Transmembrane segment 7 of human P-glycoprotein forms part of the drug-binding pocket

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P-gp (P-glycoprotein; ABCB1) protects us by transporting a broad range of structurally unrelated compounds out of the cell. Identifying the regions of P-gp that make up the drug-binding pocket is important for understanding the mechanism of transport. The common drug-binding pocket is at the interface between the transmembrane domains of the two homologous halves of P-gp. It has been shown in a previous study [Loo, Bartlett and Clarke (2006) *Biochem. J.* **396**, 537–545] that the first transmembrane segment (TM1) contributed to the drug-binding pocket. In the present study, we used cysteine-scanning mutagenesis, reaction with an MTS (methanethiosulfonate) thiol-reactive analogue of verapamil (termed MTS-verapamil) and cross-linking analysis to test whether the equivalent transmembrane segment (TM7) in the C-terminal-half of P-gp also contributed to drug binding. Mutation of Phe⁷²⁸ to cysteine caused a 4-fold decrease in apparent affinity for the drug substrate verapamil. Mutant F728C also

showed elevated ATPase activity (11.5-fold higher than untreated controls) after covalent modification with MTS-verapamil. The activity returned to basal levels after treatment with dithiothreitol. The substrates, verapamil and cyclosporin A, protected the mutant from labelling with MTS-verapamil. Mutant F728C could be cross-linked with a homobifunctional thiol-reactive cross-linker to cysteines I306C(TM5) and F343C(TM6) that are predicted to line the drug-binding pocket. Disulfide cross-linking was inhibited by some drug substrates such as Rhodamine B, calcein acetoxymethyl ester, cyclosporin, verapamil and vinblastine or by vanadate trapping of nucleotides. These results indicate that TM7 forms part of the drug-binding pocket of P-gp.

Key words: cysteine-scanning mutagenesis, disulfide cross-linking analysis, drug-binding pocket, P-glycoprotein, protein modification, transmembrane segment 7 (TM7).

INTRODUCTION

The human multidrug resistance P-gp (P-glycoprotein, ABCB1) is a 170 kDa plasma membrane protein that uses ATP to pump hydrophobic molecules out of the cell [1,2]. The protein is expressed in relatively high levels in the apical membranes of epithelial cells of intestine, kidney, liver and blood–brain/testes barriers [3]. Its physiological role might be to protect us from cytotoxic compounds in our diets and environment [4]. P-gp is clinically important because it can potentially undermine cancer and AIDS/HIV chemotherapies [5].

The 1280 amino acids of P-gp are organized as two homologous halves (43 % amino acid identity) that are joined by a linker region [6]. Each half begins with a TMD [TM (transmembrane) domain] containing six TM segments followed by a hydrophilic NBD (nucleotide-binding domain) [7]. The two halves of the molecule, however, do not have to be covalently linked for function [8]. The two ATP molecules bind at the interface between the Walker A sites and LSGGQ sequences between the NBDs [9]. ATP hydrolysis probably occurs by an alternating mechanism [10]. This is supported by the findings that both NBDs can hydrolyse ATP but inhibition at either site inhibits the activity of the protein [11,12].

Similarly, the drug-binding pocket is at the interface between the TMDs [13–16]. P-gp retains the ability to bind drug substrates even after deleting the two NBDs [8]. An interesting question about P-gp is how it can recognize so many structurally diverse

compounds. One suggestion is that P-gp has distinct binding sites. Indeed, studies have suggested that P-gp may have up to four distinct drug-binding sites [17–20]. By contrast, we have proposed that a common drug-binding pocket lies at the interface between the TMDs. Substrates bind through a ‘substrate-induced fit’ mechanism [15]. Binding of a substrate would involve various combinations of residues from different TMDs and structurally different substrates could share the same residues during drug binding. The common drug-binding pocket is relatively large [14,21] and can accommodate different substrates simultaneously [20,22].

To determine the mechanism of P-gp, it is important to identify TM segments that line the drug-binding pocket. In our initial model of P-gp, we predicted that the last three TM segments of each TMD (TMs 4–6 and 10–12 respectively) formed the drug-binding pocket [23], whereas TMs 5 and 8 and TMs 2 and 11 [24,25] formed the gates for entry of drug substrates [26]. In a recent study, however, it was shown that TM1 also lines the drug-binding pocket [27]. Therefore the predicted model of P-gp was modified such that TM1 was placed close to TMs 4–6. The revised model is consistent with the crystal structure of the bacterial transporter MsbA [28]. The revised model of P-gp also predicts that the first TM segment in the second TMD (TM7) is also close to the drug-binding pocket.

In the present study, we have used cysteine-scanning mutagenesis, reaction with a thiol-reactive analogue of verapamil and cross-linking analysis to test whether residues in TM7 contribute to the drug-binding pocket.

Abbreviations used: AM, acetoxymethyl ester; DTT, dithiothreitol; HEK, human embryonic kidney; M5M, 1,5-pentanedithiol bismethanethiosulfonate; M8M, 3,6-dioxaoctane-1,8-diyl bismethanethiosulfonate; M11M, 3,6,9-trioxadecane-1,11-diyl bismethanethiosulfonate; M14M, 3,6,9,12-tetraoxatetradecane-1,14-diyl bismethanethiosulfonate; M17M, 3,6,9,12,15-pentaoxaheptadecane-1,17-diyl bismethanethiosulfonate; MTS, methanethiosulfonate; NBD, nucleotide-binding domain; P-gp, P-glycoprotein; TBS, Tris-buffered saline; TM, transmembrane; TMD, TM domain; TMD1, N-terminal TMD containing TM segments 1–6; TMD2, C-terminal TMD containing TM segments 7–12.

