Drug Binding in Human P-glycoprotein Causes Conformational Changes in Both Nucleotide-binding Domains

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Running Title: Cross-talk between ATP- and drug-binding sites of P-gp
Summary

The human multidrug resistance P-glycoprotein (P-gp, ABCB1) uses ATP to transport many structurally diverse compounds out of the cell. It is an ABC transporter with two nucleotide-binding domains (NBD) and two transmembrane domains. Recently, we showed that the LSGGQ motif in one NBD (531^LSGGQ_535 in NBD1; 1176^LSGGQ_1180 in NBD2) is adjacent to the Walker A sequence (1070^GSSGCGKS_1077 in NBD2; 427^GNSGCGKS_434 in NBD1) in the other NBD (Loo, T.W., et al. (2002) J. Biol. Chem. 277, 41303-41306). Drug substrates can stimulate or inhibit the ATPase activity of P-gp. Here, we report the effect of drug-binding on cross-linking between the “LSGGQ signature” and “Walker A” sites (C431(NBD1)/C1176C(NBD2) and C1074(NBD2)/L531C(NBD1)). Seven drug substrates (calcein-AM, demecolcine, cis(Z)-flupentixol, verapamil, cyclosporin A, Hoechst 33342 and trans(E)-flupentixol) were tested for their effect on oxidative cross-linking. Substrates that stimulated the ATPase activity of P-gp (calcein-AM, demecolcine, cis(Z)-flupentixol and verapamil) increased the rate of cross-linking between C431(NBD1-Walker A)/C1176C(NBD2-LSGGQ) and between C1074(NBD2-Walker A)/L531C(NBD1-LSGGQ) when compared to cross-linking in the absence of drug substrate. By contrast, substrates that inhibited ATPase activity (cyclosporin A, Hoechst 33342 and trans(E)-flupentixol) decreased the rate of cross-linking. These results indicate that interaction between the LSGGQ motifs and Walker A sites must be essential for coupling drug-binding to ATP hydrolysis. Drug-binding in the TM domains can induce long-range conformational changes in the NBDs,
such that compounds that stimulate or inhibit ATPase activity must decrease and increase respectively, the distance between the Walker A and LSGGQ sequences.
Introduction

P-glycoprotein (P-gp) is an ATP-dependent drug pump that transports numerous structurally diverse compounds of different sizes out of the cell (recently reviewed in (1,2)). Therefore, P-gp can complicate cancer and AIDS chemotherapy because many therapeutic compounds are substrates of P-gp (3,4).

P-gp is a single polypeptide of 1280 amino acids. It is organized as two repeating units of 610 amino acids that are joined by a linker region of about 60 amino acids (5). Each repeat has six transmembrane (TM) segments and a hydrophilic domain containing an ATP-binding site (6,7). P-gp functions as a monomer (8), but the two halves of the molecule do not have to be covalently linked for function (9,10). The transmembrane domains alone are sufficient to mediate drug-binding (10), but both ATP-binding sites must be functional for drug efflux activity (11-14).

An important aspect in understanding the mechanism of P-gp is how drug transport is coupled to ATP hydrolysis. The observations that drug binding to P-gp can either stimulate or inhibit ATP hydrolysis suggests that drug-binding and ATP hydrolysis must be tightly regulated (12,15,16).

The “signature” sequence (LSGGQ) in each NBD appears to be an important region in P-gp. Although the signature sequences are present in all ABC transporters (17), their function is unknown. We recently showed that the LSGGQ in one NBD was close to the Walker A sequence in the other NBD (18). We postulated that the LSGGQ sequence might play a role in conveying conformational changes from the drug-binding site to the ATP-binding sites.
In this study, we examined the effect of drug substrates on cross-linking between the LSGGQ motifs and the Walker A sites.
Materials and Methods

Construction of Mutants – A histidine-tagged Cys-less P-gp was constructed and then used for making mutants containing pairs of cysteines (6,19,20). Two mutants that contained a cysteine in the LSGGQ site and another in the Walker A site were constructed (18). One mutant (L531C/C1074) contained a cysteine in the NH2-terminal \textsuperscript{531}LSGGQ\textsuperscript{535} site and an endogenous cysteine (C1074) in the COOH-terminal Walker A site (\textsuperscript{1070}GSSGCGKC\textsuperscript{1077}). The other mutant (C431/L1176C) contained the endogenous C431 in the NH\textsubscript{2}-terminal Walker A site (\textsuperscript{427}GNSGCGKS\textsuperscript{434}) and another cysteine in the COOH-terminal \textsuperscript{1176}LSGGQ\textsuperscript{1180} site.

