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P-glycoprotein ATPase activity requires lipids to activate a switch at the first transmission interface

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ABSTRACT

P-glycoprotein (P-gp) is an ABC (ATP-Binding Cassette) drug pump. A common feature of ABC proteins is that they are organized into two wings. Each wing contains a transmembrane domain (TMD) and a nucleotide-binding domain (NBD). Drug substrates and ATP bind at the interface between the TMDs and NBDs, respectively. Drug transport involves ATP-dependent conformational changes between inward-(open, NBDs far apart) and outward-facing (closed, NBDs close together) conformations. P-gps crystallized in the presence of detergent show an open structure. Human P-gp is inactive in detergent but basal ATPase activity is restored upon addition of lipids. The lipids might cause closure of the wings to bring the NBDs close together to allow ATP hydrolysis. We show however, that cross-linking the wings together did not activate ATPase activity when lipids were absent suggesting that lipids may induce other structural changes required for ATPase activity. We then tested the effect of lipids on disulfide cross-linking of mutants at the first transmission interface between intracellular loop 4 (TMD2) and NBD1. Mutants L443C/S909C and L443C/R905C but not G471C/S909C and V472C/S909C were cross-linked with oxidant when in membranes. The mutants were then purified and cross-linked with or without lipids. Mutants G471C/S909C and V472C/S909C cross-linked only in the absence of lipids whereas mutants L443C/S909C and L443C/R905C were cross-linked only in the presence of lipids. The results suggest that lipids activate a switch at the first transmission interface and that the structure of P-gp is different in detergents and lipids.

1. Introduction

P-glycoprotein (P-gp) is an ABC (ATP-binding cassette) protein that can pump a wide variety of cytotoxic compound out of the cell. Overexpression of P-gp can lead to the phenomenon of multidrug resistance during chemotherapy [1]. P-gp contains two homologous halves with each half containing six transmembrane (TM) segments and a nucleotide-binding domain (NBD) [1]. The two halves are inactive when expressed as separate proteins but will form an active transporter when the two halves are co-expressed in the same cell [2]. The two halves form two wings. One wing consists of NBD1 and transmembrane (TM) segments 1, 2, 3, 6, 10 and 11 while the other consists of NBD2 and TMs 4 5, 7, 8, 9 and 12. The intracellular (ICL) loops between TMs 2/3 (ICL1) and 10/11(ICL4) interact with NBD1 to form the first transmission interface while the loops between TMs 4/5 (ICL2) and 8/9 (ICL3) interact with NBD2 to form the second transmission interface [3]. Drug substrates bind at the interface between the TMDs [4]. P-gp acts as a membrane 'vacuum cleaner' [5] that extracts drug substrates out of the inner leaflet of the lipid bilayer [6]. Drug substrates activate ATPase activity [7]. The catalytic cycle of P-gp transport is predicted to be similar to other ABC exporters [8]. The mechanism involves conformational changes occurring when drug substrate is bound to P-gp in the open state and when it is released to the cell exterior during the closed state (Fig. 1A).

Crystal structures for mouse [9] and Caenorhabditis elegans P-gps [10] in open inward-facing conformations are consistent with the results of many biochemical studies [11–15]. The crystal structures however, were determined in the presence of detergent. A recent study on MsbA suggested that ABC crystal structures obtained in detergent may not be physiological because of the large separation of the NBDs [16].

We have observed that purified human P-gp exhibits negligible basal ATPase activity in the absence of lipids. It is possible that the

Abbreviations: ABC, ATP-binding cassette; P-gp, P-glycoprotein; NBD, nucleotide-binding domain; HEK, human embryonic kidney; TM, transmembrane; TMD, transmembrane domain; ICL, intracellular loop.

