Structures of P-glycoprotein reveal its conformational flexibility and an epitope on the nucleotide-binding domain

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P-glycoprotein (P-gp) is one of the best-known mediators of drug efflux-based multidrug resistance in many cancers. This validated therapeutic target is a prototypic, plasma membrane resident ATP-Binding Cassette transporter that pumps xenobiotic compounds out of cells. The large, polyspecific drug-binding pocket of P-gp recognizes a variety of structurally unrelated compounds. The transport of these drugs across the membrane is coincident with changes in the size and shape of this pocket during the course of the transport cycle. Here, we present the crystal structures of three inward-facing conformations of mouse P-gp derived from two different crystal forms. One structure has a nanobody bound to the C-terminal side of the first nucleotide-binding domain. This nanobody strongly inhibits the ATP hydrolysis activity of mouse P-gp by hindering the formation of a dimeric complex between the ATP-binding domains, which is essential for nucleotide hydrolysis. Together, these inward-facing conformational snapshots of P-gp demonstrate a range of flexibility exhibited by this transporter, which is likely an essential feature for the binding and transport of large, diverse substrates. The nanobody-bound structure also reveals a unique epitope on P-gp.

membrane protein structure | ABC transporter |
nanobody-transporter complex

The mammalian adenosine triphosphate (ATP)-binding cassette (ABC) transporter P-glycoprotein (P-gp) is present in many tissues (1) and can detoxify cells by pumping xenobiotics across the membrane (2). A hallmark feature of this transporter is its ability to bind and transport an array of structurally diverse molecules ranging in size from 100 to 4,000 daltons (Da) (molecular mass) (3). In fact, P-gp was discovered in a cell line where it prevented the permeation of several different drugs (4). Although its physiological function is not fully understood, the well-recognized role of P-gp in mediating multidrug resistance (MDR) in many types of cancers has made it a valid therapeutic target (5). P-gp also plays a significant role in the pharmacokinetics and bioavailability of drugs by mediating their transport in the liver, intestines, and across the blood–brain barrier (1, 2, 6).

P-gp is a ~170-kDa molecule comprised of two pseudosymmetric halves, each containing a nucleotide-binding domain (NBD) and a transmembrane domain (TMD). Currently, the “alternating access” model is the most widely accepted paradigm explaining the mechanics of transport by ABC transporters (7, 8). According to this model, binding of ATP at the NBDs drives conformational changes in the TMDs and switches the transporter’s overall conformation from inward-facing to outward-facing (inward/outward refer to the opening of the drug-binding pocket relative to the cell). This ATP-driven switch results in the vectorial

transport of substrates out of the cell. The hydrolysis of ATP and release of Pi/ADP are essential for resetting the transporter back to the inward-facing conformation.

The overall protein topology of the inward-facing conformation of P-gp has been established (9) and is reiterated in the X-ray structures of related bacterial and mammalian ABC transporters found in the ABCB subfamily (8, 10, 11), including the human ABCB10 [Protein Data Bank (PDB) ID codes 3ZDQ, 4AYX, 4AYT, and 4AYW] (12). Furthermore, the inward-facing conformation has been biochemically validated in bacterial ABC exporters, using electron paramagnetic resonance on multicopy suppressor of Htrb mutations (MsbA) (13) and LmrA (14), cysteine cross-linking of MsbA (15), and hydrogen/deuterium exchange coupled to mass spectrometry of BmrA (16). The inward-facing conformation is a key intermediate in the alternating access mechanism (7), as it allows the transporter to scan the inner leaflet of the membrane for substrates. This notion is supported by the inward-facing X-ray structures of P-gp, which have cyclic peptide inhibitors bound to the substrate-binding pocket (9).

Owing to its role in MDR, several small molecule inhibitors of P-gp have been developed during the last four decades (2, 17), but none have yet been approved for clinical use (18). In recent years, antibody-derived therapeutics have become increasingly popular because of their specific affinities and relatively high tolerance in humans. Nanobodies (Nbs) are small (~15 kDa) single-domain proteins that are derived from, and contain, the unique structural and functional properties of natural heavy chain-only antibodies found in camelids (19). Like conventional antibodies, Nbs achieve antigen-specificity by using three loops called complementarity determining regions (CDRs 1–3).