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MATERIALS AND METHODS

Construction of mutants

The seven endogenous cysteines at positions 137, 431, 717, 956, 1074, 1125 and 1227 were replaced with alanines to create cysteine-less (Cys-less) P-gp [7]. None of the cysteines were important for function [7]. Single cysteine residues were re-introduced at each position in TM7 (residues 711–731) in the Cys-less P-gp. All of the mutants contained a His₁₀ tag at the C-terminal end to facilitate purification of the mutant P-gp by nickel-chelate chromatography [29]. For cross-linking analysis, the double cysteine mutants L65C(TM1)/F728C(TM7), I306C(TM5)/F728C(TM7) and F343C(TM6)/F728C(TM7) were constructed as described previously [15].

Expression of mutants, and purification and measurement of ATPase activity

The mutant P-gps were transiently expressed in HEK (human embryonic kidney)-293 cells as described previously [29]. HEK-293 cells expressing mutant P-gp were also grown in the presence of 10 μ M cyclosporin A for 24 h because it acts as a pharmacological/specific chemical chaperone to increase the yield of mature enzyme [30]. To prevent potential interference of cyclosporin A in subsequent labelling or disulfide cross-linking studies, the cells were grown in the absence of cyclosporin A and at 30°C for 24 h before harvest. His-tagged P-gp was isolated by nickel-chelate chromatography as described previously [29]. A sample of the isolated His-tagged P-gp was mixed with an equal volume of 10 mg/ml sheep brain phosphatidylethanolamine (Type II-S; Sigma) or 10 mg/ml *Escherichia coli* lipids (Avanti Polar Lipids) that had been washed and suspended in TBS [Tris-buffered saline consisting of 10 mM Tris/HCl (pH 7.4) and 150 mM NaCl]. The sample was sonicated and ATPase activity measured in the absence of drug substrate, in the presence of various concentrations of verapamil (1–3000 μ M), vinblastine (0.6–60 μ M), colchicine (0.1–10 mM) or in the presence of saturating levels of calcein-AM (calcein acetoxymethyl ester; 0.6 mM), demecolcine (3 mM), verapamil (1 mM), cyclosporin A (0.2 mM) or *trans*-(E)-flupentixol (0.6 mM) [31]. The samples were incubated for 30 min at 37°C and the amount of inorganic phosphate released was determined [29].

Reaction of P-gp mutants with MTS (methanethiosulfonate)-verapamil

His-tagged TM7 single cysteine mutants were expressed in 30 (10-cm diameter) plates of HEK-293 cells in the absence of cyclosporin A. The cells were then incubated at 30°C for 24 h. The cells were washed three times with PBS [10 mM sodium phosphate (pH 7.4) and 150 mM NaCl] and then suspended in a total volume of 1.5 ml of NTA/PBS buffer [10 mM Tris/HCl (pH 8.0), 100 mM sodium phosphate and 150 mM NaCl]. The cells were solubilized by addition of an equal volume of NTA/PBS buffer containing 2% (w/v) *n*-dodecyl- β -D-maltoside. Insoluble material was removed by centrifugation at 16 000 *g* for 15 min at 4°C. DNA was removed from the supernatant by passage through a miniprep plasmid DNA spin column (Qiagen). Half of the supernatant (1.3 ml) was then incubated with the desired concentration of MTS-verapamil (0.01–10 mM) for 10 min at 20°C, whereas the remaining sample served as an untreated control. In the drug protection studies, the solubilized material was preincubated with 3 mM verapamil or 0.2 mM cyclosporin A (saturating concentrations) for 10 min at 20°C prior to labelling with MTS-verapamil. The samples were then cooled in an ice-bath, followed by addition of 0.15 ml of 3 M NaCl and 0.05 ml

Figure 1 Schematic models of P-gp and composition of TM7

The 12 TMs of P-gp are shown as numbered cylinders. The branched lines represent glycosylation sites. NBD1 and NBD2 represent the nucleotide-binding domains. The amino acids in predicted TM7 (positions 711–731) are shown as an α -helical net. The amino acids identical with those in the predicted TM1 structure are shaded in grey.

of 1 M imidazole (pH 7.0). His-tagged P-gp was then isolated by nickel-chelate chromatography as described previously [29]. The recovery of P-gp was monitored by immunoblot analysis with a rabbit anti-P-gp polyclonal antibody [32].

Disulfide cross-linking analysis

The double cysteine mutants L65C(TM1)/F728C(TM7), I306C(TM5)/F728C(TM7) and F343C(TM6)/F728C(TM7) were transiently expressed in HEK-293 cells [31]. The cells were harvested and washed three times with PBS (pH 7.4) and the membranes prepared as described previously [23]. The membranes were suspended in TBS. A sample of the membrane was then treated with zero-length cross-linker (1 mM copper phenanthroline) or with 0.2 mM of homobifunctional MTS cross-linkers with spacer arms of various lengths: M5M [1,5-pentanediy bismethanethiosulfonate, 9.1 Å spacer arm (1 Å = 0.1 nm)]; M8M (3,6-dioxaoctane-1,8-diyl bismethanethiosulfonate, 13 Å spacer arm); M11M (3,6,9-trioxaundecane-1,11-diyl bismethanethiosulfonate, 16.9 Å spacer arm); M14M (3,6,9,12-tetraoxatetradecane-1, 14-diyl bismethanethiosulfonate, 20.8 Å spacer arm) or M17M (3,6,9,12,15-pentaoxaheptadecane-1,17-diyl bismethanethiosulfonate, 24.7 Å spacer arm) (Toronto Research Chemicals) for 15 min at 4°C [14]. The reactions were stopped by addition of 2 $\times$ SDS sample buffer [125 mM Tris/HCl (pH 6.8), 20% (v/v) glycerol and 4% (w/v) SDS] containing 50 mM EDTA and no reducing agent. The reaction mixtures were then subjected to SDS/PAGE (7.5% gels) and immunoblot analysis was performed using a rabbit polyclonal antibody against P-gp [32]. Intramolecular disulfide cross-linking between TMD1 (the N-terminal TMD containing TM segments 1–6) and TMD2 (the C-terminal TMD containing TM segments 7–12) can be detected because the cross-linked product migrates with a slower mobility on SDS polyacrylamide gels [31].