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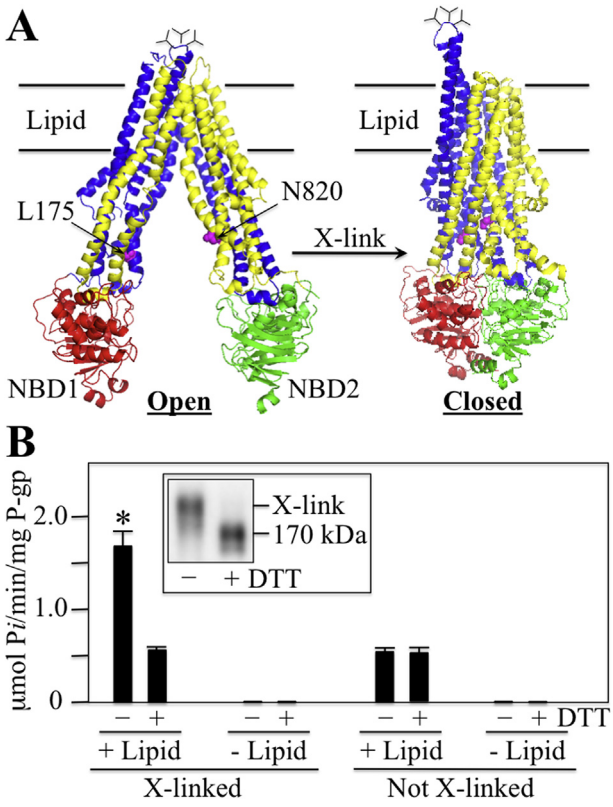

Fig. 1. Activation of P-gp ATPase activity after clamping the wings together requires lipid. (A) Predicted structures of human P-gp in the open [10] and the closed conformations [38]. The models were viewed using the PyMol system [39]. TMs 1–6 are shown in blue and TMs 7–12 are shown in yellow. The branched lines between TM segments 1 and 2 represent glycosylated sites. The L175C(ICL1)/N820C(ICL3) cross-linkable cysteines are located in the cytoplasmic loops that connects the TMDs to the NBDs. (B) Membranes prepared from HEK 293 cells expressing mutant L175C/N820C were treated in the absence (Not X-linked) or presence (X-linked) of M4M cross-linker and the P-gps were isolated by nickel-chelate chromatography. Samples were subjected to immunoblot analysis after treatment with (+) or without (−) 10 mM dithiothreitol (DTT). The locations of mature (170 kDa) and cross-linked (X-link) P-gps are indicated (inset). The ATPase activities of the cross-linked and uncross-linked P-gps were determined in the presence (+Lipid) or absence (- Lipid) of lipids after treatment with (+) or without (−) 10 mM dithiothreitol (DTT). Each value is the mean ± S.D. (n = 3). The asterisk indicates significant difference ($P < 0.001$) relative to the sample treated with DTT as well as to those measured without lipid.

Walker A and the opposing LSGGQ sites in the NBDs are too far apart (>30 Å) in detergent for ATP hydrolysis [10]. To test if high levels of ATPase activity could be obtained in the absence of lipids, we cross-linked P-gp in a closed conformation. We found that clamping P-gp in a closed conformation activated ATPase activity only in the presence of lipids. The results suggested that lipids affected other domain–domain interface(s) such as the transmission interfaces connecting the NBDs to the TMDs that are required for ATPase activity. The first transmission interface was previously shown to be less sensitive to mutations than the second transmission interface [17,18] suggesting that the first transmission interface might be quite malleable. Here we show that cross-linking of residues at the first transmission interface in purified P-gp is affected by the presence of lipids.

2. Materials and methods

2.1. Construction of mutants

Mutations were introduced into Cys-less P-gp cDNA containing a 10-histidine tag [19] to introduce pairs of cysteines by site-directed mutagenesis as described by Kunkel [20]. Mutant L175C/N820C was used to test the effects of cross-linking the two wings together [21]. The L175C cysteine is located in the N-terminal wing (Fig. 1A) and N820C is located in the other wing. The G471C/S909C, V472C/S909C, L443C/S909C, L443C/R905C mutants were constructed to monitor cross-linking at the first transmission interface between NBD1 and intracellular loop 4 (ICL4) in TMD2.