Nbs are mostly monomeric, encoded by single genes, highly soluble, and can be efficiently produced in prokaryotic and eukaryotic hosts, including bacteria and yeast. They are also

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capable of penetrating many tissues including the human gut (19). These characteristics provide Nbs with great promise because therapeutics against a number of diseases (19). Additionally, Nbs can bind to cryptic epitopes and potentially stabilize proteins in certain conformations. These properties make Nbs useful as chaperones for inducing lattice formation during protein crystallization (20).

Here, we present three unique X-ray structures of mouse P-gp (*ABCB1a*) with different inward-facing conformations, all wider than those originally published (9). One crystal form comprises P-gp complexed with a single Nb, called Nb592, bound to the first NBD (NBD1). Consistent with its binding location, we show that Nb592 is a potent inhibitor of P-gp's ATP hydrolysis activity. Taken together, these different structures of P-gp reveal a unique epitope and demonstrate the flexibility of the transporter to sample a range of inward-facing conformations.

Results

Structure Determination. To structurally map the conformational flexibility of P-gp in the absence of nucleotides and substrates, we determined three X-ray structures (Table S1) that crystallized in two different crystal forms (crystal forms A and B; Table S1). Depending on the crystal growth condition, crystal form A yielded crystals that had variations in unit cell dimensions (Table S1) with changes mostly in the c- and a-cell edges (crystal 1 and crystal 2). To reduce bias, we determined an experimentally derived electron density map of crystal form A by generating a heavy atom derivative using ethyl-mercury-chloride (Table S1, labeled "Experimental Map Crystal") and obtained protein phases by using the multiple anomalous dispersion technique (MAD). An initial model was built by using this electron density map to 4.5 Å (Fig. S1). A higher resolution native crystal (crystal 1; Fig. 1A and Table S1) later diffracted to 3.8 Å and was solved by the molecular replacement method. The structure revealed that the distance between the NBDs, as measured by the C α atom of residues 626 (C-term of NBD1) and 1271 (C-term of NBD2), is ~ 31 Å, which is much larger than the ~ 13 Å observed in the previously published mouse P-gp structure (9). This represents a large (~ 18 Å) range of displacement between the NBDs that P-gp can conformationally sample while in the inward-facing state.

A second native crystal of P-gp (crystal 2) diffracted to 4.0 Å and was determined by molecular replacement using our model derived from crystal 1. This structure of P-gp is the widest, with a distance of ~ 36 Å between residues 626 and 1271. Crystals 1 and 2 represent the more extreme ranges of the variations observed for crystal form A. The two X-ray structures revealed distinct inward-facing conformations of P-gp, with the overall structure similar to the one published (9) and some smaller local changes. For example, a break in the helical structure of transmembrane helix (TM) 12 in the original more closed inward-facing conformation is fully helical in these structures. The linker (residues 627–683) between NBD1 and TM7 is not observed and presumed to be disordered. Residues 1–33 and 1272–1276 at the N- and C- termini are also not observed.

To further validate the models of P-gp (crystals 1–2) derived from crystal form A, which is critical at these moderate diffraction resolutions (3–4.5 Å), we made 17 single-site cysteine substitution mutants in P-gp and labeled them with ethyl mercury chloride. These mutants included residues S80C, S176C, M188C, A216C, S218C, A250C, R272C, L283C, G284C, S305C, A309C, G342C, A344C, K730C, S876C, S889C, and S975C distributed throughout the TM helices. Fig. 1B shows a composite view of all experimentally validated positions mapped on to the crystal 1 model, whereas Fig. S2 provides close-up shots of each mercury-labeled single-site mutant. Combined with seven wild-type cysteine residues of mouse P-gp previously identified (9) and

Fig. 1. Structure of inward-facing P-gp. (A) Open inward-facing conformation (crystal 1) with the NBDs far apart. The N-terminal half of the protein (blue) and the C-terminal half (yellow) are connected by a flexible linker region (black dashed line) that is disordered in the structure. (B) Stereo view of superposition of anomalous Fourier mercury peaks validating the position of knocked-in and wild-type cysteine residue positions. Relative peak positions were determined by aligning the corresponding TMD or NBD subdomains of the model derived from the mutant diffraction data (Table S2) with the model derived from crystal 1. The locations of the 24 anomalous mercury peaks (mesh, sigma values) were used to confirm the registration of amino acids in the structure.

observed in these data, we were able to experimentally validate the accuracy of our model by using a total of 24 positions.