The amount of cross-linking was quantified by scanning the gel lanes, followed by analysis with the NIH (National Institutes of Health) Image program (available at <http://rsb.info.nih.gov/ni-image>).

RESULTS

TM segments 1 and 7 are the first TM segments in each of the two TMDs (Figure 1) and initiate insertion of each of the TMDs

Table 1 Concentrations of substrates required for activation of ATPase activity of TM7 cysteine mutants by 50% (S_{50})

ND, not determined because of very low expression.

Mutant	Verapamil S_{50} (μ M)	Vinblastine S_{50} (μ M)	Colchicine S_{50} (μ M)
F711C	9.8 $\pm$ 1.1	2.3 $\pm$ 0.2	410 $\pm$ 20
V712C	10.0 $\pm$ 0.8	1.9 $\pm$ 0.2	320 $\pm$ 40
V713C	9.9 $\pm$ 1.3	1.8 $\pm$ 0.2	340 $\pm$ 40
G714C	14.1 $\pm$ 2.1	1.9 $\pm$ 0.3	410 $\pm$ 30
V715C	9.8 $\pm$ 1.3	1.8 $\pm$ 0.3	330 $\pm$ 40
F716C	10.1 $\pm$ 1.0	2.1 $\pm$ 0.1	340 $\pm$ 30
C717C	10.0 $\pm$ 1.5	2.3 $\pm$ 0.3	390 $\pm$ 20
A718C	10.5 $\pm$ 1.3	2.0 $\pm$ 0.1	330 $\pm$ 30
I719C	10.5 $\pm$ 1.5	1.9 $\pm$ 0.2	370 $\pm$ 30
I720C	12.4 $\pm$ 0.7	2.4 $\pm$ 0.2	390 $\pm$ 10
N721C	13.0 $\pm$ 1.2	1.7 $\pm$ 0.2	380 $\pm$ 40
G722C	ND	ND	ND
G723C	11.1 $\pm$ 0.4	2.0 $\pm$ 0.3	410 $\pm$ 20
L724C	12.0 $\pm$ 1.5	2.4 $\pm$ 0.3	340 $\pm$ 30
Q725C	11.7 $\pm$ 1.1	2.0 $\pm$ 0.1	390 $\pm$ 20
P726C	11.9 $\pm$ 1.0	1.9 $\pm$ 0.2	450 $\pm$ 30
A727C	21.2 $\pm$ 3.1	1.9 $\pm$ 0.2	480 $\pm$ 40
F728C	48.7 $\pm$ 2.2	2.7 $\pm$ 0.3	830 $\pm$ 90
A729C	34.3 $\pm$ 3.0	2.3 $\pm$ 0.2	760 $\pm$ 80
I730C	12.0 $\pm$ 1.1	2.0 $\pm$ 0.1	420 $\pm$ 30
I731C	13.5 $\pm$ 1.9	1.9 $\pm$ 0.2	410 $\pm$ 30
Cys-less	11.6 $\pm$ 1.3	2.1 $\pm$ 0.3	400 $\pm$ 40

into the endoplasmic reticulum during synthesis of the protein. The amino acid sequences of the two TM segments show 43 % amino acid identity. Previous evidence suggested that the two TM segments could play similar roles because mutations to conserved amino acids at equivalent positions (Gly⁵⁴, Ala⁵⁸ and Gly⁶² of TM1 and Gly⁷¹⁴, Ala⁷¹⁸ and Gly⁷²² of TM7) caused misprocessing of P-gp [33,34]. The two TMs were also partly interchangeable because TM1/TM1 and TM7/TM7 chimaeras of P-gp could still confer drug resistance [34]. TM7 appeared, however, to be more important for activity because replacement of TM1 with TM7 (TM7/TM7 chimaera) yielded a protein that retained about 70 % activity whereas replacement of TM7 with TM1 (TM1/TM1 chimera) yielded a protein that had 20 % of the activity of wild-type P-gp [34]. Since we previously showed that TM1 plays a role in drug-binding [27], the results with the chimeric molecules suggested that TM7 might also play an important role in drug-binding.

We wanted to test whether TM7 lines the drug-binding pocket by using cysteine-scanning mutagenesis, reaction with a thiol-reactive analogue of verapamil and cross-linking analysis. The first step was to determine whether mutation of the residues in TM7 to cysteine affected the affinity of P-gp for drug substrates. Drug substrates such as vinblastine, colchicine and verapamil are useful compounds because of their ability to stimulate the ATPase activity of P-gp by 6- to 20-fold [35]. Accordingly, each residue in TM7 (positions 711–731) was changed to cysteine and the His-tagged mutants were expressed in HEK-293 cells with 10 μ M cyclosporin A, because it acts as a pharmacological/specific chemical chaperone to increase the yield of protein by promoting maturation of P-gp [30]. The mutant proteins were then isolated by nickel-chelate chromatography, mixed with lipid and ATPase activity was determined in the presence of various concentrations of verapamil, colchicine or verapamil. Table 1 shows that two mutants, F728C and A729C, showed about 4.2- and 3-fold decreases respectively, in the apparent affinity for verapamil when compared with Cys-less P-gp. Both mutants also showed about a 2-fold reduction in apparent affinity for colchicine

with little or no change in apparent affinity for vinblastine (Table 1). The other mutants had apparent affinities for verapamil, vinblastine and colchicine that were similar to Cys-less P-gp (< 2-fold change). Therefore, it appeared that TM7 might play an important role in binding to verapamil.

