Multidrug Resistance Protein (P-Glycoprotein; MDR1)

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Abbreviations: ABC, ATP-binding cassette; ALLN, N-acetyl-leucyl-leucyl-norleucinal; AML, acute myelogenous leukemia; AUC, area under the curve; CNS, central nervous system; DSC, differential scanning calorimetry; EM, electron microscopy; EPR, electron paramagnetic resonance; FRET, fluorescence resonance energy transfer; GSL, glycosphingolipid; H33342, Hoechst 33342; MDR, multidrug resistant/resistance; MIANS, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid; NB, nucleotide-binding; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; PC, phosphatidylcholine; Pgp, P-glycoprotein; QSAR, quantitative structure-activity relationship; rhodamine 123, R123; SNP, single nucleotide polymorphism; ^{99m}Tc-MIBI, technetium-99m-sestamibi; TM, transmembrane; TMR. tetramethylrosamine; TNP, 2′(3′)-*O*-(2,4,6-trinitrophenyl)

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The P-glycoprotein gene family

P-Glycoprotein (Pgp) was one of the first members of the ATP-binding cassette (ABC) superfamily to be studied. Overexpression of Pgp was linked to multidrug resistance (MDR) in mammalian cell lines and human cancers, evoking intense interest first from molecular and cell biologists, and later, when purified Pgp became available, from biochemists and biophysicists. Today this fascinating protein, which is proposed to operate as an ATP-powered drug efflux pump, remains one of the most studied membrane transporters. Pgp genes from human, mouse, and Chinese hamster, among others, have been cloned and sequenced, and homologs have been identified in other species, including *Drosophila melanogaster* and *C. elegans*.[1,2] Pgp in higher mammals forms a small gene family, with two isoforms expressed in humans, and three isoforms in rodents. The Class I and III isoforms (human *MDR1/ABCB1*, mouse *mdr1/Abcb1a* and *mdr3/Abcb1*) are drug transporters, while the Class II isoforms (human *MDR2/3/ABCB4*, mouse *mdr2/Abcb4*) carry out export of phosphatidylcholine (PC) into the bile.[3] The two human genes arose from a duplication event, and are adjacent to each other on the chromosome. The drug-transporting isoform shares 78% amino acid sequence identity with the PC-exporting isoform, suggesting that they share similar structures and mechanisms of action. For the rest of this chapter, the term Pgp will be used to indicate the ABCB1 gene product.

Tissue distribution of P-glycoprotein

Early studies of Pgp distribution in human[4] and rodent[5] tissues showed that the protein is expressed at low levels in most tissues, but is found in much higher amounts at the apical surface of epithelial cells lining the colon, small intestine, pancreatic ductules, bile ductules and kidney proximal tubules, and the adrenal gland. Thus, epithelial cells with excretory roles generally express Pgp. The transporter is also located in the endothelial cells of the blood brain barrier,[6] the blood testis barrier,[7] and the blood-mammary tissue barrier,[8] and has recently been found to play a role in the blood-inner ear barrier, where it is expressed in the capillary endothelial cells of

the cochlea and vestibule.[9] Thus the role of Pgp in the blood-brain and blood-tissue barriers is likely to protect these organs from toxic compounds that gain entry into the circulatory system. Pgp is expressed at high levels at the luminal surface of secretory epithelial cells in the pregnant endometrium,[10] as well as the placenta,[11] where it may provide protection for the fetus[12]. The protein is also found on the surface of hematopoietic cells, where its function remains enigmatic. The ABCB4 protein is expressed at high levels on the bile canalicular membrane of hepatocytes, in accordance with its proposed role in transport of PC into the bile.[13]

Role of P-glycoprotein in human physiology

The tissue localization of Pgp suggests that the protein plays a physiological role in the protection of susceptible organs like the brain, testis and inner ear from toxic xenobiotics, the secretion of metabolites and xenobiotics into bile, urine, and the lumen of the gastrointestinal tract, and possibly the transport of hormones from the adrenal gland and the uterine epithelium. These ideas have been strongly supported by studies on transgenic knockout mice lacking one or both of the genes encoding the drug-transporting Pgps, Abcb1a and Abcb1b. Both the single and double knockout mice are fertile, viable, and phenotypically indistinguishable from wild-type mice under normal conditions. So Pgp does not appear to fulfill any essential physiological functions. However, Pgp knockout mice showed radical changes in the way that they handled a challenge with many drugs.[14] mdr3 knockout mice displayed a disrupted blood-brain barrier, and were 100-fold more sensitive to the pesticide ivermectin, which was neurotoxic to the animals.[15] This Pgp isoform appears to play the major role in preventing accumulation of drugs in the brain.[15,16] The double-knockout mouse has proved useful in evaluating the effect of Pgp-mediated transport on drugs that are targeted to the central nervous system.[17] Certain dogs of the Collie lineage,[18] and several other dog breeds,[19,20] have a naturally-occurring lack of Pgp due to a frame-shift mutation in the MDR1 gene, and are also hypersensitive to ivermectin. To date, no human null alleles have been reported, despite widespread use of drugs that are Pgp substrates.

Pgp in the intestinal epithelium plays an important role in the extrusion of many drugs from the blood into the intestinal lumen, and in preventing drugs in the intestinal lumen from entering the bloodstream. Pgp activity can, therefore, reduce the absorption and oral bioavailability of those drugs that are transport substrates.

One important goal in clinical medicine has been the development of techniques for *in vivo* functional imaging of Pgp-mediated drug transport in normal tissues and tumors, and its inhibition by specific Pgp modulators. The radiopharmaceutical, technetium-99m-sestamibi (^{99m}Tc-MIBI) has been validated as a Pgp transport substrate. Scintigraphic studies of human subjects showed rapid clearance of the radiotracer from normal liver and kidneys *in vivo*, however, it was selectively retained in these organs after administration of the Pgp modulator, PSC833.[21] Later studies have shown the prognostic value of this approach in different types of tumors, including breast and lung cancer, sarcoma and lymphoma.[22] The activity of Pgp at the human blood brain barrier has also been imaged using positron emission tomography using ^{11}C-labeled verapamil or carvedilol (Pgp transport substrates).[23]

P-glycoprotein substrates and modulators

Pgp has the ability to interact with literally hundreds of structurally diverse substrates (see Table 10-1), which are generally nonpolar, weakly amphipathic compounds, and include natural products, anticancer drugs, steroids, fluorescent dyes, linear and cyclic peptides, ionophores, *etc*. The unusual promiscuity of the transporter has made it difficult to find "non-substrates". Potential physiological substrates for Pgp could include peptides, steroid hormones, lipids, and small cytokines, such as interleukin-2, intereukin-4, and interferon-γ. However, there is little information on the extent to which endogenous compounds are transported by Pgp *in vivo*. Identification of a specific compound as a Pgp substrate is often indirect, although more specific spectroscopic approaches now allow measurement of binding affinity.[24] Direct measurement of Pgp-mediated transport has been carried out for only a small fraction of these substrates. Work

with reconstituted Pgp has shown that it is an active transporter, generating a substrate concentration gradient across the membrane.[25,26] In intact cells, the drug concentration in the cytosol is kept low enough to circumvent cytotoxicity, and they thus become multidrug-resistant.