A second, entirely different crystal form (crystal form B) of P-gp was obtained by cocrystallization of mouse P-gp with the nanobody Nb592 (Fig. 2 and Table S1). Size exclusion chromatography demonstrated that the P-gp and Nb592 form a complex that comigrates and elutes as a single peak (Fig. S3). Fractions from this sample peak were pooled together, concentrated, and crystallized. The X-ray structure of the P-gp–Nb592 complex (crystal 3) was determined to a resolution of 4.1 Å (Table S1) by molecular replacement using the model derived from crystal 1 and a model of Nb592 generated as described in *Materials and Methods*. The CDR loops of Nb592 were found to bind NBD1, thereby potentially precluding the NBDs from dimerizing. This inward-facing conformation is the least wide among the three structures presented here, with a distance of ~ 30 Å between the C α atoms of residues 626 and 1271.

The interface between Nb592 and NBD1 of P-gp is significant and constitutes ~ 745 Å² of the buried surface area. CDR3 (residues 98–107 of Nb592) penetrates into a pocket formed by three helices in NBD1 (Fig. 2B). The P-gp residues within 5 Å of Nb592 include S555, T559, E562, A563, H583, R584, L585, S586, T587, R589, H608, M612, F619, L621, V622, M623, T624, Q625, and T626 on NBD1 and K1260, F1264, S1265, and

Fig. 2. X-ray structure of P-gp in complex with Nb592. (A) Overview of the entire structure. Nb592 (red) binds to the C terminus of NBD1. There are additional interactions with NBD2 (yellow). The binding site of the nanobody precludes the ABC domains from coming together, explaining its potent ATPase inhibition properties. (B) Close up view of Nb592 binding site on P-gp. The view is rotated 180° from A. The complementarity determining regions (CDR1: residues 25–33 for Nb592, blue; CDR2: residues 51–57 for Nb592, yellow; CDR3: residues 98–107, green) of the nanobody all interact with the C-terminal portion of NBD1. CDR3 inserts into a shallow pocket formed by three helices in NBD1. The walker-A motif (residues 423–430) located on NBD1 is colored in cyan. The conserved H583 is also shown in violet.

S1268 on NBD2 (Fig. S4A). T626 is the last resolved residue at the C terminus of NBD1 and is proceeding a flexible linker region presumed disordered but could also potentially interact with Nb592.

The Pgp-Nb592 structure (crystal 3) elucidates a unique epitope on the NBDs of P-gp. A protein sequence alignment between mouse and human P-gp reveals that the two proteins are similar in the epitope/Nb592-binding site (Fig. S4B). The only differences are at residues 623 and 624 (mouse numbering): mouse, Met-Thr; human, Thr-Met. Although these residues are within 5 Å of Nb592, T624 is pointing away from Nb592 and M623 is oriented toward the conserved portion of the Nb just before and after CDR2 (residues 551–557 of Nb592). Interestingly, Nb592 does not bind to mouse NBD2, which also has a high protein sequence similarity in this region (Fig. S4B).

Inhibition of P-gp ATPase by Nb592. The NBDs of ABC transporters are responsible for the binding and hydrolysis of ATP that drives drug transport through the TMDs. The structure of P-gp with Nb592 bound at NBD1 (Fig. 2) indicates that this Nb could significantly affect the catalytic function of P-gp. We tested this hypothesis by using two biochemical approaches. First, we measured the drug (verapamil)-stimulated ATPase activity of purified mouse P-gp, in the presence of increasing concentrations of Nb592. Our results revealed that Nb592 is a strong inhibitor of ATP hydrolysis with half-maximal inhibition achieved in the nanomolar range (IC_{50} of 520 ± 57 nM) (Fig. 3A). This IC_{50} value is comparable to or lower than those reported for most small molecule P-gp inhibitors (2, 9, 17, 21). Nb592 also potently inhibited basal ATPase activity (in the absence of drug) with $90 \pm 5\%$ inhibition seen at saturating amounts of $7.5 \mu M$ Nb592. Moreover, the inhibition was fully retained in the presence of 5 mM DTT (Fig. 3B), indicating that Nb592 is a robust protein that can withstand reducing agents at concentrations that are typical of the cytoplasm.