Expression, Disulfide Cross-linking Analysis and Purification – The mutant cDNAs were expressed in HEK 293 cells in the presence of cyclosporin A to promote maturation of P-gp (21,22). Membranes were prepared as described previously (19,23). For disulfide cross-linking analysis, aliquots of membranes were added to equal volumes of TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 1 mM \textsuperscript{2+}Cu(phenanthroline)\textsubscript{3}. The samples were incubated at 21 °C or 4 °C for various intervals and the reactions stopped by addition of 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 subjected to SDS-PAGE (7.5% polyacrylamide gels) and immunoblot analysis with a rabbit polyclonal antibody against P-gp (8).
To test the effect of nucleotide or vanadate on cross-linking, the membranes were incubated with an equal volume of TBS containing the following: 1) 12 mM ATP, 24 mM MgCl₂ and 0.6 mM sodium orthovanadate; 2) 12 mM ATP and 24 mM MgCl₂; 3) 12 mM ATP; 4) 24 mM MgCl₂; 5) 0.6 mM sodium orthovanadate; 6) 12 mM ADP, or 7) 12 mM AMP-PNP. Sodium orthovanadate was prepared from Na₃VO₄, pH 10 (24) and boiled for 2 min to break down polymeric species (25). The samples were incubated for 10 min at 37 °C and then cooled in an ice-bath before treatment with oxidant at 21 °C. At this temperature, there is almost complete cross-linking in both mutants (18).

To test the effect of drug substrates on cross-linking, the mutant P-gps were preincubated with drug substrate for 10 min at 21 °C, then chilled at 4 °C for 10 min and then treated with oxidant. At 4 °C, the rate of cross-linking is also slowed and this allowed us to detect changes in cross-linking.

Purification of histidine-tagged P-gp mutants and assay of drug-stimulated ATPase activities were done as described previously (23,26) except that the isolated samples were mixed with *Escherichia coli* lipid rather than sheep brain phosphatidylethanolamine. *Escherichia coli* lipids were used because basal P-gp ATPase activity is higher in these lipids than with sheep brain phosphatidylethanolamine. This made measurement of inhibition of P-gp ATPase activity much easier. Also drug-stimulated ATPase activity of P-gp reconstituted with *Escherichia coli* lipids is similar to that measured in isolated mammalian plasma membranes that are enriched in P-gp (15).
Results

We previously showed by disulfide cross-linking analysis that the contact between the NBDs of Pgp could occur between the LSGGQ signature sequence in one NBD and the Walker A site in the other NBD (18). Mutants in which the leucine residue in the LSGGQ site is replaced with cysteine can be oxidatively cross-linked with the endogenous cysteine in the opposing Walker A sequence ((L531C(NBD1-LSGGQ)/C1074(NBD2-Walker A) or C431(NBD1-Walker A)/L1176C(NBD2-LSGGQ)). Fig. 1 shows that that cross-linking was almost complete in mutants L531C/C1074 and C431/L1176C when treated with 0.5 mM copper phenanthroline for 15 min at 21 °C. We previously showed that cross-linking occurred in the active molecule because cross-linking resulted in an inactive molecule. Activity was restored, however, after addition of dithiothreitol (18).

To determine whether cross-linking between the NBDs could be disrupted, the mutants were pre-treated with nucleotide or subjected to vanadate trapping. P-gp traps nucleotide in the presence of vanadate plus Mg.ATP and results in a transition state (27,28). Vanadate traps ADP at either NBD by occupying the position of the γ-phosphate adjacent to ADP. Vanadate trapping at one site then inhibits ATP hydrolysis at the second ATP-binding site (27). Fig. 1 shows that inhibition of cross-linking in mutants L531C/C1074 and C431/L1176C was observed only after treatment with vanadate plus Mg.ATP. Inhibition of cross-linking was not observed when the mutants were pre-treated with ATP, MgCl₂, vanadate, Mg.ATP, ADP or with the non-hydrolyzable ATP
analog AMP-PNP. It is unlikely that vanadate trapping of nucleotide denatures the protein since trapping of nucleotide is reversible (27).

We had proposed that the "LSGGQ' motifs might participate in transmitting conformational changes from the transmembrane domains to the NBDs (18). Mutations in the LSGGQ motifs do not prevent ATP binding or vanadate-trapping of nucleotide (29,30). One way of inducing different conformational changes in the TMDs is to use drug substrates with different structures. P-gp is interesting in that some drug substrates stimulate while others inhibit the ATPase activity of P-gp. Therefore, it is possible that the conformational changes in the TMDs may be monitored by changes in the cross-linking patterns in mutants L531C/C1074 and C431/L1176C.