A second class of compounds exists which interact with Pgp, the so-called modulators (also known as MDR chemosensitizers, reversers or inhibitors; see Table 10-1). Modulators are able to reverse MDR in intact cells *in vitro*, by interfering with the ability of Pgp to efflux drug and thus generate a drug concentration gradient. The ability to selectively block the action of Pgp is of importance clinically, whether the goal is to achieve more efficacious cancer chemotherapy, improve drug bioavailability and uptake in the intestine, or deliver drugs to the brain. Numerous pharmacologic agents have been identified as Pgp modulators, many by serendipity or trial and error (see Table 10-1). Modulators are as diverse structurally as substrates.[27] They appear to interact with the same binding site(s) as drugs, and compete with them for transport. Many modulators (*e.g.* verapamil, cyclosporin A, *trans*-flupenthixol) are themselves transported by the protein. Cells are generally not resistant to killing by modulators, but they are killed by MDR drugs in combination with modulators. The way in which modulators exert their action at the molecular level is still not well understood. .

P-glycoprotein structure

Like many other ABC proteins,[28,29] Pgp comprises two membrane-bound domains, each made up of six transmembrane (TM) helices, and two cytoplasmic nucleotide-binding (NB) domains which bind and hydrolyze ATP (Figure 10-1A). The topology of Pgp was established using molecular biological methods such as Cys mutations and insertion of glycosylation sites.[30,31] Earlier studies using various heterologous expression systems suggested alternate topologies in which putative TM segments were displaced outside the membrane, however, it seems likely that these arrangements were the result of misfolding, and do not reflect the true topology of the transporter *in vivo*.[32] The TM regions from both halves of Pgp form the drug-

binding region of the protein,[33] and drugs enter this binding pocket from the lipid bilayer.[34]

High resolution X-ray crystal structures of two ABC proteins, the catalytic domains of the DNA repair enzyme Rad50cd[35] and the vitamin B12 importer BtuCD,[36] showed that the two NB domains were in close contact to form a dimeric structure. Two molecules of ATP were bound at the dimer interface, with each binding site comprising the Walker A and B motifs of the *cis*-NB domain, and the LSGGQ signature C motif of the *trans*-NB domain. This so-called "sandwich dimer" structure has also been observed for the isolated NB domain of the ABC protein MJ0796, which forms a stable dimer when the ATPase activity of the protein is inactivated by the mutation E171Q.[37] It seems likely that this dimerization process plays a critical role in the catalytic cycle of the ABC proteins, and may be closely tied to the power stroke.[29]

No high resolution X-ray crystal structure is available for Pgp. Early work by Rosenberg *et al*. using electron microscopy (EM) single particle image analysis of purified Pgp produced a very low resolution structure which suggested the existence of a large, 5 nm diameter, central pore in the protein.[38] This pore was closed at the cytoplasmic face of the membrane, forming an aqueous chamber within the membrane from which entry points to the membrane lipid were observed. Two widely-separated 3 nm lobes on the cytoplasmic side of the membrane were thought to represent the NB domains. This structure was at odds with both biochemical studies, which suggested kinetic cooperativity between the two catalytic sites, and the high-resolution X-ray crystal structures of other ABC proteins described above, which showed close physical association of the two NB domains. Fluorescence resonance energy transfer (FRET) studies in which two different fluorescent probes were covalently linked to each Walker A motif Cys residue also indicated that the positioning of the two NB domains is compatible with the sandwich dimer model (Figure 10-1B),[39] and Urbatsch *et al.* found that the two Walker A Cys residues could readily crosslink spontaneously.[40] In addition, Loo *et al.* showed that Cys residues in the Walker A motifs could be crosslinked at low temperatures to Cys residues in the LSGGQ

motif, indicating that the signature sequences in one NB domain are adjacent to the Walker A site in the other NB domain.[41] Later work by Rosenberg and co-workers showed that nucleotide binding causes a repacking of the TM regions of Pgp,[42] which could open the central pore to allow access of hydrophobic drugs directly from the lipid bilayer.[43] It was proposed from this reorganization that ATP binding, not hydrolysis, drives the conformational changes associated with transport.[42] The vanadate-trapped complex of Pgp, which is thought to resemble the catalytic transition state structurally, displayed a third distinct conformation of the protein, suggesting that rotation of TM α-helices had taken place.[42] Mouse Pgp has also been studied by EM and image analysis of 2D crystals of purified protein in a lipid bilayer.[44] The resulting low resolution projection structure (22 Å) was compact, and suggested that the two cytoplasmic NB domains interact closely.

More recently, a higher resolution EM structure was obtained for human Pgp which shows close association of the NB domains,[45] and bears a much greater resemblance to the mouse Pgp structure (Figure 10-1C), so it seems likely that the NB domains indeed form the "sandwich dimer" observed for other ABC proteins. This structure also clearly showed the existence of 12 TM segments, supporting the proposed topology of the protein, but the resolution was not high enough to discern further details. The packing arrangement of the TM helices of Pgp has been systematically explored by Clarke and co-workers, who introduced Cys residues into defined positions within a Cys-less Pgp construct, and then carried out cross-linking studies.[46] The observed pattern suggested that TM6 is close to TM10, 11, and 12, whereas TM12 is close to TM4, 5, and 6. Recent work showed that the ends of TM2 and TM11 are close together on the cytoplasmic side of the membrane,[47] as are the cytoplasmic ends of TM5 and TM8.[48]

Subcellular systems for studying P-glycoprotein

Much early work on the molecular basis of MDR was carried out on intact cells selected for MDR by growth in high concentrations of drugs, such as colchicine and vinblastine.

However, the difficulties involved in dissecting such a complex system soon led to attempts to use simpler subcellular systems to study the MDR phenomenon. Native plasma membrane vesicles isolated from MDR cells expressing high levels of Pgp have proved to be very useful. When compared to membrane preparations from the drug-sensitive parent cell line, they often display much higher levels of ATPase activity, which are attributable to the presence of large amounts of Pgp in the plasma membrane.[49,50] In addition, membrane vesicles were found to be labeled by photoaffinity analogs of both MDR drugs[51] and nucleotides,[52] providing some of the first biochemical evidence that Pgp binds these molecules. Since then, membrane vesicles have been used for sophisticated kinetic studies of substrate binding using radiolabeled drugs.[53]

Plasma membrane vesicles have also proved useful in studies of Pgp-mediated drug transport. Most vesicle preparations consist of a mixture of right-side-out and inside-out vesicles,[54] and if they are well-sealed, the latter population can transport drug from the external medium into the vesicle lumen when provided with ATP. When using a vesicle system where other membrane-bound ATPases are present, it is often necessary to add an ATP-regenerating system, such as creatine kinase and creatine phosphate, to prevent rapid depletion of ATP in the external solution. Substrate uptake into the vesicle interior can be measured in one of two ways. If drug is available in radioactive form (*e.g.* [^{3}H]-colchicine, [^{3}H]-vinblastine, [^{125}I]-peptide), it is added to the vesicle preparation at time zero, together with ATP and a regenerating system, and vesicles are removed at various times (typically ranging up to 30 minutes) and collected by rapid filtration.[54] Drug uptake into the vesicles increases with time, usually reaching a plateau value which represents a steady-state. This steady-state is a result of two competing processes; active transport of drug by Pgp into the vesicle lumen (up a concentration gradient) and passive diffusion of the hydrophobic drug out of the vesicle (down a concentration gradient). Addition of excess unlabeled drug to the vesicle exterior once the steady-state has been reached results in very rapid exchange with labeled drug in the vesicle interior.[54] Ruetz and Gros expressed all

three mouse Pgps in the yeast mutant strain sec 6-4, which accumulates large numbers of secretory vesicles because of a trafficking defect.[55] These vesicles contained sufficient Pgp for characterization of the drug transport process using a rapid filtration approach.