To investigate the interaction of TM7 with verapamil further, we measured activation of ATPase activity in the TM7 cysteine mutants (residues 711–731) after reaction with a thiol-reactive analogue of verapamil, termed MTS-verapamil. We have previously shown that modification of specific cysteines in TM1 (L65C) and in TM5 (I306C) with MTS-verapamil caused permanent activation of P-gp ATPase activity (an 8- to 11-fold increase in activity compared with untreated P-gp) [27,36]. Permanent activation of P-gp ATPase activity after covalent attachment of verapamil to Cys⁶⁵(TM1) or Cys³⁰⁶(TM5) suggested that the covalently attached verapamil occupies the drug-binding pocket in a conformation that mimics the interaction of P-gp with unmodified verapamil. Therefore the His-tagged TM7 single cysteine mutants were expressed in HEK-293 cells in the absence of cyclosporin A. Maturation of the TM7 cysteine mutants was promoted by incubating the cells at low temperature (30 °C) rather than with cyclosporin A. This was to avoid the potential problem of cyclosporin A inhibiting the labelling of cysteine mutants with MTS-verapamil because cyclosporin A is a high-affinity substrate of P-gp [36]. The transfected cells were then solubilized with *n*-dodecyl- β -D-maltoside, treated with or without 0.3 mM MTS-verapamil and P-gp was isolated by nickel-chelate chromatography. This concentration of MTS-verapamil was sufficient to completely modify Cys⁶⁵(TM1) [27] and Cys³⁰⁶(TM5) [36], and to ensure that it remained selective for sulfhydryl groups. Samples of the isolated P-gp were mixed with lipid and assayed for ATPase activity and compared with untreated P-gp. All of the mutants, except Q725C, P726C, F728C and A729C, were unaffected by treatment with MTS-verapamil (Figure 2A). Mutant P726C, however, showed about a 50 % reduction in basal activity after treatment with MTS-verapamil. By contrast, the ATPase activity of mutants Q725C, F728C and A729C increased 2.6-, 4.7- and 4.5-fold respectively, compared with untreated mutant P-gp.

Although mutants Q725C, F728C and A729C showed increased ATPase activity after treatment with MTS-verapamil, the activities were less than 50 % of the verapamil-stimulated ATPase activity of Cys-less P-gp (maximum 11.1-fold stimulation; Figure 2B). We have shown previously that mutants Q725C, F728C and A729C were stimulated more than 10-fold with verapamil [37]. These results suggested that the mutants' interactions with verapamil were likely to be similar to those of Cys-less P-gp. Therefore it was possible that reaction of MTS-verapamil with mutants Q725C, F728C and A729C was incomplete or that they were completely modified but the bound verapamil was in a sub-optimal conformation for activation of ATPase activity. To distinguish between these two possibilities, mutants Q725C, F728C and A729C (activated by 0.3 mM MTS-verapamil) were treated with or without 3 mM MTS-verapamil. His-tagged P-gps were then isolated by nickel-chelate chromatography, mixed with lipids and ATPase activity was determined in the presence or absence of verapamil. Figure 2(B) shows that increasing the level of MTS-verapamil from 0.3 mM to 3 mM did not decrease or increase the basal ATPase activity of mutant A729C. In addition, the activity of MTS-verapamil-modified mutant A729C could not be activated further by unmodified verapamil (Figure 2B). Therefore MTS-verapamil modification of mutant A729C inhibits its verapamil-stimulated ATPase activity. In contrast, labelling of mutants Q725C or F728C with 3 mM MTS-verapamil increased their ATPase activities further

Figure 2 Effect of MTS-verapamil on basal ATPase activity of TM7 cysteine mutants

(A) His-tagged Cys-less or mutants F711C/I731C P-gps were expressed in HEK-293 cells and solubilized with *n*-dodecyl- β -D-maltoside. Insoluble material was removed by centrifugation and the supernatants were treated with or without 0.3 mM MTS-verapamil. His-tagged P-gps were then isolated by nickel-chelate chromatography. Equivalent amounts of P-gp were mixed with lipid, sonicated and assayed for ATPase activity in the absence of drug substrate. ND, not determined because of low expression. (B) Cys-less, Q725C, F728C and A729C P-gp mutants were treated with or without 3 mM MTS-verapamil and His-tagged P-gp isolated by nickel-chelate chromatography. Equivalent amounts of P-gp were mixed with lipid and ATPase activity determined in the presence (+) or absence (–) of 1 mM verapamil (Ver). The fold-stimulation is the ratio of activity of a sample treated with MTS-verapamil to that of an untreated sample. Each value is the mean $\pm$ S.D. ($n = 3$).

(> 11-fold stimulation; Figure 2B). Addition of unmodified verapamil did not increase their activities any higher. Therefore mutants Q725C and F728C were chosen for further investigation because their labelling with MTS-verapamil appeared to mimic the interaction of P-gp with unmodified verapamil.