2.2. Disulfide cross-linking analysis

Cys-less P-gp mutants containing pairs of cysteines were expressed in HEK 293 cells in the presence of 5 μM cyclosporine A at 37 °C in the presence of 5 mM sodium butyrate for 24 h and then incubated at 30 °C for 24 h. Sodium butyrate is a histone deacetylase inhibitor that enhances expression of proteins in HEK 293 cells [22]. Cyclosporine A and low temperature incubation promote maturation of the mutants [23].

To test the effects of clamping the wings together, membranes were prepared from fifty 10 cm plates of cells expressing histidine-tagged L175C/N820C P-gp as described previously [21]. The membranes were suspended in 1.5 ml of Tris-buffered saline (TBS), pH 7.4. Equivalent amounts of membranes were treated with or without 0.02 mM 1,4-butanediyl bismethanethiosulfonate (M4M, spans 4.9–6.5 Å) cross-linker at 20 °C for 5 min. A sample of the reaction mixtures was added to 2 X SDS sample buffer (125 mM Tris–HCl, pH 6.8, 20% (v/v) glycerol and 4% (w/v) SDS) containing 25 mM EDTA and no reducing agent. The reaction mixtures were then subjected to SDS-PAGE (6.5% (w/v) polyacrylamide gels) and immunoblot analysis with a rabbit polyclonal antibody against P-gp [24]. Intramolecular disulfide cross-linking between domains can be detected because the cross-linked product migrates with a slower mobility on SDS-PAGE gels [25].

2.3. Measurement of ATPase activity

Histidine-tagged P-gp mutant L175C/N820C was treated with or without M4M and isolated by nickel-chelate chromatography as described previously [26]. Recovery of P-gp was monitored by immunoblot analysis with rabbit anti-P-gp polyclonal antibody [24]. A sample of the isolated histidine-tagged P-gp in 0.1% (w/v) n-dodecyl-β-D-maltoside was mixed with an equal volume of 10 mg/ml total Escherichia coli (E. coli) lipids (Avanti Polar Lipids, Inc.) (lipid-rich detergent micelles) or TBS, pH 7.4. The P-gp:lipid mixture was then sonicated for 45 s at 4 °C (bath type probe, maximum setting, Branson Sonifier 450, Branson Ultrasonic, Danbury, CT) and ATPase activity determined in the presence or absence of 10 mM dithiothreitol (DTT) as described previously [17]. E. coli lipids were used because basal ATPase activity is significantly higher in these lipids than in sheep brain phosphatidylethanolamine used in previous studies [27] and is often used to test if a compound is an inhibitor of (basal) P-gp ATPase activity [27]. E. coli lipids are functionally relevant because drug-stimulated ATPase activity of P-gp reconstituted with this lipid is similar to that measured in isolated mammalian plasma membranes (from drug-resistant cell lines) that are enriched in P-gp [28].

To test for NBD1/ICL4 interactions, histidine-tagged Cys-less P-gp, mutants G471C/S909C, V472C/S909C, L443C/S909C or L443C/R905C were transiently expressed in HEK 293 cells. Membranes were prepared and samples were treated with or without oxidant (1 mM copper phenanthroline) for 5 min at 20 °C. The reactions were stopped by addition of EDTA (10 mM final concentration). The samples were then mixed with 1 volume of 2X SDS sample buffer containing 25 mM EDTA with no reducing agent and samples subjected to immunoblot analysis as described above.

Uncross-linked P-gps were also isolated by nickel-chelate chromatography and samples assayed for ATPase activity in the presence or absence of 2 mg/ml lipids. Samples of isolated P-gps were mixed with 2 mg/ml lipids or TBS, pH 7.4 and treated with or without oxidant (1 mM copper phenanthroline) for 5 min at 20 °C. The reactions were stopped by addition of EDTA (10 mM final concentration) followed by an equal volume of 2X SDS sample buffer containing 25 mM EDTA but no reducing agent. The samples were then subjected to SDS-PAGE and immunoblot analysis as described above.