Caution should be taken when using the fixed time-point rapid filtration approach since transport can become non-linear within 1 minute, making estimation of the initial rates of Pgp-mediated transport difficult. In these situations, maximal uptake of drug is measured instead, however, steady-state uptake values cannot be treated as kinetic data, and do not allow, for example, determination of K_M or V_{max} for the drug transport process. In addition, this approach consumes relatively large amounts of membrane vesicles and radiolabeled drug. Fluorescence approaches have been developed that circumvent these problems, and allow continuous real-time monitoring of Pgp-mediated drug transport in native membrane vesicle systems. For example, fluorescence quenching of daunorubicin transported into the interior of DNA-loaded plasma membrane vesicles allowed kinetic characterization of Pgp-mediated drug transport.[56]

Biochemical characterization of Pgp required purification of the protein in a functional state. This has been accomplished by several research groups, using a variety of drug-selected MDR cell lines, and cells transfected with the MDR1 gene, as the source of protein.[57-61] In general, expression of Pgp in heterologous systems (*E. coli*, baculovirus-infected insect cells, and yeast) has been fraught with difficulties, and has not led to the widespread use of this approach. The use of *E. coli* as a host cell for expression was shown to lead to misfolding of the protein.[32] Overexpression in the yeast, *Pichia pastoris*, is the exception, and has led to the purification of milligram amounts of both wild-type and mutant Pgps.[62] This system has also proved very useful for overexpression of other ABC transport proteins.[63] Purified Pgp has been characterized with respect to both its ATPase and drug transport activities (see below), and various biophysical studies have been carried out to assess its structure and conformation, using CD spectroscopy,[64] fluorescence spectroscopy,[24] and EM.[44] Pgp has been successfully reconstituted into

proteoliposomes, so that both its ATPase and drug transport functions are retained.[25,26,58,65-68]

ATP binding and hydrolysis by P-glycoprotein

ATP hydrolysis supplies the energy for active drug transport. In most ATP-driven transporters, ATP hydrolysis is tightly coupled to substrate transport, so that it is hydrolyzed only when substrate is concurrently transported. However, Pgp is unusual in displaying a high level of constitutive (basal) ATPase activity, which is observed in the absence of added drugs for plasma membrane vesicles from MDR cells[49,50] and insect cells overexpressing recombinant Pgp,[69,70] and purified Pgp.[57-59] Constitutive ATPase activity has since been reported for other eukaryotic ABC proteins, including MRP1 (ABCC1), CFTR (ABCC7), ABCA1, ABCR (ABCA4) and several bacterial ABC transporters. Purified Pgp has a maximal basal ATPase activity as high as 3-5 μmol/min per mg protein, depending on the presence of detergent, lipids and drugs.[62,71]

The K_M for ATP hydrolysis by membrane-bound and purified Pgp reported by several laboratories is quite high (in the range 0.4-0.8 mM), indicating that Pgp has a relatively low nucleotide affinity compared to other transporters. A divalent cation is necessary for ATP hydrolysis. Physiologically, this ion is Mg^{2+}, although both Mn^{2+} and Co^{2+} can support ATP hydrolysis at lower rates.[72] Several inhibitors of Pgp ATPase activity have been identified, including ortho-vanadate and various sulfhydryl-modifying agents, including maleimides, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), *p*-chloromercuribenzenesulfonate, $HgCl_2$, *etc*. Sulfhydryl reagents covalently modify two Cys residues, one in each Walker A motif (Cys 431 and 1074 in human Pgp),[73] and thereby inhibit catalysis, although ATP binding still takes place.[74] These Cys are not required for ATPase activity, since a Cys-less Pgp protein is still active,[30] and the loss of activity when they are modified likely results from steric interference.

The basal ATPase activity of Pgp is modulated by drug substrates and modulators in a complex and puzzling fashion. Three different patterns have been observed. Many drugs display a biphasic pattern, with stimulation of ATPase activity at low concentrations, and varying

degrees of inhibition at higher concentrations. Some compounds have been observed to only stimulate activity; for example, many linear peptides, cyclic peptides, and ionophores stimulate Pgp ATPase activity up to 2.5-fold.[75] On the other hand, some substrates appear to produce only inhibition of activity. The molecular basis of these differences in ATPase modulation is not known. The biphasic pattern might arise from the presence of a "stimulatory" drug binding site and an overlapping "inhibitory" drug binding site,[76] but why such an arrangement would be intrinsic to the mechanism of Pgp is not clear. To complicate matters further, extremely variable results have been seen from one research lab to another. For example, vinblastine stimulated the ATPase activity of human Pgp,[77] but inhibited the ATPase activity of hamster Pgp.[57-59] Modulation of Pgp ATPase activity by drugs and modulators is also highly dependent on the detergent used to isolate the protein, or the surrounding lipid environment.[62,65,78]

The ATPase activity of Pgp is rapidly and completely inhibited by the P_i analog, ortho-vanadate (V_i) in the presence of ATP. V_i is trapped after a single catalytic turnover in only one NB domain,[72] as the complex $ADP{\cdot}V_i{\cdot}M^{2+}$, where M^{2+} is a divalent cation, usually Mg^{2+}. The trapped complex can also form from ADP and V_i, but at a lower rate. The V_i-trapped complex displays no ATPase activity, suggesting that both catalytic sites must be functional for ATP hydrolysis to take place. Based on these observations, Senior *et al.* proposed that Pgp operates by an alternating sites mechanism, whereby only one catalytic site can be in the transition state conformation at any time, and the two sites alternate in catalysis.[79] Based on studies of myosin and other nucleotide-utilizing proteins, the V_i-trapped complex is believed to structurally resemble the catalytic transition state.[80] However, the V_i-trapped complex of Pgp is very stable; V_i and ADP dissociate slowly from the catalytic site, and ATPase activity is regained.[72]

Nucleotide binding to Pgp is of relatively low affinity, making it difficult to measure by classical techniques. Binding of both unmodified nucleotides[74,81] and fluorescent TNP-labeled nucleotides[71] to purified Pgp has been quantitated using fluorescence spectroscopic approaches.[24]

Recently, an electron paramagnetic resonance (EPR) spectroscopy study also examined binding of a spin-labeled ATP analog.[82] These studies were consistent in showing a K_d value for ATP or ADP binding in the range 0.2-0.4 mM. TNP-labeled nucleotides bind with higher affinity (K_d of 30-40 μM), likely because the nitrophenyl ring engages in additional interactions with hydrophobic residues in the nucleotide-binding site.[71] The stoichiometry of ATP binding is normally 2 (i.e. both catalytic sites are occupied).[82,83] In the V_i-trapped complex, the second untrapped catalytic site can still bind ATP with the same affinity.[83]

Drug binding to P-glycoprotein

Several different approaches have been used to characterize the binding of drugs and modulators to Pgp. Photoaffinity labeling by analogs of substrates and modulators, such as [^{3}H]azidopine and [^{125}I]iodoarylazidoprazosin, has been widely used to study the drug-binding properties of Pgp.[84,85] Competition experiments with photoactive substrate analogs have given an indication of binding affinity, and demonstrated interactions between substrates and modulators. However, labeling stoichiometry is often very low, complicating interpretation of the results. In addition, kinetic analysis of binding, and quantitation of dissociation constants, is not possible.