We then tested whether drug substrates could protect mutants Q725C and F728C from being labelled by MTS-verapamil. HEK-293 cells expressing mutants Q725C or F728C were solubilized with *n*-dodecyl- β -D-maltoside and then treated with or without saturating levels of verapamil (3 mM), cyclosporin A (0.2 mM), colchicine (10 mM) or *trans*-(E)-flupentixol (0.6 mM). The samples were then treated with or without 0.3 mM MTS-verapamil for 10 min at 20°C. His-tagged P-gp was isolated by nickel-chelate chromatography to remove the drug substrate. Immunoblot analysis showed that the presence of drug substrate did not affect the yield of His-tagged P-gp (results not shown). The isolated P-gps were mixed with lipid and assayed for ATPase activity. Figure 3 shows that the substrates verapamil and cyclosporin A protected mutant F728C, but not mutant Q725C, from labelling with MTS-verapamil. Colchicine or *trans*-(E)-flupentixol, however, did not protect either mutant from labelling by MTS-verapamil. These results suggest that the verapamil covalently attached to Cys⁷²⁸ occupies the drug-binding pocket of P-gp, whereas labelling of Cys⁷²⁵ was non-specific. Therefore mutant F728C was selected for further analysis because some drug substrates protected it from labelling by MTS-verapamil.

The reaction of mutant F728C with higher concentrations of MTS-verapamil showed that maximum stimulation (11.5-fold) occurred in the presence of 3–10 mM MTS-verapamil (see Figure 1 of supplementary data at <http://www.BiochemJ.org/bj/399/bj3990351add.htm>). Half-maximal stimulation of ATPase activity occurred at 0.37 mM MTS-verapamil. We then confirmed that activation of mutant F728C was indeed due to covalent attachment of MTS-verapamil to Cys⁷²⁸. HEK-293 cells expressing mutant F728C were treated with 3 mM MTS-verapamil for 10 min at 20°C and His-tagged P-gp isolated by nickel-chelate chromatography. The isolated P-gp was mixed with lipid and was assayed for ATPase activity in the presence or absence of DTT (dithiothreitol) or in the presence of DTT and verapamil. Exposure of the MTS-verapamil labelled F728C mutant to DTT caused a 75% decrease in ATPase activity (see

Figure 3 Inhibition of MTS-verapamil labelling of mutants Q725C and F728C by substrates

HEK-293 cells expressing mutants Q725C or F728C were solubilized with *n*-dodecyl- β -D-maltoside. Insoluble material was removed by centrifugation. Equivalent amounts of supernatant were incubated for 10 min at 20°C in the presence of no drug (None), 3 mM verapamil (Ver), 0.2 mM cyclosporin A (Cyclo), 10 mM colchicine (Colch) or 0.6 mM *trans*-(E)-flupentixol (T-Flu). The mixtures were then incubated with or without 0.3 mM MTS-verapamil for 10 min at 20°C. His-tagged P-gps were isolated by nickel-chelate chromatography, mixed with an equal volume of lipid and assayed for ATPase activity. The activities are expressed relative to that of a sample not treated with MTS-verapamil. Each value is the mean $\pm$ S.D. ($n = 3$).

Supplementary Figure 2 at <http://www.BiochemJ.org/bj/399/bj3990351add.htm>). The decrease in ATPase activity was due to release of the covalently bound verapamil rather than denaturation of the protein, because the ATPase activity was restored when the DTT-treated mutant was assayed in the presence of verapamil (see Supplementary Figure 2 at <http://www.BiochemJ.org/bj/399/bj3990351add.htm>).

We then tested whether other drug substrates could stimulate further the ATPase activity of MTS-verapamil-treated mutant F728C. Drug substrates were selected that were expected to stimulate P-gp ATPase activity further (calcein-AM, demecolcine or verapamil) or inhibit its ATPase activity [cyclosporin A or *trans*-(E)-flupentixol] [35]. Mutant F728C was treated with or without

Figure 4 Effect of drug substrates and inhibitors on the ATPase activity of mutants F728C and Q725C before and after labelling with MTS-verapamil

HEK-293 cells expressing His-tagged mutants F728C or Q725C were solubilized with *n*-dodecyl- β -D-maltoside and then incubated in the absence (A and C) or presence (B and D) of 3 mM MTS-verapamil. P-gp was then isolated by nickel-chelate chromatography, mixed with *E. coli* lipids and ATPase activity measured in the absence (No Drug) or presence of 0.6 mM calcein-AM (CAM), 3 mM demecolcine (Deme), 1 mM verapamil (Ver), 0.2 mM cyclosporin A (Cyclo) or 0.6 mM *trans*-(E)-flupentixol (T-Flu). The fold-stimulation is the ratio of activity with drug substrate to that of the unlabelled sample assayed without drug substrate. Each value is the mean $\pm$ S.D. ($n = 3$).

3 mM MTS-verapamil and His-tagged P-gp was isolated by nickel-chelate chromatography. The isolated P-gp was mixed with lipids prepared from *E. coli* and assayed for ATPase activity in the presence of saturating concentrations of calcein-AM (0.6 mM), demecolcine (3 mM), verapamil (1 mM), cyclosporin A (0.2 mM) or *trans*-(E)-flupentixol (0.6 mM). We used *E. coli* lipids because the composition of lipid surrounding P-gp has been shown to influence its activity [38]. P-gp has higher basal activity in *E. coli* lipids that makes it easier to detect inhibition of ATPase activity, but drug-stimulated ATPase activity is not as high as observed with sheep brain lipid [38]. The activity of untreated mutant F728C was stimulated 6.0-fold in the presence of verapamil (Figure 4). Calcein-AM and demecolcine stimulated the ATPase activity by 7.8- and 7.3-fold respectively. Cyclosporin A and *trans*-(E)-flupentixol, however, reduced the ATPase activity below basal levels. When mutant F728C was treated with MTS-verapamil, its activity could not be increased or decreased further with calcein-AM, demecolcine or *trans*-(E)-flupentixol (Figure 4B). Cyclosporin A, however, caused a slight reduction (5.0-fold compared with 6.3-fold) in ATPase activity. These results suggest that attachment of MTS-verapamil to Cys⁷²⁸ either blocks entry of other drug substrates into the drug-binding pocket or that other drug substrates can still enter the drug-binding pocket but that the covalently-bound verapamil has a more dominant effect on ATPase activity. To distinguish between these possibilities, we compared the properties of mutant Q725C with mutant F728C. The activity of mutant Q725C was very similar to that of mutant F728C when assayed in the presence of calcein-AM, demecolcine, verapamil, cyclosporin A or *trans*-(E)-flupentixol before and after labelling with MTS-verapamil (Figures 4C and 4D). These results suggest that substrates may still be able to enter the drug-binding pocket of these mutants, but covalently-bound verapamil has a more dominant effect on ATPase activity.