2.4. Data analysis

ChemiDoc™ XRS$^+$ with Image Lab™ software (Bio-Rad Lab. Inc., Mississauga, Ontario) was used to record and quantify the signals from the immunoblots. The results were expressed as an average of triplicate experiments ± standard deviation (S.D.). The Student's two-tailed t-test was used to determine statistical significance ($P < 0.001$).

3. Results and discussion

We previously found that isolated human P-gp shows negligible ATPase activity in the absence of lipids (also see Fig. 1B). One explanation is that P-gp adopts a wide-open conformation in detergent without lipids as reported in the crystal structures [9,10,29] such that the NBDs are too far apart (greater than 30 Å apart) to form a dimer that is necessary for ATP binding and hydrolysis. Lipids may promote formation of a more closed conformation as observed for MsbA [16]. Does P-gp show high ATPase activity in the absence of lipids if it was locked in a closed conformation? To address this question we used mutant L175C/N820C that has a cysteine in each wing of P-gp (Fig. 1A). Cross-linking mutant L175C/N820C with a short cross-linker (M4M; 4.9–6.5 Å) traps P-gp in a closed conformation that exhibits high ATPase activity in the presence of lipids [30]. If lipids simply enhance formation of a closed conformation, then cross-linked L175C/N820C would be expected show high ATPase activity in the absence of lipids.

Membranes prepared from HEK 293 cells expressing histidine-tagged L175C/N820C P-gp were treated with or without M4M cross-linker. Both cross- and uncross-linked P-gps were then isolated by nickel-chelate chromatography. Cross-linking causes P-gp to migrate slower on SDS-PAGE gels (see Fig. 1B) and was abolished when treated with DTT (Fig. 1B). The isolated P-gps were then mixed with E. coli lipids or TBS, pH 7.4 and ATPase activity measured. As shown in Fig. 1B, uncross-linked mutant had no detectable ATPase activity in the absence of lipids. The activity increased to about 0.5 μmol/min/mg P-gp in the presence of lipids regardless of whether the sample was treated with DTT. In contrast, cross-linking increased the activity of L175C/N820C to 1.7 μmol/min/mg P-gp in the presence of lipids (Fig. 1B). The increase in activity was due to cross-linking as treatment with DTT reduced the activity to that of uncross-linked P-gp (about 0.5 μmol/min/mg P-gp). Without lipids however, cross-linked L175C/N820C showed little ATPase activity in the presence or absence of DTT. Therefore lipids do not simply promote formation of a more closed conformation but also induce other structural changes to switch P-gp into a high ATPase activity mode.

Stimulation of P-gp ATPase activity may be a two-step process. The first step might be to promote interaction between the two wings to bring the NBDs together to allow ATP molecules to bind at the NBD1/NBD2 interface followed by switching of the transmission interfaces into active modes. The transmission interfaces of ABC transporters [31] consist of loops that connect cytoplasmic extensions of TM segments in the TMDs to the NBDs. It is thought that conformational changes in the NBDs as a result of ATP binding/hydrolysis are transmitted to the TMDs through these interfaces (NBD1/ICL1/ICL4 at the first transmission interface and NBD2/ICL2/ICL3 at the second transmission interface). The NBD1/ICL4 and NBD2/ICL2 contact points appear to be more important for activity in ABC drug pumps than the NBD1/ICL1 and NBD2/ICL3 contact points because some drug pumps (e.g. BCRP; ABCG2) do not have the latter [32].

We then tested whether lipids affected the disulfide cross-linking patterns at the first transmission interface (NBD1/ICL4) (Fig. 2A). The NBD1/ICL4 transmission interface was selected rather than the NBD2/ICL2 interface because it is much less sensitive to mutations [17]. The NBD2/ICL2 interface is highly sensitive to point mutations with the majority of mutations severely inhibiting folding and/or activity of P-gp [17]. By contrast, mutations in the first transmission interface do not seem to affect assembly and activity of the protein.