Direct binding studies using radioactive drugs and modulators have been carried out using native plasma membrane vesicles containing Pgp.[53,86-89] Such an approach is technically difficult because of the high levels of non-specific background binding obtained with hydrophobic drugs, which arises from non-specific partitioning into the membrane. Detailed kinetic analysis led to the estimation of K_d values for binding, and rates of association/dissociation could also be quantitated. Complex allosteric interactions were found between multiple drug binding sites.[53]

Fluorescence quenching approaches have been developed to monitor binding and obtain quantitative estimates of K_d for binding of drugs and modulators to purified Pgp.[24,90] These techniques can measure equilibrium binding without the need to separate Pgp-bound drug from free drug. The first approach used Pgp labeled at the two Walker A motif Cys residues with 2-(4′-

maleimidylanilino)naphthalene-6-sulfonic acid (MIANS).[74] Saturable quenching of MIANS fluorescence was obtained with nucleotides, drugs and modulators, and fitting of the data led to an estimate of the K_d value for binding. More recently, saturable quenching of the intrinsic Trp fluorescence of purified Pgp was observed with nucleotides, drugs and modulators, and again led to quantitation of the binding affinity.[81] Values of K_d for a large number of different drugs and modulators range from 37 nM for paclitaxel (a high affinity substrate), to 158 μM for colchicine (a low affinity substrate).[24,91] Thus, the substrate binding affinity of Pgp covers a range of 10^4.

P-glycoprotein-mediated drug transport

The transport activity of Pgp can be studied in intact cells, or in simpler subcellular systems, such as plasma membrane vesicles and reconstituted proteoliposomes. In general, it has proved difficult to characterize the transport properties of Pgp in complex intact cell systems. However, one exception to this has been the use of polarized epithelial cells (such as MDCK, LLC-PK1 or Caco-2 cells) grown as monolayers on permeable filters that allow separate access to the basal and apical compartments.[92] Transfection of Pgp results in expression of the protein at the apical surface, and quantitative measurements of basal-to-apical and apical-to-basal fluxes of a drug can be made.[93-95] This approach can be very useful for direct determination of whether a drug is transported by Pgp, and showed that many MDR modulators are themselves transport substrates.[94,96-98] However, these cell lines also show endogenous expression of drug transporters, although at low levels, which may complicate interpretation of experimental data.

Plasma membrane vesicles from MDR cells have been used extensively for measurements of Pgp-mediated drug transport. Inside-out vesicles (present in variable amounts in plasma membrane preparations) transport drug into the lumen when supplied with ATP and an ATP-regenerating system.[54,56,99-103] Radiolabeled drugs, such as [^{3}H]vinblastine, [^{3}H]daunorubicin or [^{3}H]colchicine, are usually employed. Some early work purporting to measure drug binding to membrane vesicles did not differentiate between binding and transport, since ATP was included

in the samples (at the time, it was not known if ATP was required for drug binding). These studies probably measured drug transport rather than ATP-dependent drug binding. Osmotic sensitivity is a useful test to differentiate between transport and binding, and has been used for both plasma membrane vesicles[54,99,100] and reconstituted systems.[54] One additional difficulty is the high background levels of drug often observed for hydrophobic drugs like vinblastine.[54]

In general, drug transport into plasma membrane vesicles or proteoliposomes is saturable at high drug concentrations, and requires ATP hydrolysis; non-hydrolysable analogs do not support transport. A drug concentration gradient is generated across the membrane, which can usually only be estimated indirectly.[54] Drugs/modulators block transport of other drugs with varying degrees of effectiveness. Reconstituted proteoliposomes containing fully or partially purified Pgp have also been used to characterize drug transport. An ATP-regenerating system is often not required, since other membrane-bound enzymes do not deplete ATP. Similar approaches using radiolabeled substrates have been used to monitor transport in proteoliposomes. In this more defined system, the magnitude of the drug concentration gradient was estimated more precisely; Pgp built up a 5- to 6-fold gradient of colchicine[25] and NAc-LLY-amide.[26]

Real-time fluorescence assays can continuously monitor the Pgp-mediated transport of fluorescent substrates. H33342 is highly fluorescent when partitioned into the membrane, but loses fluorescence after export into the aqueous solution, allowing the initial rate of movement of the dye out of plasma membrane vesicle to be quantitated in real time.[104] The same system was used to demonstrate H33342 transport by purified Pgp reconstituted into liposomes in an inside-out orientation.[66] Tetramethylrosamine (TMR), which loses its fluorescence when transported into the interior of reconstituted vesicles containing Pgp, was used to measure the initial rate of transport over times as short as 30 seconds.[105] Kinetic analysis of TMR transport showed that Pgp obeyed Michaelis-Menten kinetics with respect to both ATP (K_M = 0.48 mM, close to the K^M for ATP hydrolysis) and TMR (K_M = 0.3 μM).

The stoichiometry of ATP hydrolysis relative to the number of drug molecules transported by Pgp is a controversial issue that has still not been resolved, mainly because of the high basal levels of ATP hydrolysis. Sharom *et al.* estimated that 3-4 additional molecules of ATP were hydrolysed for every molecule of colchicine transported,[25] while Ambudkar *et al.* reported that 2.8 ATP molecules were hydrolysed for every molecule of vinblastine transported.[106] Shapiro and Ling estimated that the apparent rate of transport of H33342 from the membrane was 50-fold lower than the rate of ATP hydrolysis.[66] It seems likely that the true turnover rate of Pgp transport will always be underestimated by conventional transport experiments, since the rate of *net* drug accumulation inside a vesicle or proteoliposome is measured. Lipophilic drugs that are moved from the membrane into the lumen immediately repartition into the bilayer, where they are then re-exported, resulting in futile cycling of the transporter that does not result in a net increase in drug moved across the membrane. A transport system using the ionophore valinomycin (a Pgp substrate) with bound $^{86}Rb^{+}$ circumvented this problem.[107] Pgp mediated the ATP-dependent uptake of valinomycin-$^{86}Rb^{+}$ complex into the proteoliposome lumen, where the radioactive cation accumulated to a concentration of 8 mM, since it is not lipid-soluble. When the ATPase and transport activities of Pgp were measured under the same conditions, comparable rates of valinomycin transport and ATP hydrolysis were found, with 0.5-0.8 ionophore molecules transported/ATP molecule hydrolyzed.[67]

Substrate specificity of P-glycoprotein and nature of the drug-binding site

Pgp displays a remarkable ability to interact with, and transport, a large variety of compounds, ranging from chemotherapeutic drugs to peptides. Most preferred substrates are amphipathic and relatively hydrophobic, although some are not (colchicine, for example, is quite water-soluble). Pgp substrates range in size from large complex molecules, such as paclitaxel and vinblastine, to smaller drugs such as daunorubicin and doxorubicin. Pgp also interacts with linear and cyclic peptides and ionophores, including gramicidin D, valinomycin, N-acetyl-leucyl-

leucyl-norleucinal (ALLN), leupeptin, pepstatin A and several bioactive peptides.[75,108] Small tripeptides, such as NAc-LLY-amide, are excellent transport substrates.[26] Even nonionic detergents, such as Triton X-100 and nonylphenol ethoxylates, interact with Pgp.[91,109,110] Many substrates, but not all, contain planar aromatic rings and positively-charged tertiary N atoms. No highly conserved recognition elements have been found in Pgp substrates and modulators.