Another approach to test whether Cys⁷²⁸ lines the drug-binding pocket is through disulfide cross-linking with cysteine residues in other TM segments facing the drug-binding pocket. Potential

Figure 5 Disulfide cross-linking of P-gp mutants

Membranes prepared from HEK-293 cells expressing mutants (A) L65C(TM1)/F728C(TM7), (B) I306C(TM5)/F728C(TM7), (C) F343C(TM6)/F728C(TM7), (D) L65C(TM1)/Q725C(TM7), (E) I306C(TM5)/Q725C(TM7) or (F) F343C(TM6)/Q725C(TM7) were treated with 1 mM copper phenanthroline (CuP), 0.2 mM of the homobifunctional disulfide cross-linker M5M, M8M, M11M, M14M or M17M, or no cross-linker (None) for 15 min at 4 °C. The reactions were stopped by addition of SDS sample buffer containing no reducing agent, and samples were subjected to immunoblot analysis on SDS/PAGE (7.5 % gels). The positions of the cross-linked (X-link) and mature (170 kDa) P-gps are indicated.

cysteines facing the drug-binding pocket that may be cross-linked with F728C are L65C(TM1), I306C(TM5) and F343C(TM6) because they were covalently modified with MTS-verapamil (L65C and I306C) or with MTS-Rhodamine (F343C). Labelling of these residues was also inhibited by drug substrates [27,36,39]. Cross-linking between cysteines in different domains of P-gp can be readily detected because the cross-linked protein migrates with altered mobility on non-reducing SDS/PAGE [40]. Accordingly, mutants L65C(TM1)/F728C(TM7), I306C(TM5)/F728C(TM7) and F343C(TM6)/F728C(TM7) were constructed and transiently expressed in HEK-293 cells. Membranes were prepared and treated with or without a zero-length cross-linker (copper phenanthroline) or with homobifunctional cross-linkers (M5M, M8M, M11M, M14M and M17M) containing spacer arms of 5–17 residues [14] at 4 °C for 15 min. Cross-linking was performed at 4 °C to reduce thermal motion in the protein. The reactions were stopped by addition of SDS sample buffer containing no reducing agent, and samples were subjected to immunoblot analysis. Figure 5(A) shows that little cross-linked product was detected with mutant L65C(TM1)/F728C(TM7). Mutant I306C(TM5)/F728C(TM7) showed about 50 % cross-linking with all of the homobifunctional cross-linkers (Figure 5B). Mutant F343C(TM6)/F728C(TM7) was most efficiently (> 80 %) cross-linked with M11M, M14M and M17M (Figure 5C). None of the mutants were cross-linked by copper phenanthroline (Figures 5A–5C). Mutant F343C(TM6)/F728C(TM7) was then selected for

Figure 6 Effect of substrates on cross-linking of mutants F343C(TM6)/F728C(TM7) or F343C(TM6)/Q725C(TM7)

(A and C) Membranes prepared from HEK-293 cells expressing mutants F343C(TM6)/F728C(TM7) or F343C(TM6)/Q725C(TM7) were pre-incubated for 10 min at 20 °C with no drug (None), 10 mM colchicine (Colch), 0.05 mM cyclosporin A (Cyclo), 2 mM Rhodamine B (Rhod), 1 mM MTS-verapamil (Ver), 0.2 mM vinblastine (Vin), 3 mM demecolcine (Deme), 0.3 mM Hoechst 33342 (Hoech), 0.6 mM *trans*-(E)-flupentixol (T-Flu), 0.6 mM *cis*-(Z)-flupentixol (C-Flu) or 0.6 mM calcein-AM (Calcein). The samples were cooled to 4 °C and then treated with (+) or without (–) 0.2 mM M14M cross-linker for 2 min at 4 °C. The reactions were stopped by addition of SDS sample buffer containing no reducing agent, and samples were subjected to immunoblot analysis on SDS/PAGE (7.5 % gels). The positions of the cross-linked (X-link) and mature (170 kDa) P-gps are indicated. (B and D) The relative amount of cross-linking was determined by scanning the gel lanes, and the results expressed as the amount of cross-linked product in the presence of drug substrate to that cross-linked with no drug substrate present (None taken as 100 %).

further study, as it was the most efficiently cross-linked mutant. Cross-linking was due to linkage between Cys³⁴³ and Cys⁷²⁸ because cross-linked product was not detected on SDS/PAGE when membranes prepared from HEK-293 cells transfected with the F343C or F728C single cysteine mutant cDNAs, or co-transfected with the F343C and F728C mutant cDNAs, were treated with the homobifunctional cross-linkers (results not shown).