We made further measurements that suggested that Nb592 prevents dimerization of the NBDs. ATP hydrolysis requires intimate interactions of residues from both *cis* and *trans* NBDs with bound ATP to facilitate the hydrolytic attack on the γ -phosphate (22). Sodium orthovanadate (Vi) is an inorganic phosphate (Pi) analog that can trap ATP in the posthydrolysis state (ADP•Pi) (23), resulting in an outward-facing conformation with dimerized NBDs (8). Using 8-azido- $[\alpha^{32}P]$ -ATP, we

show that 8-azido- $[\alpha^{32}P]$ -ADP is trapped in mouse P-gp by Vi, in the presence of the ATP hydrolysis activator, verapamil (Fig. 3C, lanes 1–3) (24). At increasing concentrations of Nb592, the hydrolysis of ATP is effectively inhibited as indicated by the reduced amount of trapped 8-azido- $[\alpha^{32}P]$ -ADP in the catalytic sites of P-gp (Fig. 3C, lanes 4–10).

Taken together, both biochemical experiments show that the binding of Nb592 to mouse P-gp inhibits its ATP hydrolysis activity by hindering the formation of an ATP hydrolysis-competent NBD dimer sandwich. These results complement the structure of the Pgp-Nb592 complex (Fig. 2), suggesting a molecular basis for the inhibition of ATPase activity. This unique mechanism of inhibiting P-gp by specifically targeting the NBDs contrasts the mode of inhibition by several small molecule inhibitors/drugs, which target the substrate-binding cavity in the TMDs (2, 9, 17).

Discussion

The overall topology and protein fold of the three unique P-gp X-ray structures described in this work are similar to those published (9), with some small localized differences. When the N-terminal halves of the P-gp models in this study were structurally aligned (residues 33–209, 852–961, and 320–626), the C-terminal halves showed a significant displacement relative to one another, most notably in the relative positions of NBD2 (Fig. 4). The overall change in the structures can be described as a hinge movement where a small angular change at the pivot point causes larger changes farther away toward the NBDs. The movement near the pivot or “TM-hinge region” on the extracellular side is smaller, and comprises Loop 3–4 (L3-4; residues 206–208) and Loop 5–6 (L5-6; residues 319–324), and the

Fig. 3. Nb592 is a strong inhibitor of P-gp's ATPase activity. (A) Nb592 inhibits verapamil-stimulated ATPase activity with an IC_{50} of 520 ± 57 nM. Data points indicate the average activity $\pm$ SEM from four independent experiments, relative to P-gp's activity in the absence of Nb592. Lines represent nonlinear regression analysis of the data points; R^2 value for the fit was 0.95. (B) Inhibition of verapamil-stimulated ATPase activity in the presence of 5 mM DTT, without or with $7.5 \mu M$ Nb592 ($n = 4$). (C) Nb592 prevents vanadate-induced 8-azido- $[\alpha^{32}P]$ -ADP trapping in P-gp's catalytic sites. P-gp was incubated with $100 \mu M$ verapamil (VER) and $200 \mu M$ orthovanadate (Vi) in the 8-azido- $[\alpha^{32}P]$ -ATP hydrolysis/trapping reaction as indicated above the lanes. Upper, autoradiogram; Lower, Coomassie-stained SDS/PAGE gel showing the presence of P-gp (loading control).

Fig. 4. Conformational changes by P-gp. (A) Stereoview of the three P-gp structures described in this study (crystal 1, red; crystal 2, green; P-gp-Nb592 complex [crystal 3], blue) aligned by using residues in TMD1 and NBD1 (residues 33–209, 852–961, and 320–626; designated as “Half Aligned”). The rmsd on C α atoms for the aligned portion was 0.11 Å between crystal 2 and crystal 1 and 0.28 Å between P-gp-Nb592 complex (crystal 3) and crystal 1. The “TM-hinge regions” for this half of the molecule (L3-4 and L5-6 as described in the text) are marked. The relatively large displacement of the other half of the molecule, including NBD2, is clearly shown. The relative position of the Nb592 is marked in interaction with NBD1. It also makes a smaller contact with NBD2. The P-gp-Nb592 complex is the most closed inward-facing conformation described in this study. (B) Same structural alignment as A except turned 180° to show the opposite side of the transporter. The “TM-hinge region” comprised of L9-10 and L11-12 is indicated.

corresponding Loop 9–10 (L9-10; residues 849–851) and Loop 10–11 (L10-11; residues 963–968) on the other half of the molecule.