Many attempts have been made over the years to develop a quantitative structure-activity relationship (QSAR) for Pgp substrates and modulators, to link various properties of these molecules (physical, chemical or structural) with their biological activity.[111] One problem in achieving this goal has been the wide variety of biological assays (many indirect) used to infer interaction with Pgp, the use of limited series of structurally related compounds, and the lack of consistency in the molecular descriptors used. Very few studies have measured binding affinity directly, and various surrogates, such as inhibition of drug transport or stimulation of ATPase activity, have been used instead. An additional complication has been the likely existence of at least two binding regions within the drug binding pocket of the protein, which interact with each other allosterically.[112] Hydrophobic peptides appear to differ from other Pgp substrates (for example, they much smaller than a typical substrate, and often have no aromatic rings or tertiary N atoms), and most studies on common pharmacophores have not considered them.

Seelig *et al.* defined a set of structural elements that are required for interaction of a compound with Pgp,[113] consisting of two or three electron donors (hydrogen bond acceptors) arranged in a fixed spatial separation. Any molecule containing at least one of these structural units was predicted to be a Pgp substrate, and binding affinity was predicted to increase with hydrogen bond strength. The TM domains of Pgp contain a high fraction of amino acids with side chains capable of acting as hydrogen bond donors to interact with substrates. A more recent 3D-QSAR study supported these ideas, and suggested that interaction of the substrate with one or more sites within the protein plays a key role in efflux.[114] Substrate recognition was proposed to

be based on the dimensions of the drug molecule, and the presence of two types of recognition elements, two hydrophobic groups 16.5 Å apart and two hydrogen bond acceptors 11.5 Å apart. Another 3D-QSAR approach was used to generate a Pgp pharmacophore consisting of one hydrogen bond acceptor, one aromatic ring, and two hydrophobic units.[115] Pajeva and Wiese proposed a pharmacophore model consisting of two hydrophobic units, three hydrogen bond acceptors, and one hydrogen bond donor.[116] They concluded that drug binding affinity depends on the number of points simultaneously involved in the interaction with Pgp, and proposed that different drugs can be involved in different binding modes with these points. QSAR studies have also been carried out for Pgp modulators,[117,118] and attempts have been made to classify them based on their structures.[119] Artificial neural networks have also been used,[120] with the aim of employing such analysis as a predictive tool to identify new MDR-reversing agents.

Many questions remain about how Pgp can bind and transport so many structurally diverse compounds. Biochemical studies have been used to argue for a single common drug binding site, or two or more separate sites. Based on ATPase inhibition studies, it was proposed that drugs, peptides and modulators all competed for a common drug-binding site,[121] whereas another group concluded that two separate pharmacophores existed.[122] Photoaffinity labeling studies suggested that Pgp contained two non-identical drug binding sites, one in each half of the protein.[123] Binding studies using radiolabeled drug supported the existence of multiple binding sites, which displayed complex allosteric interactions and could switch between high- and low-affinity conformations.[53] Shapiro and Ling demonstrated the existence of two "functional" transport sites within Pgp; the H-site showed preference for the drug Hoechst 33342 (H33342), while the R-site showed preference for rhodamine 123 (R123).[112] The two sites interacted with each other allosterically, such that H-site and R-site drugs mutually stimulated each other's transport, while two H-site drugs inhibited each other's transport, as did two R-site drugs. Later work suggested the existence of a third allosterically linked drug transport site.[124]

Soluble bacterial transcription factors that bind multiple drugs, QacR, BmrR and MarR, have provided intriguing insights into how a single drug binding site can accommodate many structurally diverse compounds.[125] Crystallographic studies of QacR complexes with 6 drugs showed that the protein contains a large, flexible binding pocket, rich in aromatic amino acids, but also containing some polar residues.[126] Van der Waal's and hydrophobic interactions play a major role in drug binding, augmented by electrostatic interactions between charged groups on the drug and charged amino acid side chains. The size and flexibility of the binding pocket allow drugs with different structures to establish interactions with different subsets of residues. Two distinct, but partially overlapping, binding pockets were observed. Later studies showed that two drugs could bind to the protein simultaneously.[127] Structural studies of the human xenobiotic nuclear receptor, PXR, showed that the same drug can bind within a large hydrophobic cavity in three different orientations, each stabilized by a different complement of polar side chains.[128]

Multidrug transport proteins such as Pgp likely bind their substrates using principles similar to those observed for soluble multidrug-binding proteins.[129] The crystal structure of the bacterial RND-family multidrug efflux pump, AcrB, binding 4 structurally diverse drugs, showed that 3 ligand molecules bind simultaneously to a large central cavity, primarily by hydrophobic, aromatic stacking and van der Waal's interactions.[130] Each drug binds to AcrB using a different subset of amino acid residues. Studies using Cys mutants and thiol-reactive substrate analogs support the idea of a common hydrophobic pocket within Pgp, and show that residues from multiple TM segments contribute to the binding region.[131-134] Cys crosslinking experiments showed that the packing of the TM segments of Pgp is altered when drugs bind, in a different way for each substrate.[135] This "induced-fit"-type mechanism can explain how the binding pocket accommodates such a broad range of structurally diverse compounds.

Like the transcriptional regulator proteins, the drug-binding pocket of Pgp appears to be able to accommodate more than one compound simultaneously. Based on their crosslinking data,

Loo *et al.* proposed that a thiol-reactive substrate and a second drug molecule could simultaneously occupy different regions of the binding pocket.[136] More recently, fluorescence approaches showed that LDS-751 and R123 could both bind to the R-site of Pgp at the same time, interacting in a non-competitive fashion.[137] The dimensions of the drug binding pocket, determined using a thiol-reactive crosslinking substrate, also suggest that it is large enough to accommodate two substrates at the same time.[138]

Several approaches have been used to locate and characterize the regions of Pgp that form the drug binding pocket. Labeling of the protein with various photoactive drug analogs, followed by chemical or proteolytic cleavage and identification of the labeled peptides showed that several TM segments in both halves of Pgp were involved in substrate binding.[139-142] Different regions of the protein were labeled by different drug analogs, suggesting that they did not all bind at exactly the same location. Mutagenesis studies indicated that residues in TM 4, 5, and 6 in the N-terminal half of Pgp and TM 9, 10, 11 and 12 in the C-terminal half were involved in forming the drug binding pocket.[143] Loo and Clarke systematically inserted single Cys residues by site-directed mutagenesis into 252 positions in the TM segments, and then reacted them with either a thiol-reactive substrate or drug analog.[131,132,144] Overall, the drug binding pocket is envisioned as funnel-shaped, narrower at the cytoplasmic side of the membrane[145] where TM2/TM11 and TM5/TM8 come together.[47,48] They concluded that the drug-binding pocket is found at the interface between the two TM "halves" of Pgp. This was confirmed by Pleban *et al*., who used propafenone-type substrate photoaffinity ligands, in conjunction with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, to define the substrate binding site(s) of Pgp.[146] They observed the highest labeling in TM 3, 5, 8, and 11, and when this pattern was projected onto a 3D homology model of Pgp, labeling was found to occur at the interface formed by TM3 and 11 in one half of the protein, and TM5 and 8 in the other half.