If Cys³⁴³(TM6) and Cys⁷²⁸(TM7) line the drug-binding pocket, then drug substrates should be able to inhibit cross-linking between these cysteines. Therefore membranes prepared from HEK-293 cells expressing mutant F343C(TM6)/F728C(TM7) were pre-incubated with or without the drug substrates colchicine, cyclosporin A, Rhodamine B, MTS-verapamil, vinblastine, demecolcine, Hoechst 33342, *trans*-(E)-flupentixol, *cis*-(Z)-flupentixol or calcein-AM for 10 min at 20 °C. The mixtures were then cooled to 4 °C and treated with M14M cross-linker for 2 min at 4 °C. Samples were then subjected to immunoblot analysis. Figure 6(A) shows that pre-treatment of the membranes with MTS-verapamil completely inhibited cross-linking by M14M. This indicated that cross-linking was specific to cysteine residues. A greater than 2-fold reduction in cross-linking was observed when membranes were pre-treated with cyclosporin A, Rhodamine B, vinblastine or calcein-AM (Figure 6B). Little or no inhibition of cross-linking was detected with colchicine,

demecolcine, Hoechst 33342 or the stereoisomers of flupentixol. These results suggest that linkage of Cys³⁴³(TM6) to Cys⁷²⁸(TM7) with M14M overlaps the drug-binding sites of cyclosporin A, Rhodamine B and calcein-AM, but not that of colchicine, demecolcine, Hoechst 33342 or the stereoisomers of flupentixol. The cross-linking results are consistent with the drug-protection studies (Figure 3) in that protection was observed with cyclosporin A but not with colchicine or *trans*-(E)-flupentixol.

For comparison, we tested the cross-linking characteristics of mutant Q725C with a second cysteine residue introduced at positions Leu⁶⁵, Ile³⁰⁶ or Phe³⁴³. Figure 5(D) shows that mutant L65C(TM1)/Q725C(TM7) had relatively weak cross-linking with M14M and M17M, whereas mutants I306C(TM5)/Q725C(TM7) (Figure 5E) and F343C(TM6)/Q725C(TM7) (Figure 5F) showed strong cross-linking with M8M, M11M, M14M and M17M cross-linkers. Mutants F343C(TM6)/F728C(TM7) (Figures 6A and 6B) and F343C(TM6)/Q725C(TM7) (Figures 6C and 6D) were then cross-linked with M14M in the presence of various drug substrates. Figure 6 shows that the drug substrates were less effective in protecting mutant F343C(TM6)/Q725C(TM7) from cross-linking by M14M when compared with mutant F343C(TM6)/F728C(TM7). These results are consistent with those in Figure 3, and suggest that Cys⁷²⁸ lines the drug-binding pocket.

We then tested whether cross-linking of mutant F343C(TM6)/F728C(TM7) by M14M cross-linker was affected by vanadate

Figure 7 Effect of vanadate trapping of nucleotide on cross-linking of mutant F343C(TM6)/F728C(TM7)

(A) Membranes prepared from HEK-293 cells expressing mutant F343C(TM6)/F728C(TM7) were pre-incubated for 20 min at 4°C with no addition (None) or with 5 mM ATP (ATP), 10 mM MgCl₂ and 5 mM ATP (Mg.ATP), 10 mM MgCl₂ and 5 mM ADP (ADP), 10 mM MgCl₂ and 0.2 mM sodium orthovanadate (VO₄) or 10 mM MgCl₂ and 5 mM adenosine 5'-[β , γ -imino]triphosphate (AMP.PNP). One sample was incubated with 10 mM MgCl₂, 5 mM ATP and 0.2 mM sodium orthovanadate (ATP/VO₄) at 37°C for 10 min to convert P-gp into the vanadate-trapped transition state. The samples were cooled to 4°C and then treated with (+) 0.2 mM M14M cross-linker for 2 min at 4°C. The reactions were stopped by addition of SDS sample buffer containing no reducing agent, and samples were subjected to immunoblot analysis on SDS/PAGE (7.5% gels). The positions of the cross-linked (X-link) and mature (170 kDa) P-gps are indicated. (B) The relative amount of cross-linking was determined by scanning the gel lanes, and the results expressed as the amount of cross-linked product in the presence of drug substrate to that cross-linked with no drug substrate present (None taken as 100%).

trapping of nucleotides. Vanadate traps ADP at either NBD by mimicking the transition state of the γ -phosphate of ATP during ATP hydrolysis. Vanadate trapping at one NBD inhibits hydrolysis at the second site [41]. Accordingly, membranes prepared from HEK-293 cells expressing mutant F343C(TM6)/F728C(TM7) were pre-incubated with or without ATP plus vanadate for 10 min at 37°C to promote vanadate trapping of nucleotides. Incubation of P-gp under these conditions causes over 90% inhibition of P-gp ATPase activity [31]. The samples were chilled to 4°C and then treated with M14M cross-linker for 2 min at 4°C. The reactions were stopped by addition of SDS sample buffer containing no reducing agent, and samples were subjected to immunoblot analysis. Figure 7 shows that vanadate trapping of nucleotides inhibited cross-linking by about 80%. Inhibition of cross-linking was due to vanadate trapping because little

Figure 8 Residues in TM1 and TM7 arranged as α -helices

The residues of TM1 and TM7 are arranged as cylindrical helices and alignment is based on amino acid identity. Residues that show alterations in their ATPase activity after reaction with MTS-verapamil or show disulfide cross-linking are shown occupying one face of the helix (enclosed in a large circle). Black circles indicate the positions of residues that are fully activated after reaction with MTS-verapamil and whose labelling by MTS-verapamil is blocked by drug substrate. The dark grey circle indicates the position of a cysteine that is highly activated by MTS-verapamil, but whose labelling by MTS-verapamil is not protected by drug substrate. The light grey circles indicate the positions of cysteines that show inhibition of verapamil-stimulated (729) or basal ATPase activity (726) after reaction with MTS-verapamil. Residues 68 and 69 in TM1 (in bold) when changed to cysteine could be cross-linked to cysteines in TM11 during ATP hydrolysis [43].

inhibition (< 20%) was observed in the presence of ATP, ADP, vanadate or the non-hydrolysable ATP analogue, adenosine 5'-[β , γ -imino]triphosphate (AMP.PNP) (Figure 7).