The most striking feature of these P-gp structures is a much larger separation between NBD1 and NBD2, suggesting that the transporter can adopt a much wider inward-facing conformation than previously described. The overall range of distances between residues 626 (C-term of NBD1) to 1271 (C-term of NBD2) sampled by the three inward-facing conformations presented here is ~29–36 Å, whereas the corresponding distance in the original published structure (9) is only ~13 Å. Such dynamic conformational flexibility in the inward-facing state has also been observed in biochemical, biophysical, and molecular dynamics simulations experiments on bacterial ABC transporters, such as MsbA (13) and BmrA (16), and recently on mouse P-gp (25). The enlargement of the portals (formed from TM4/TM6 and TM10/TM12) facing the inner-membrane leaflet side may be required for larger substrates like β -amyloid

(~4 kDa in size) (26) to enter and bind inside the substrate-binding pocket.

The wideness of our P-gp structures is comparable to recently published structures of other ABC transporters having similar ABC B-like protein folds (8, 11, 12, 27). Interestingly, the structures of human ABCB10 and *Thermotoga maritima* TM287/288 (10) also have nucleotide analogs bound to separated NBDs, suggesting a state of these transporters just before the formation of the ABC sandwich that is essential for the structural transition to the outward-facing conformation. The degree of NBD separation might also be influenced in part by crystal lattice contacts and may ultimately be constrained in the physiological context by the thickness of the hydrophobic section of the lipid bilayer and the TMDs of the transporter.

The discovery and structural elucidation of unique epitopes on therapeutic targets has tremendous value in the pharmaceutical industry. Antibodies like UIC2 that have been developed against P-gp target epitopes on the TMDs/extracellular surface (28). The structure of P-gp complexed with Nb592 (crystal 3) reveals an epitope located on NBD1 (Fig. 2B) that is away from the Walker-A or conserved histidine (H583). The interaction between Nb592 and NBD1 of P-gp appears to be quite specific, as the CDR loops of Nb592 do not bind NBD2, despite a relatively high level of sequence conservation between NBD1 and NBD2 (Fig. 2D). The binding location of Nb592 on NBD1 (Fig. 2) suggests that it sterically prevents the NBDs from dimerizing and provides a structural basis to support and explain our biochemical observations that Nb592 (i) strongly inhibits ATPase activity, and (ii) completely abolishes Vi-induced ADP-trapping, in mouse P-gp (Fig. 3) signifying that NBD dimerization, followed by ATP “occlusion,” is essential to the formation of the hydrolysis-competent state during the transport cycle. Future biochemical studies may establish whether Nb592 can bind and inhibit other ABC transporters via a similar mechanism.

Nb592 is a robust protein and will likely bind to its intracellular epitope even when exposed to the reducing glutathiones in the cytoplasm, as our studies suggest (Fig. 3). This result is quite remarkable considering the presence of two highly conserved cysteines, C22 and C96, that form a disulfide bond in the Nb, as observed in our structure. Further validation of these types of inhibitors in whole-cell settings will be necessary to assess their penetration through cell membranes and cross-reactivity with other cellular ATPases. Cell penetration can be improved through innovations such as small peptide/chemical tags (29), whereas specificity may be enhanced through molecular scaffold engineering. Although the IC₅₀ of Nb592 (520 nM) is better than the QZ59 inhibitors that cocrystallized in the original P-gp structures (9), it is an order of magnitude less than Tariquidar (43 nM), a P-gp inhibitor that has been used in clinical trials (18, 30). Thus, although Nb592 itself might not directly be used as a clinical inhibitor, it provides a template for the future development of this type of molecular scaffold. Several techniques may also be applied to improve the binding affinity of Nb592 (31).

The X-ray structures of P-gp described here, along with those published (9), will be useful for the molecular modeling of conformational trajectories to understand how the substrate-binding pocket changes during the transport cycle (25). Because human and mouse P-gp share nearly 87% protein sequence identity, our structures present experimentally derived checkpoints useful for docking simulations. The development of these algorithms to more accurately model and predict substrate binding will provide a useful screening tool, complementing *in vivo* and cell-based studies, for understanding P-gp’s role in drug pharmacokinetics. Collectively, these models could facilitate the development of therapeutically important compounds evading P-gp in several clinically relevant contexts, like penetration of drugs through the blood–brain barrier.