Pawagi *et al.* proposed that aromatic amino acid residues may play an important role in

the binding and transport path for drug substrates.[147] Studies of intrinsic Pgp fluorescence also showed that Trp residues were highly quenched by binding of certain drugs, and FRET from Trp to substrate molecules took place with high efficiency,[81,148] suggesting that Trp residues are located close to the sites of drug binding.

P-glycoprotein as a hydrophobic vacuum cleaner or drug flippase

Pgp substrates are typically hydrophobic, and are expected to partition into the membrane. The substrate binding sites of Pgp appear to be contained within its TM regions, and drugs gain access to these sites after partitioning into the lipid bilayer (Figure 10-2).[34] The idea that the transporter acts as a "vacuum cleaner" for hydrophobic molecules present in the membrane was first suggested by Higgins and Gottesman[149], and has found widespread acceptance. In intact cells, Pgp substrates entering the cell from the extracellular medium are intercepted at the plasma membrane and extruded to the exterior without entering the cytosol.[150] Lipid bilayers are amphipathic multilayered structures, and do not behave like an organic solvent such as octanol in terms of drug partitioning. After entering the membrane, Pgp substrates (which are generally amphipathic in nature) are not distributed uniformly in the hydrophobic core of the lipid bilayer, but tend to concentrate in the interfacial regions of the membrane.[151]

Several studies suggested that the drug binding pocket of Pgp is probably located within the cytoplasmic leaflet of the membrane. Drugs appear to gain access to this binding site after moving to the cytoplasmic leaflet by spontaneous "flip-flop" from the outer leaflet, which can be a slow process for many compounds that are Pgp substrates.[152,153] Transport by reconstituted Pgp of the fluorescent dyes, H33342 and LDS-751, suggested that they were likely extracted from the cytoplasmic leaflet of the membrane.[154,155] The positively charged compound, N-methyl-dexniguldipine, which is unable to flip-flop to the inner leaflet, could interact with Pgp if added to cell fragments and membrane vesicles where the cytoplasmic membrane face is accessible, but not if added to intact cells.[156] Similarly, some peptide modulators cannot interact with Pgp in

intact cells if supplied in the extracellular medium, but can do so in membrane vesicles where they can reach the cytoplasmic leaflet.[157] More recently, a FRET approach estimated the distance separating bound H33342 from a fluorescent probe covalently linked to the catalytic sites, and the results clearly place the drug binding site within the cytoplasmic membrane leaflet.[148] The binding site for another fluorescent substrate, LDS-751, was also localized by FRET to the cytoplasmic half of the bilayer, although closer to the membrane surface in the interfacial region.[158] H33342 binds to the H-site and LDS-751 to the R-site, so it appears that both of these "functional" drug transport sites are in Pgp domains within the cytoplasmic membrane leaflet.

It was proposed that Pgp may operate as a drug flippase, moving hydrophobic drug molecules from the inner to the outer leaflet of the membrane (Figure 10-2).[149] Given the high level of amino acid similarity between Pgp and its close relative, the MDR3/ABCB4 protein, which functions as a PC-specific phospholipid flippase, this suggestion seems reasonable. The location of the drug-binding pocket in the cytoplasmic leaflet of the membrane is also compatible with this idea. If Pgp maintains a higher drug concentration in the outer leaflet than the inner leaflet, then equilibration of drug between the membrane and the aqueous phase on each side would result in the observed drug concentration gradient. Such partitioning/equilibration of nonpolar drugs between lipid bilayers and water is a very fast process, limited only by diffusion.[159] In fact, it is not possible to distinguish experimentally between a transport process in which drugs are moved from the inner to the outer leaflet, followed by rapid partitioning into the aqueous phases on each side, and one in which drugs are moved from the inner leaflet directly to the extracellular aqueous phase, followed by re-partitioning of drug into the outer leaflet.

Several studies have indicated that Pgp can move fluorescent NBD-labeled phospholipid derivatives from the inner to the outer leaflet of the plasma membrane in intact cells overexpressing the protein,[160,161] and glycosphinglipids (GSL) have also been considered as substrates.[162,163] Since then, reconstituted Pgp in lipid bilayer vesicles has been shown to act as

an outwardly-directed flippase for NBD-labeled phospholipids and simple GSL, such as glucosyl-, galactosyl- and lactosylceramide.[164,165] The lipid translocation process shares many biochemical features with drug transport; it requires ATP hydrolysis, it is inhibited by V_i, and drug substrates compete with flippase activity.[164,165] Thus, both drugs and membrane lipids appear to follow the same transport route through the Pgp molecule, increasing the likelihood that drug transport takes place via a flippase-like mechanism. It is possible that Pgp plays a physiological role in flipping glucosylceramide from the cytoplasmic leaflet to the luminal leaflet of the Golgi apparatus, which is a required step in the biosynthesis of complex GSL.[165,166]

Role of the lipid bilayer in P-glycoprotein function

The hydrophobic vacuum cleaner model proposes that drugs and modulators partition into the membrane before interacting with the transporter. Pgp substrates generally have high lipid:water partition coefficients,[152,167] and accumulate within the membrane to high concentrations (Figure 10-2). Pgp would thus experience a much higher drug concentration than that nominally added to the aqueous phase, by 300- to 20,000-fold.[152,167] The role of the lipid bilayer is thus to concentrate the drug; Pgp itself may have a relatively low intrinsic substrate binding affinity. The kinetics of transport of the lipophilic dye, H33342, out of the membrane were measured using a fluorescence approach.[104] The transport rate was directly proportional to the amount of H33342 in the lipid phase and inversely proportional to its concentration in the aqueous phase, thus demonstrating that Pgp removes the dye from the lipid bilayer.