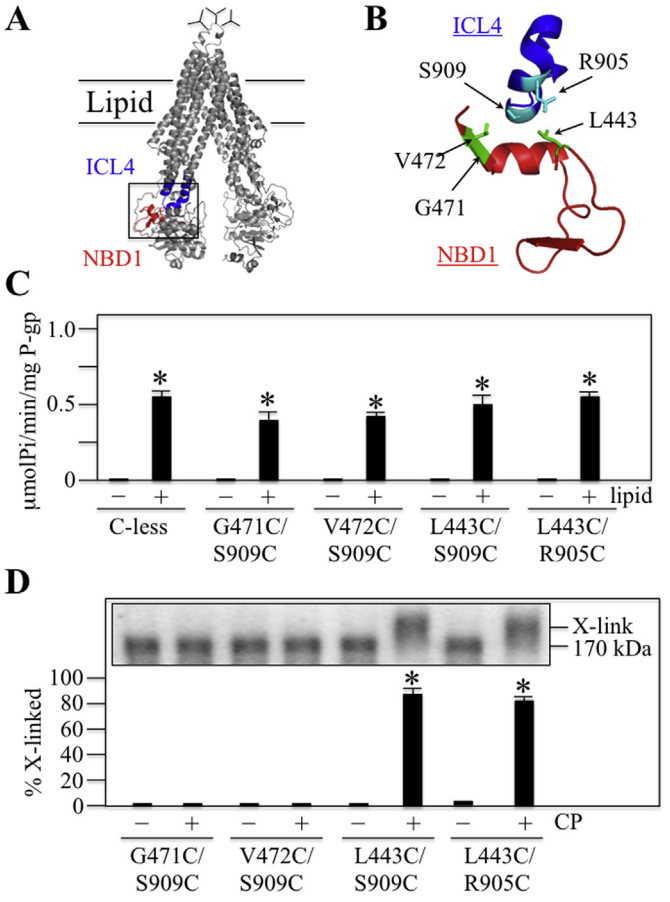

Fig. 2. Cross-linking of double cysteine mutants at the first transmission interface. (A) The location of the first transmission interface (boxed) between ICL4/NBD1 is shown in a model of human P-gp in the open conformation [9] and (B) the residues Leu^{443}(NBD1), Gly^{471}(NBD1), Val^{472}(NBD1), Arg^{905}(ICL4) and Ser^{909}(ICL4) to be mutated to cysteine at the interface. (C) ATPase activities of the histidine-tagged uncross-linked double cysteine mutants isolated by nickel-chelate chromatography in the presence (+) or absence (−) of lipids. Each value is the mean ± S.D. ($n = 3–5$) An asterisk indicated significant difference ($P < 0.001$) relative to a sample assayed without lipid. (D) Membranes prepared from cells expressing the mutants were treated with (+) or without (−) copper phenanthroline (CP). Samples were subjected to immunoblot analysis (inset). The position of cross-linked (X-link) and mature (170 kDa) P-gp is shown. The amount of cross-linked P-gp relative to total was determined. Each value is the mean ± S.D. ($n = 3–5$). An asterisk indicates significant difference ($P < 0.001$) relative to a sample not treated with CP.

Accordingly, we constructed four histidine-tagged double cysteine mutants (G471C (NBD1)/S909C (ICL4), V472C(NBD1)/S909C(ICL4), L443C(NBD1)/S909C(ICL4), L443C(NBD1)/R905C(ICL4)) at the NBD1/ICL4 interface [10] (Fig. 2B). The R905C and S909C mutations are located in intracellular helix loop 4 (IH4) that links the cytoplasmic extensions of TM segments 10 and 11. The cysteine replacements at positions G471C and V472C in NBD1 lie close to the Q-loop (residue Gln^{475} is predicted to co-ordinate with the γ-phosphate of ATP) [33] while L443C lies close to the Walker A sequence for binding of ATP. The mutants were expressed in HEK 293 cells, isolated by nickel-chelate chromatography, mixed with lipids and assayed for ATPase activity. All mutants had negligible basal ATPase activity in the absence of lipids (Fig. 2C). In the presence of lipids, however, the mutants exhibited basal ATPase activities that were similar to the Cys-less parent (Fig. 2C).