Materials and Methods

P-gp Protein Expression and Purification. Gene-optimized mouse P-gp (ABCB1a, GenBank JF834158) was expressed as described (9, 32) in 10-L cultures of *Pichia pastoris* grown in a bioreactor (Bioflow 415; New Brunswick Scientific). Protein expression was induced by addition of methanol (3.6 mL/h per liter of culture volume) overnight. Cells were lysed by a single pass through a cell disrupter (TS-Series; Constant Systems) at 40,000 psi. Cell wall and debris were removed by centrifugation (3,500 × *g*, 35 min, 4 °C), and crude plasma membranes were obtained by centrifugation at 35,267 × *g* for 2–3 h at 4 °C. The purification procedure is similar to that described (9) with some modifications. Membranes containing P-gp were resuspended in cold buffer (100 mM NaCl, 15% glycerol, 20 mM Tris at pH 8.0, and Sigma protease inhibitors) and solubilized with a final percentage of ~4.5% (vol/vol) Triton X-100 for 1–2 h at 4 °C. Insoluble material was removed by centrifugation at 38,400 × *g*, 4 °C for 30–60 min, and the supernatant was poured over a metal resin (Ni-NTA Superflow; Qiagen) by FPLC (AKTA; GE Life Sciences). Immobilized P-gp was buffer exchanged into 20 mM Hepes, 20 mM imidazole, 0.04% sodium cholate (Sigma), and 0.0675% β-dodecyl maltoside (DDM). Protein was eluted with buffer containing 200 mM imidazole at pH 7.5. The eluted protein was then diluted 1:10 in buffer containing 20 mM Hepes at pH 8.0, 100 mM NaCl, 0.2 mM tris(2-carboxyethyl)phosphine (TCEP), 0.04% sodium cholate, 0.065% β-DDM, and rebound to a new Ni-NTA column. The column was washed with buffer containing 20 mM imidazole, eluted with 200 mM imidazole, the protein was concentrated (Centricon YM-50 or YM-100; Millipore), spun at 95,000 rpm (TLA120.1 rotor) for 30–60 min at 4 °C, and subjected to gel filtration chromatography (Superdex200 16/60; GE Healthcare) at 4 °C.

Elicitation of Nanobodies Against P-gp. To generate the nanobodies, 2 mg of purified, detergent-solubilized mouse P-gp was injected into a llama (*Llama glama*) over a period of 6 wk to elicit an immune response. The immunization, library construction, and nanobody selection have been performed by following standard procedures according to ref. 33, and a C-terminal His6-tagged nanobody library in pMES4 (GenBank accession no. GQ907248) of 2×10^8 independent clones was established by using the PstI/BstEII site. P-gp-specific phages were recovered by incubating P-gp-coated wells with 100 mM triethylamine at pH 11.0, for 10 min. The P-gp-coated wells were then washed once with Tris-HCl at pH 6.8, and several times with PBS. Finally, freshly grown TG1 cells were added to the wells to recover the noneluted phage. After two rounds of selection, individual colonies were screened for the expression of P-gp-specific nanobodies: Maxisorb 96-well plates were coated overnight at 4 °C with 1 μg/mL P-gp in sodium bicarbonate buffer at pH 8.2. Residual protein-binding sites in the wells were blocked for 2 h at room temperature with 2% milk in PBS. Detection of P-gp-bound nanobodies was performed with a mouse anti-His tag monoclonal (Serotec). Subsequent detection of the mouse anti-tag antibodies was done with an alkaline phosphatase anti-mouse IgG conjugate (Sigma). The absorption at 405 nm was measured 30–60 min after adding the enzyme substrate 4-nitrophenyl phosphate. Plasmids were extracted from the positive clones and transformed in *Escherichia coli* WK6 strains.

