation changes that occur during the catalytic cycle of P-gp. An example is the change in the pattern of crosslinking between TM6 and TM12 in the presence of substrates or ATP. The crosslinking pattern seen in mutants F343C/M986C, G346C/G989C and P350C/S993C was inhibited by the presence of substrates such as verapamil, vinblastine, cyclosporin A or colchicine [78]. It seemed that the presence of substrates increased the distance between these crosslinkable residues. An alternative explanation is that the substrates occupy the space between the cysteines. The presence of ATP had a different effect on crosslinking between TMs 6 and 12. Mutant L332C (TM6)/L975C (TM12) is normally not crosslinkable except in the presence of ATP. Hydrolysis of ATP is required since the presence of ATP+vanadate, a non-hydrolyzable ATP analog (AMP.PNP), or ADP did not induce crosslinking [78]. Data from these types of experiment can be incorporated into a model as shown in Fig. 8. In the absence of drug substrates, the residues lining one face of TM6 are close to one face of TM12. Binding of drug substrates by P-gp leads to an increase in the distance between TM6 and TM12. Hydrolysis of ATP induces conformational changes that result in movement between TM6 and TM12 and subsequent drug efflux. If TM6 and TM12 are locked in a fixed position by crosslinking before addition of drug substrate and ATP, then drug-stimulated ATPase activity is not detected [78]. In summary, cysteine-scanning mutagenesis combined with thiol-modification chemistry has contributed much to our understanding of the structure and mechanism of P-gp.

Future studies will answer questions such as: (1) Do all substrates bind to the same region of P-gp? (2) Which model best explains how the TM segments are packed in the membrane? (3) Do other TM segments show conformation changes upon binding of substrate or during ATP hydrolysis? (4) Why do some mutations inhibit biosynthesis? (5) How do substrates correct processing defects?

Acknowledgements

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