Drug substrates (1) that either stimulated (calcein-AM, demecolcine, cis(Z)-flupentixol and verapamil) or inhibited (cyclosporin A, Hoechst 33342 and trans(E)-flupentixol) the ATPase activity of Cys-less P-gp (data not shown) were identified. The flupentixol isomers are interesting in that the cis(Z)-isomer stimulates, while the trans(Z)-isomer inhibits the ATPase activity of wild-type P-gp (31). These drug substrates were then tested on mutants L531C/C1074 and C431/L1176C. Fig. 2 shows that the ATPase activity of mutant L531C/C1074 was stimulated by calcein-AM, demecolcine, cis(Z)-flupentixol and verapamil (6.4-, 6.8-, 3.5- and 4-fold, respectively). Half-maximal stimulation of ATPase activity with calcein-AM, demecolcine, cis(Z)-flupentixol and verapamil occurred at 30 µM, 163 µM, 29 µM and 9 µM, respectively. Cyclosporin A, Hoechst 33342 and trans(E)-flupentixol inhibited the activity of mutant L531C/C1074 with 50% inhibition occurring at concentrations of about 0.12, 0.67 and
1.1 mM, respectively. Similar results were obtained with mutant C431/L1176C (data not shown).

We then tested whether the structurally different stimulatory and inhibitory drug substrates affected cross-linking in the NBDs. Accordingly, membranes from mutant L531C/C1074 were preincubated for 10 min at 21 °C with 1 mM calcein-AM, 2 mM demecolcine, 1 mM cis(Z)-flupentixol, 0.2 mM verapamil, 0.5 mM cyclosporin A, 0.5 mM Hoechst 33342 or 1 mM trans(E)-flupentixol. These concentrations were required for maximal stimulation or inhibition of ATPase activity (Fig. 2). The membranes were then treated with oxidant at 4 °C for various intervals. The rationale for doing the cross-linking at 4 °C was that thermal motion in the protein would be reduced and that subtle changes caused by drug substrate binding may be detected. Fig. 3 shows the effect of substrate on cross-linking of mutant L531C/C1074. In the absence of drug substrate, about 50% of the mutant protein was cross-linked by 16 min. In the presence of compounds (calcein-AM, demecolcine, cis(Z)-flupentixol and verapamil) that stimulate the ATPase activity of P-gp, however, the rate of cross-linking was significantly increased so that 50% cross-linking occurred by 2 min. The presence of the inhibitory compounds (cyclosporin A, Hoechst 33342 and trans(E)-flupentixol) had the opposite effect. In the presence of these inhibitors, less than 50% cross-linking was observed at 32 min (Fig. 3).

We then tested the effect of drug substrates on cross-linking of mutant C431/L1176C. In the absence of drug substrates, 50% of the mutant protein was cross-linked with oxidant after 16 min (Fig. 4). In the presence of the stimulatory drug
substrates (calcein-AM, demecolcine, cis(Z)-flupentixol and verapamil), the rate of cross-linking was increased since 50% of the mutant protein was cross-linked by 2-4 min. Drug substrates that inhibited ATPase activity (cyclosporin A, Hoechst 33342 and trans(E)-flupentixol) of the mutant P-gp also inhibited the cross-linking of the mutant protein. Fig. 4 shows that in the presence of these compounds, less than 50% cross-linking occurred at 32 min.

The effect of substrates on mutants L531C/C1074 and C431/L1176C were very similar. Compounds that stimulated the ATPase activity also stimulated the rate of cross-linking of the mutants, while those that inhibited ATPase activity also inhibited the rate of cross-linking. It is unlikely that drug substrates are binding directly to the ATP-binding sites, since it has been shown that drug binding does not alter the affinity for ATP (15,16,32).
Discussion

Disulfide cross-linking between adjacent cysteines in the Walker A sequence of one NBD and the “LSGGQ” site in the other NBD is a useful approach for monitoring changes in the NBDs. A condition that dramatically affects cross-linking between these two sites occurs after vanadate trapping of nucleotide (Fig. 1). Hydrolysis of ATP in the presence of vanadate was essential for inhibition of cross-linking because cross-linking was not inhibited in the presence of the non-hydrolyzable ATP analog, AMP.PNP. Similarly, cross-linking was not inhibited when the mutants were pre-incubated with only, ATP, MgCl₂, ADP, vanadate or Mg.ATP. These results indicate that the trapped vanadate either occupies the space between the cross-linkable cysteines in the LSGGQ and Walker A sites in the two NBDs or that its presence causes the two NBDs to move apart.