Nanobody Expression and Purification. Nb592 protein was produced in the *E. coli* WK6 strain described above. Bacteria were grown in terrific broth to an OD₆₀₀ of 0.7 and then expression was induced by 1 mM IPTG overnight at 28 °C. Bacteria were then pelleted at 7,500 × *g* for 15 min at room temperature. Pellets were resuspended in 15 mL of TES buffer (0.2 M Tris at pH 8.0, 0.5 mM EDTA, and 0.5 M sucrose) and kept under slow agitation for 1 h at 4 °C. Thirty milliliters of fourfold diluted TES buffer was added, and the sample was osmotically lysed under slow agitation for 45 min at 4 °C. Samples were then centrifuged for 30 min at 4 °C and 6,000 × *g*. Supernatant was used for purification on Ni-NTA resin (Qiagen). Binding to the Ni-NTA resin was performed at 4 °C for 1 h. The column was washed with 50 mM phosphate buffer at pH 6.0, 1 M NaCl, then eluted with 50 mM sodium acetate buffer at pH 4.5 and 1 M NaCl. The protein solution was neutralized by using 1 M Tris at pH 7.5. The eluted protein was then subjected to gel filtration (Superdex75 10/300GL; GE Healthcare) at 4 °C.

Formation of P-gp–Nanobody Complex. The P-gp–Nb592 complex was generated by incubating threefold stoichiometric excess nanobody with P-gp purified from the metal resin elution step for 30 min at 4 °C. The complex was then subjected to gel filtration chromatography as described above to remove excess Nb592.

ATPase Inhibition. Purified P-gp was reduced with 1 mM DTT for 30 min on ice, and then excess DTT was removed by passage through 1-mL Sephadex G-50 centrifuge columns equilibrated in 20 mM Hepes at pH 7.4, 10% glycerol, 250 mM NaCl, and 0.1% DDM as described in ref. 34. Protein was activated with 1% (wt/vol) *E. coli* polar lipids (Avanti) for 10 min at room temperature. Lipid-activated P-gp (0.5–2 μg) was preincubated with increasing amounts of Nb592 for 30 min on ice, then ATP hydrolysis assayed with 10 mM MgATP and 100 μM verapamil in a final volume of 50 μL for 15 min at 37 °C. Release of inorganic phosphate was determined by the Malachite green method (34). Negative controls containing P-gp, Nb592, or both were assayed for 1 min at 37 °C and were subtracted as background values. In some experiments, P-gp was substituted for Cys-less P-gp (35), purified in the absence of reducing agents, which gave essentially the same results. The data were fitted by using nonlinear regression on Sigmaplot (v11) with the equation: $Y = d - (a \times x^b / (c^b + x^b))$, where *a* is the verapamil-stimulated activity in the absence of Nb592, *b* is the Hill coefficient, *c* is the concentration for half-maximal inhibition (IC₅₀), *d* is the maximal inhibition, and *x* is the concentration of Nb592.

For vanadate trapping experiments, P-gp was activated with 1 mM DTT and *E. coli* lipids (2:1, wt/wt) for 10 min at room temperature (34). Lipid-activated P-gp (3.3 μg, 3–5 μL) was preincubated with increasing concentrations of Nb592 for 5 min, and reacted with 200 μM 8-azido-α[32P]-ATP, 2 mM Mg²⁺, and/or 100 μM verapamil and/or 200 μM orthovanadate in a final volume of 100 μL for 15 min at 37 °C. Unbound nucleotide was removed by passage through centrifuge columns, and the eluates UV cross-linked for 7 min on ice as described in ref. 34. Samples were resolved on 10% SDS/PAGE gels stained with Coomassie Brilliant Blue, and the dried gels were exposed to film.

Crystallization, Data Collection, and Structure Determination of P-gp and P-gp–Nb592 Complex. Purified P-gp or P-gp–Nb592 were isolated after gel filtration chromatography at a protein concentration of 1–2 mg/mL and subjected to reductive methylation (36). Freshly made borane and formaldehyde were added to the protein solution at final concentrations of 50 mM and 100 mM, respectively, and incubated in the dark for 2 h at 4 °C with gentle shaking. The reaction was quenched with the addition of ice cold 2.5 mM glycine and incubated for 30 min at 4 °C. Methylated P-gp was then concentrated to 1 mL (Centricon YM-50 or YM-100; Millipore 4) and subsequently diluted with 9 mL of quench buffer (20 mM Tris at pH 7.5, 100 mM NaCl, 0.2 mM TCEP, 0.04% sodium cholate, and 0.065% β-DDM). The concentration/dilution step was repeated two times. In some cases, 2 mM methyl-β cyclodextrin was added to the dilute protein and the mixture was concentrated for crystallization.