When membranes prepared from cells expressing the mutants were treated with oxidant, it was observed that only mutants L443C/R905C and L443C/S909C showed robust cross-linking (Fig. 2D). The results were surprising since the crystal structures and models of human P-gp [9,10,29] showed that the distance between the α-carbons in G471C/S909C and V472C/S909C are closer to each other (7.4–8.6 Å) than in mutants L443C/R905C or L443C/S909C (8.3–11.6 Å) (Table 1).

It was possible that the mutants with cysteines predicted to be farther apart were cross-linked (Fig. 2D) because the presence of lipids alters interaction at this transmission interface. To address this possibility the histidine-tagged mutants G471C/S909C, V472C/S909C, L443C/S909C or L443C/R905C were expressed in HEK 293 cells and isolated by nickel chromatography to remove the lipids. The purified mutants were then cross-linked with oxidant in the presence or absence of lipids. In the absence of lipids, mutants G471C/S909C and V472C/S909C showed robust cross-linking (Fig. 3A and B). By contrast, mutants L443C/S909C and L443C/R905C were cross-linked only in the presence of lipids (Fig. 3A and B). All four double-cysteine mutants retained over 95% of their activity when they were left without lipids for 45 min at 20 °C and then assayed for ATPase activity in the presence of lipid (data not shown). This suggests that the cross-linking results were not due to the stabilizing effect of lipids.

The results of this study suggest that lipids cause conformational changes at the first transmission interface to activate ATPase activity (Fig. 2C and D). Without lipids, P-gp shows little ATPase activity as residue Ser^{909} in IH4 lies close to Gly^{471} and Val^{472} in NBD1. The presence of lipids activates a switch to promote ATPase activity by moving Ser^{909} closer to Leu^{443} and away from Gly^{471} and Val^{472}. Conformational changes at the first transmission interface are critical for activity as cross-linking of mutant L443C/S909C inhibited drug-stimulated ATPase activity by more than 90% [34].

Our results are consistent with studies on MsbA [35] showing that lipids are required to activate the ATPase activity of ABC export proteins. It was found that lipids did not alter the structure of the NBDs but suggested that they altered functional interactions between domains. A LRET spectroscopy study of MsbA showed that lipids caused a significantly smaller separation between the NBDs