DISCUSSION

The results from three different methods suggest that the extracellular end of TM7 may play a direct role in P-gp-drug substrate interactions. Introduction of cysteine residues at positions 728 and 729 reduced the apparent affinity for drug substrates, especially verapamil. Covalent modification of Cys⁷²⁸, and to a lesser extent Cys⁷²⁵ and Cys⁷²⁹, activated P-gp ATPase activity. Finally, cross-linking between mutant F343C(TM6)/F728C(TM7) could be inhibited by some drug substrates.

TM7, like TM1 in TMD1 [27], appears to form part of the drug-binding pocket of P-gp. When the residues in TM7 and TM1 are arranged as α -helices, the thiol-reactive residues in TM7 and TM1 are clustered towards the extracellular ends of the TM segments (Figure 8). Each TM segment contained one cysteine residue (Cys⁶⁵ in TM1 and Cys⁷²⁸ in TM7) that was covalently labelled with MTS-verapamil in a drug-protectable manner, resulting in highly stimulated ATPase activity.

The orientation of the covalently attached verapamil may play an important role in the activation of P-gp ATPase activity. Attachment of MTS-verapamil to Cys⁷²⁸ caused greater than 10-fold activation of ATPase activity (Figure 2B) and is comparable with the level of activity observed when unmodified P-gp is assayed in the presence of verapamil. This is in contrast with the activity of MTS-verapamil-treated mutant F729C which was activated only about 4.5-fold (Figure 2B). The lower activity observed in this mutant was not due to incomplete reaction with MTS-verapamil because the activity was not increased further

after the reaction with 3 mM MTS-verapamil or when the mutant was modified with 3 mM MTS-verapamil and assayed with a saturating concentration of verapamil (1 mM) (Figure 2B). These results suggest that the verapamil covalently linked to Cys⁷²⁹ is in a sub-optimal orientation for stimulation of ATPase activity. Labelling of one mutant, P726C, with MTS-verapamil resulted in inhibition of the basal activity of P-gp (Figure 2A). Figure 8 shows that residue 726 is predicted to lie on another face of the helix and away from the predicted drug-binding pocket. Therefore an explanation for inhibition of ATPase activity after MTS-verapamil treatment of mutant P726C is that the bound verapamil molecule interferes with TM conformational changes associated with ATP hydrolysis [42–44].

Labelling of Cys⁷²⁵ appeared to be non-specific because labelling could not be blocked with verapamil. Residue Gln⁷²⁵ did not appear to directly participate in drug binding since the mutant Q725C did not show any changes in apparent affinity for verapamil, vinblastine or colchicine (Table 1). It is also possible that the binding sites for verapamil and MTS-verapamil are not identical because of the addition of the thiol-reactive arm in MTS-verapamil.

Inhibition of cross-linking of mutant F343C(TM6)/F728C(TM7) after vanadate trapping of nucleotides (Figure 7) indicates that TM7 undergoes conformational changes during the reaction cycle and that there is cross-talk between the TMDs and NBDs of P-gp. It is unlikely that covalent modification of Cys⁷²⁸ with MTS-verapamil changed the affinity of P-gp for ATP. We found that the K_m of mutant F728C for ATP remained at about 1 mM before and after modification with MTS-verapamil (results not shown). These results are similar to those observed when I306C(TM6) was modified with MTS-verapamil [36]. A large change in affinity for ATP may not be expected with verapamil since it has been shown recently by fluorescence resonance energy transfer [45] or electron spin resonance [46] that verapamil binding to P-gp increased the affinity for ATP by only 20–40%. Binding of some drugs such as colchicine to P-gp, however, can increase the affinity of ATP 4-fold.

A recent structure of the bacterial ABC transporter MsbA complexed with ADP/vanadate and lipopolysaccharide further supports the prediction that TM1 and TM7 of P-gp are involved in binding of drug substrates [47]. MsbA has one TMD (six TM segments) and one NBD but functions as a homodimer. MsbA has amino acid sequence and reaction characteristics such as substrate-stimulation and vanadate-inhibition of ATPase activity that are similar to those of P-gp [48]. An important observation in the structure of MsbA bound to substrate was that the two bound lipopolysaccharide molecules showed extensive contacts with the two TM1 segments of the homodimer. What does the ability of MTS-verapamil to permanently activate P-gp ATPase activity tell us about the mechanism of P-gp? It indicates that binding of only one drug substrate molecule is sufficient to stimulate ATPase activity and that it can do so by binding in several different orientations as long as it does not interfere with movement of the TM segments during the reaction cycle. Modification of residues such as Cys⁷²⁸ with MTS-verapamil resulting in highly-stimulated ATPase activity suggests that P-gp is an unusual ATP-dependent transporter in that its ATPase activity can be uncoupled from drug transport. Indeed, P-gp will continuously hydrolyse ATP at a low rate in the absence of any drug substrate [49]. It has been proposed that the low coupling efficiency of P-gp may be needed to enable it to recognize such a wide range of substrates, i.e. the broad specificity of P-gp has come about at the expense of its coupling efficiency [49].

Further studies are needed to explore whether the low coupling efficiency of P-gp could be exploited during chemotherapy of

cancers that overexpress P-gp. ATP is inefficiently generated in cancer cells through the Warburg effect [50]. It may be possible to uncouple P-gp ATPase activity from drug transport so that its high rate of ATPase activity could deplete ATP levels within these cells and make them more susceptible to chemotherapeutic agents.

This work was supported by grants from the National Cancer Institute of Canada through the Canadian Cancer Society and from the Canadian Institutes of Health Research. D. M. C. is the recipient of the Canada Research Chair in Membrane Biology.

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Received 16 May 2006/20 June 2006; accepted 3 July 2006

Published as BJ Immediate Publication 3 July 2006, doi:10.1042/BJ20060715