Immediately before crystallization, P-gp and the P-gp–Nb592 complex were concentrated to 8–12 mg/mL P-gp-only crystals (crystal form A) described in this work were grown by using the sitting drop method at 4 °C by combining protein and precipitant at 1:1 (volume:volume). Crystal 1 and crystal 2, and all point mutants of Pgp (Table S2), were grown at a protein concentration of 10–12 mg/mL by using 0.1 M Hepes (pH 7–8), 50 mM lithium sulfate, 10 mM EDTA, and 25–29.5% (wt/vol) PEG 600 at 4 °C. These crystals typically appeared after 1–3 d and continued to grow to full size in approximately 2 wk. Crystals of the P-gp–Nb592 complex were grown by using 0.1 M Hepes (pH 7–8) and 22–27% (wt/vol) PEG 600.

X-ray diffraction data were collected on cryo-cooled crystals at the Stanford Synchrotron Radiation Laboratory (BL 11–1), the Advanced Light Source (ALS) (BL5.0.1) and the Advanced Photon Source (23-ID-B, and 23-ID-D). Datasets were processed with HKL2000 (HKL Research, Inc.) and mosflm (37). Experimentally derived protein phases were obtained via the multiple anomalous dispersion technique by using diffraction data collected on the mercury LIII edge and inflection point (Table S1) calculated by the program PHASES. The overall combined phasing power was 1.9, with a figure of merit of 0.732. The experimental electron density map revealed that there was only one molecule in the asymmetric unit. An initial P-gp model was positioned by using PDB ID code 3GSU using the program Coot (38). The structures of crystal 1 and crystal 2 were determined by molecular replacement using the package MolRep as part of the CCP4 suite (37). Similar to the original published structures of P-gp, the N terminus (residues 1–33) was not visualized and no electron density was present for most of the linker region (residues 627–683), which is likely a flexible region connecting the two halves of P-gp.

To further validate these structures of P-gp (crystal 1 and crystal 2), we introduced single-site cysteine mutants throughout the TM regions (Table S2). PCR-based mutagenesis was performed with pairs of complementary mutagenic primers that carry the desired cysteine (codon TGT) by using the *pLIC-Opti-Pgp* expression plasmid (32) as a template, *Pfx50* DNA polymerase (Invitrogen), and the In-Fusion HD cloning kit (Clontech). All plasmids were verified by sequencing. Each mutant was expressed in *P. pastoris* strain

KM71H (9, 32), purified, and crystallized as described above. Crystals of P-gp mutants were soaked with 5 mM ethyl-mercury chloride for 1–2 h, flash cooled, and X-ray data was collected at synchrotrons and by using our in-house X-ray source (Bruker). The identity and position of the corresponding mercury-labeled cysteine residues yielded peaks in the anomalous difference Fourier (Fig. 1B and Fig. S2).

For the structure determination of the P-gp–Nb592 complex, the molecular replacement method was used followed by rigid body refinement of the TMD and NBD regions using crystal 1 as the model. The position of Nb592 was determined by using a $2F_o - F_c$ difference map using phases derived from only a P-gp model. A homology model for Nb-592 was built from chain A of PDB ID code 1HCV by using Swiss Model (39) and idealized by using the Chiron server (<http://troll.med.unc.edu/chiron/login.php>). The preliminary nanobody model was manually fit into difference density and refined against the X-ray data by using rigid body refinement.

Crystallographic refinement using native data from all crystals of P-gp was accomplished by using the simulated annealing protocol (mlf target; CNS v1.3) and later using Phenix v1.8.2–1309 (40). A final round of group B-factor refinement and bulk solvent correction produced chemical models for crystals 1–3. The chemical geometry of the refined P-gp and P-gp–Nb592 complex structures were corrected and checked with molprobity (41), yielding models with no violations in the Ramachandran phi-psi plot and good bond

angle/bond-length geometry (Table S1) comparable with other structures in this moderate resolution range. All structures of P-gp were validated by using a sigma-A weighted $2F_o - F_c$ composite simulated annealing (SA) omit map (iteratively omitting 5% of the model) and multiple $F_o - F_c$ SA difference maps. The $F_o - F_c$ maps were calculated by using CNS v1.3 systematically omitting 11 consecutive residues throughout the model, generously omitting a neighboring sphere size of 4.0 Å, and a map cushion surrounding the omitted region of 2.0 Å. All models of P-gp and P-gp–Nb592 were also validated by using $F_o - F_c$ difference maps Figs. S5–S7. Figures were generated by using PyMOL (14) and Adobe Photoshop 7.0.

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