The drug-binding site in the TMDs (33,34) and the ATP binding sites in P-gp must be quite far apart. Fluorescence resonance energy transfer studies indicate that the ATP-binding sites are about 40 Angstrom from the drug-binding site (35). Our results show that binding of drug substrates must induce conformational changes in the drug-binding site that are transmitted distally to the NBDs. Similarly, conformational changes in the NBDs are also transmitted to the drug-binding site in the TMDs (25,28). Therefore, there must be continuous “cross-talk” among the domains of P-gp.

The LSGGQ sequence can increase or decrease the rate of ATP hydrolysis depending on its distance from the Walker A site as shown in Fig. 5. Inhibitory
substrates may cause both of the Walker A and LSGGQ sites to move farther apart and/or reduce the rate of ATP hydrolysis. In this study, the inhibitory substrates reduced the rate of cross-linking and this may occur by moving the two NBDs apart (Figs. 3 and 4). Drug substrates that stimulate ATPase activity of P-gp must bring the Walker A and LSGGQ sites closer together so that hydrolysis of ATP occurs at a faster rate.

There is no detailed crystal structure information about eukarytic ABC transporters. Recent crystal structure studies on other ABC transporters, Rad50cd (36), BtuCD (37) and MJ0796 (38), however, show that the LSGGQ sequences in these proteins are located adjacent to the γ-phosphate of ATP. It is likely that as the LSGGQ motif moves closer to the Walker A site, the rate of ATP hydrolysis increases. Our results support this idea and may explain why a substrate is a stimulator or inhibitor of P-gp. These results could form the basis for the development of better inhibitors of P-gp.
Footnotes

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**Abbreviations:** The abbreviations used are: P-gp, P-glycoprotein; ABC, ATP-Binding Cassette; NBD, nucleotide-binding domain; NBD1, NH$_2$-terminal NBD; NBD2, COOH-terminal NBD; TM, transmembrane; HEK, human embryonic kidney;
Figure Legends

Fig. 1. Cross-linking of P-gp mutants. Membranes were prepared from HEK 293 cells expressing mutants (A) L531C/C1074 or (B) C431/L1176C. The membranes were preincubated with no additions (None), or with Mg.ATP plus vanadate (Mg.ATP+Vi), ATP (ATP), MgCl₂ (Mg), vanadate (Vi), MgCl₂ plus ATP (Mg.ATP), ADP (ADP) or AMP.PNP (AMP.PNP) for 10 min at 37 °C. The membranes were then treated with oxidant for 15 min at 21 °C. The reactions were stopped by addition of SDS sample buffer containing 50 mM EDTA and no reducing agent. The mixtures were subjected to immunoblot analysis. The positions of the cross-linked (X-link) product and mature (170 kDa) P-gp are indicated. CuP; copper phenanthroline.

Fig. 2. Effect of drug substrates on the ATPase activity of P-gp mutant L531C/C1074. Histidine-tagged mutant L531C/C1074 was isolated by nickel-chelate chromatography, mixed with Escherichia coli lipids and sonicated. ATPase activity was determined in the presence of various concentrations of drug substrates. Fold-stimulation is the ratio of the activity with drug to that without drug substrate.

Fig. 3. Effect of drug substrate on cross-linking of mutant L531C/C1074. Membranes containing P-gp mutant L531C/1074 were pre-incubated with no drug, calcein-AM, demecolcine, cis(Z)-flupentixol, verapamil, cyclosporin A, Hoechst 33342 or trans(E)-flupentixol and then treated with oxidant at 4 °C for the indicated times. The
reactions were stopped by addition of SDS sample buffer containing 50 mM EDTA and no reducing agent. The mixtures were subjected to immunoblot analysis. The positions of the cross-linked (X-link) product and mature (170 kDa) P-gp are indicated.

**Fig. 4. Effect of drug substrates on cross-linking of mutant C431/L1176C.** Cross-linking on membranes containing P-gp mutant C431/L1176C were done as described in the legend to Fig. 3.

**Fig. 5. Model for coupling drug-binding to conformational changes in the NBDs.** In the absence of drug substrates, C431 in the NBD1-Walker A site is adjacent to C1176 in the NBD2-LSGGQ site, while C1074 in the NBD2-Walker A site is adjacent to the C531 in the NBD1-LSGGQ site. Binding of a stimulatory substrate brings the adjacent cysteines closer together so that cross-linking is enhanced. An inhibitory substrate causes the adjacent cysteines to move farther apart and hence reduced cross-linking.
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Fig. 1
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