The mode of action of Pgp modulators also appears to be intimately linked to the presence of the membrane, and the behavior of drugs within it. Modulators show the same structural features as substrates, interact with the drug binding pocket, and (in many cases) are also transported by Pgp, yet cells are not resistant to them, and they reverse drug resistance. The behavior of modulators has been linked to their rate of diffusion across the membrane.[168] Pgp substrates were found to cross a lipid bilayer relatively slowly (for example, R123 had a half-life

of 3 minutes), while the transbilayer movement rate of several MDR modulators was too fast to be detected.[169] Amphipathic drugs and modulators localize in the interfacial regions of the bilayer, and appear to cross membranes by a "flip-flop" mechanism.[152] Thus, it was proposed that drugs and modulators are handled similarly by Pgp; they are transported, with hydrolysis of ATP. Compounds that have been pumped out can re-enter the outer leaflet and flip-flop back into the inner leaflet (*i.e.* diffuse across the membrane), before interacting with Pgp again and being re-exported. For substrates, the rate of membrane re-entry is slow enough for efflux *via* Pgp to keep pace, and a drug gradient is established, causing resistance. For modulators, the rate of membrane re-entry is so rapid that Pgp cannot keep pace and essentially operates in a futile cycle; the transport turnover and rate of ATP hydrolysis are high, but no concentration gradient is generated, thus cells are not resistant to modulators. This model[159] suggests that effective modulators are high affinity substrates with a high transbilayer diffusion rate.

Reconstitution of Pgp into bilayers of defined lipids has been an important tool in exploring the role of specific lipids, and the membrane in general, on its activity. Differential scanning calorimetry (DSC) studies indicated that reconstituted Pgp perturbed a large number of membrane phospholipids, even at relatively high lipid:protein ratios.[170] When Pgp was reconstituted into proteoliposomes composed of three different PCs, drug substrates displayed different partition coefficients into these bilayers.[167] The affinity of drug binding, measured using fluorescence quenching, was highly correlated with the lipid:water partition coefficient, so that the K_d decreased as the partition coefficient increased (Figure 10-2) The concentration of the drug in the membrane is thus important for high affinity interaction of drugs with Pgp.

The ATPase activity of Pgp is also modulated by the lipid environment surrounding the protein. Addition of various phospholipids to purified Pgp resulted in concentration-dependent increases or decreases in activity, and protected the protein from thermal inactivation.[78] The values of both the K_M for ATP hydrolysis and K_d for ATP binding were different above and

below the melting temperature of the lipid bilayer, and the activation energies for ATP hydrolysis in the gel and liquid-crystalline phases of the bilayer were also significantly different.[171] Thus, both ATP binding and ATP hydrolysis by Pgp are affected by the phase state of the membrane in which it is reconstituted, possibly because the NB domains may interact with the bilayer surface. The profile for ATPase activation/inhibition by drugs and modulators changes when Pgp is moved from detergent solution into a lipid bilayer,[25] and also varies with the nature of the host lipid in which the protein is reconstituted.[65] This suggests that the coupling between the drug binding sites and the NB domains is affected by the lipid environment of the protein.

Pgp-mediated drug transport is also affected in an interesting way by the fluidity of the membrane. Changes in the fluidity of canalicular membrane vesicles altered Pgp-mediated transport of daunorubicin and vinblastine.[172] When lipid fluidity was increased using membrane fluidizers, drug transport was significantly inhibited, suggesting that physical state of the membrane affects Pgp transport function. This idea was explored further using Pgp reconstituted into proteoliposomes composed of two synthetic PCs with different melting points.[105] A real-time fluorescence assay used to measure the initial rate of transport found a highly unusual biphasic temperature dependence; a high rate of transport in the rigid gel phase, the maximum transport rate at the melting temperature of the bilayer, and a lower transport rate in the fluid liquid crystalline phase. This pattern suggests that the rate of drug transport by Pgp may be dominated by partitioning of drug into the bilayer, which shows similar temperature dependence.

Mechanism of action of P-glycoprotein

Much remains to be understood about how Pgp transports (or flips) drugs, and how coupled ATP hydrolysis powers transport. Transport can be broken down into several steps: entry of substrates into the binding pocket within the cytoplasmic leaflet, conformational changes in Pgp driven by ATP binding/hydrolysis, and release of drug to either the outer leaflet or the extracellular aqueous phase. Many different experimental approaches, including various

biochemical and spectroscopic techniques, have provided evidence that conformational changes take place during the catalytic cycle of Pgp and other ABC proteins.[173] It is assumed that release of drug from Pgp involves reorientation of the drug binding site from the cytosolic side of the membrane (or the inner membrane leaflet) to the extracellular side (or the outer membrane leaflet), accompanied by a switch from high to low drug binding affinity. Superimposed on the transport cycle is the ATP hydrolysis cycle, which involves ATP binding, formation of the nucleotide sandwich dimer, ATP hydrolysis, dissociation of P_i, and dissociation of ADP. A recent review discusses the drug translocation mechanism of Pgp in detail.[174]

Substrates may diffuse from the lipid bilayer into the drug-binding pocket through "gates" formed by TM segments at either end of the pocket".[175] The nature of the local environment within the drug binding pocket is still controversial. Loo *et al*. tested whether Cys residues within the drug-binding pocket of Pgp were able to react with charged thiol-reactive compounds, and concluded that the drug-binding pocket is accessible to water.[176] In contrast, the fluorescence properties of drugs bound to purified Pgp clearly indicate that the local environment of the binding site is very hydrophobic, with a polarity lower than that of chloroform.[158] Several drugs (***e.g.*** H33342, LDS-751) show large increases in the intensity of their fluorescence emission, and a substantial blue shift in their emission wavelength on binding to Pgp, both hallmarks of a hydrophobic local environment.[148,158]

Conformational communication must exist between the drug binding pocket and the NB domains, so that substrate binding activates ATP hydrolysis and initiates the transport cycle. Binding of drugs caused large changes in the fluorescence of MIANS groups covalently linked within the catalytic site of the NB domains, thus confirming this idea. More recently, the effect of drug binding on cross-linking between Cys residues in the signature C and the Walker A motifs was tested. Drug binding in the TM regions induced long range conformational changes in both NB domains, to decrease or increase the distance between these two sequence motifs.[177]

TM2/TM11[47] and TM5/TM8[48] are close together, and likely enclose the drug-binding pocket, which is located at the interface between the TM halves at the cytoplasmic side of Pgp. These regions may form the "hinges" required for conformational changes during the transport cycle. Covalent linkage of a drug analog to position 306 led to permanent activation of Pgp ATPase activity, suggesting that this region of the protein may be part of the signal that switches on ATP hydrolysis when the drugs occupy the binding pocket.[178]

A photoaffinity labeling study reported that the V_i-trapped complex of Pgp, which is thought to resemble the catalytic transition state, has drastically reduced affinity for drug substrates.[179] It was proposed that following ATP hydrolysis, drug is moved from a high to a low affinity binding site, thus promoting release from Pgp on the other side of the membrane.[180] However, this has been contradicted by quantitative fluorescence quenching measurements of drug and nucleotide binding to the V_i-trapped complex, which showed that it can bind several different drugs with high affinity.[181] High affinity substrate binding was also observed for the V_i-trapped complex of the ABC protein TAP1/TAP2.[182] Based on these results, a concerted transport mechanism was proposed rather than a multistep reaction.[181] In this model, release of drug from Pgp during the catalytic cycle is proposed to occur simultaneously with ATP hydrolysis, and precedes formation of the V_i-trapped complex.

Drug release was proposed to occur as a result of re-hydration of the substrate when it enters the drug binding pocket,[176] which was originally envisioned as a large cavity filled by water,[38] and it was suggested that hydration may prevent drug from re-partitioning into the lipid bilayer. However, recent EM structures of Pgp do not show a large water-filled cavity.[44,45] In addition, hydration of drug when it is released into an aqueous environment cannot prevent it from re-entering the membrane, since a hydrated hydrophobic molecule is an unfavourable situation entropically, and it will very rapidly partition into the lipid bilayer.