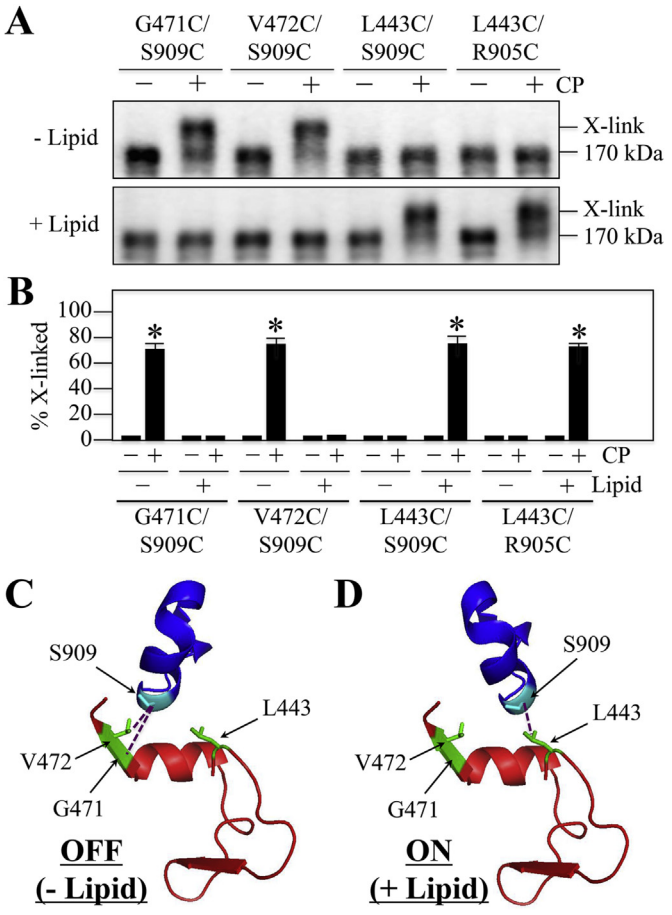

Fig. 3. Lipid activates a switch at the NBD1/ICL4 interface. (A) Histidine-tagged mutants (G471C/S909C, V472C/S909C, L443C/S909C and L443C/R905C were isolated by nickel chelate chromatography, mixed with (+Lipid) or without (- Lipid) lipids and treated with (+) or without (–) copper phenanthroline (CP). Samples were subjected to immunoblot analysis. The position of cross-linked (X-link) and mature (170 kDa) P-gp is shown. The level of cross-linked P-gp relative to total was determined (B). Each value is the mean ± S.D. (n = 3–5). An asterisk indicates significant difference ($P < 0.001$) relative to samples not treated with CP. Models of the NBD1/ICL4 interface in the absence (C) and presence (D) of lipid are shown. In the absence of lipids, P-gp adopts the 'off' conformation with negligible basal ATPase activity and with Ser^{909} close to Gly^{471} or Val^{472} while the presence of lipids causes P-gp to adopt the 'on' conformation that exhibits high ATPase activity and with S909C close to L443C. For clarity, the L443/R905 interaction is not shown.

than was observed in the crystal structure of MsbA in an open inward-facing conformation [36]. Small separations between the NBDs were also observed in fluorescence resonance energy transfer (FRET) studies of mouse P-gp incorporated into lipids [37]. The LRET and FRET studies suggest that incorporation of ABC transporters into lipids narrows the average distances between the NBDs.

The cross-linking studies suggest that lipids can induce further structural changes in P-gp. Clamping the two wings together was

Table 1
Distances between the α-carbons of residues in various structures and models of P-gp.

Residue positions	Human P-gp (open) [9]	Human P-gp (open) [10]	C. elegans P-gp (open) (4F4C) [10]	Mouse P-gp (open) (4M1M) [9]	Mouse P-gp (open) (4KSB) [29]	Human P-gp (closed) [38]
G471/S909	8.2 Å	7.9 Å	7.7 Å (A495/A950)[a]	8.2 Å (G467/S905)[a]	7.9 Å (G467/S905)[a]	8.6 Å
V472/S909	7.4 Å	7.5 Å	7.5 Å (V496/A950)[a]	7.4 Å (V468/S905)[a]	8.4 Å (V468/S905)[a]	7.3 Å
L443/S909	8.4 Å	8.9 Å	8.8 Å (Y467/A950)[a]	8.8 Å (L439/S905)[a]	11.6 Å (L439/S905)[a]	8.3 Å
L443/R905	9.4 Å	8.3 Å	8.1 Å (Y467/R946)[a]	9.4 Å (L439/R901)[a]	11.1 Å (L439/R901)[a]	9.0 Å

[a] These residues are at equivalent positions to those in human P-gp.

not sufficient to activate P-gp ATPase activity. Addition of lipids was required to activate a switch at the first transmission interface. Our study shows that lipids can also modulate NBD/TMD interactions and that the structure of P-gp may show significant differences in detergent and lipids.

In summary, our results show that the structure at the first transmission interface (NBD1/ICL4) that is important for NBD/TMD cross talk is affected by the presence of lipids. Lipids activate an intriguing and novel conformational switch for coupling substrate transport to ATP hydrolysis in ABC proteins.

Conflict of interest

No competing financial interests have been declared.

Author contributions

The manuscript was written through contributions of all authors. Both authors approve the final version of the manuscript.

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