ATP provides the energy for the “power stroke”, which consists of conformational

change(s) that drive drug transport. Two proposals exist that propose different origins for the power stroke of Pgp.[183] Based on observations that drug binding altered ATP binding affinity,[88] Higgins and Linton proposed the ATP switch model, in which dimerization of the NB domains driven by ATP binding is the source of the power stroke.[184] Tight ATP binding following drug binding is proposed to drive formation of the sandwich dimer, transmitting conformational changes to the drug binding domains that result in drug transport. ATP hydrolysis is then used to separate the NB domains and "re-set" the transporter. However, this model is controversial, since several groups have reported that drug binding does not affect ATP binding,[49,70,77] and quantitation of ATP binding affinity showed only small changes upon drug binding.[83] Senior *et al.* proposed that transport is driven by relaxation of a high-energy intermediate formed during ATP hydrolysis, which thus provides the power stroke.[79] One molecule of ATP was proposed to drive the transport of one drug molecule. Sauna and Ambudkar have proposed an alternate model in which two molecules of ATP are hydrolyzed per cycle.[185] In this model, drug and ATP binding do not influence each other, hydrolysis of one ATP molecule drives drug transport, and hydrolysis of a second ATP molecule re-sets the transporter. This model is also unsatisfactory. There has been no independent verification of the proposed requirement for two rounds of ATP hydrolysis per drug molecule transported. Sauna *et al*. reported that Pgps with mutations in the Walker B Glu residues (E556Q and E1201Q) failed to undergo the second round of ATP hydrolysis required to re-set the transport cycle.[186] However, this was contradicted by Senior and co-workers, who found that these mutants could undergo multiple catalytic turnovers. Rapid kinetic studies that dissect out various steps in the transport cycle, and define their kinetic and thermodynamic constants, may be required to fully understand the mechanism of action of Pgp.

Role of P-glycoprotein in drug therapy

Pgp substrates include many drugs that are used in the treatment of common human diseases. The protein consequently plays a central role in drug absorption and disposition *in vivo*,

and is an important determinant in the pharmacokinetic profile of many drugs, and ultimately, the clinical response.[187,188] Pgp substrates include anti-cancer drugs, HIV protease inhibitors, analgesics, calcium channel blockers, immunosuppressive agents, cardiac glycosides, anti-helminthics, antibiotics, and H_2-receptor antagonists, to name just a few (see Table 10-1).

High levels of Pgp are found in the luminal membrane of the capillary endothelial cells, where it immediately pumps drugs back into the blood. The presence of Pgp strongly reduces the brain accumulation of many different drugs, and in knockout mice, penetration of substrates into the brain is increased 10- to 100-fold. Pgp prevents the penetration of HIV protease inhibitors into the brain, limiting treatment efficacy. Anti-cancer drugs directed to brain tumours are also prevented from reaching their desired site of action.

Pgp appears to be a major player in limiting absorption of drugs from the intestinal lumen. Studies in knockout mice showed that the bioavailability of orally administered paclitaxel, a drug known for its poor solubility, increased from 11% to 35% in animals lacking Pgp.[189] Paclitaxel and other drugs are also excreted directly from the blood circulation into the intestinal lumen. However, not all Pgp substrates show compromised drug absorption. For example, digoxin, HIV protease inhibitors, verapamil, and quinidine all show high oral bioavailability, despite being good Pgp substrates.[187] Thus Pgp may not be as quantitatively important as first thought in drug absorption. It is possible that high drug concentrations in the intestinal lumen saturate the transporter, and the rate of passive drug diffusion through the intestinal epithelium is also high. Because of the increased likelihood that a drug will fail to be effective in animal and human trials if it is a Pgp substrate, many pharmaceutical companies have added interactions with Pgp to their drug discovery screening processes, in an attempt at early identification of these compounds. This is especially important for drugs targeted to the CNS.

Blockade of Pgp with modulators can have dramatic effects on systemic drug disposition, by decreasing drug elimination through the intestine, bile and urine. Initially, the focus was on

using modulators with anti-cancer drugs to improve the efficacy of chemotherapy treatment,[190] but later it was realized that modulators could be useful in altering the pharmacological behavior of many drugs, to improve their delivery. Modulators may enhance intestinal drug absorption and increase drug penetration through biologically important protective barriers, such as the blood-brain, blood-cerebrospinal fluid, and the maternal-fetal barriers. Delivery of drugs to the brain, either to treat epilepsy and other central nervous system (CNS) diseases, AIDS, or brain tumors such as gliomas, might therefore be increased by addition of an effective modulator. This has been shown to be feasible in a mouse model, using highly effective modulators such as PSC833 and GF120918.[191-193] The future development of more effective Pgp modulators may make enhanced drug delivery to the brain a realistic clinical goal.

First generation modulators (*e.g.* verapamil, quinidine) were poor Pgp inhibitors, requiring high plasma levels to reverse MDR, which could not be obtained without unacceptable patient toxicity. In addition, these drugs were used clinically to treat other medical conditions, and produced pharmacological side effects. Several advanced MDR-reversing agents are in various stages of development.[194] Second- and third-generation MDR inhibitors with good clinical potential include PSC833, GF120918, XR9576, LY335979, VX-710, and OC 144-093.

Several Pgp modulators also inhibit one or more cytochrome P450 enzymes (*e.g.* CYP3A4) that function to metabolize drugs. Thus, it has been widely observed that treatment with Pgp modulators decreases drug clearance, resulting in increased toxicity of co-administered drugs. Plasma drug levels remain higher for longer, increasing the "area under the curve" (AUC), and often necessitating a substantial reduction in drug dose to avoid toxicity. More selective third generation Pgp modulators, such as LY335979 and XR9576, do not inhibit the CYP enzymes, and show only small increases in AUC, so that dose reduction is not needed. Understanding how Pgp modulators affect the toxicity and pharmacokinetics of other drugs is important for the design of clinical trials of MDR modulation.

Modulation of P-glycoprotein in cancer treatment

A major reason for the failure of chemotherapy treatment to cure human cancers is the ability of tumor cells to become simultaneously resistant to several different anti-cancer drugs. Many mechanisms are known to contribute to MDR in tumor cells, of which the presence of multidrug efflux pumps is only one. Three ABC family members, Pgp, MRP1 (ABCC1) and BCRP (ABCG2), are likely to be the major drug efflux pumps expressed in human cancers.[195] Tumor cells are notoriously heterogeneous, and correlations between drug resistance and the expression of efflux pumps have been hard to establish. Some tumors express Pgp before chemotherapy treatment (*e.g.* colorectal and renal cancers), while in others, expression increases after exposure to MDR drugs (*e.g.* leukemias, lymphomas, myeloma, and breast and ovarian carcinomas). In general, patients with Pgp-positive tumors respond less well to chemotherapy, and have a poorer outlook and long-term survival. There is strong evidence linking Pgp expression with poor response to chemotherapy in acute myelogenous leukemia (AML).[196,197]

Studies to validate the role of MDR reversal in the treatment of various malignancies are under way; there have been some partial successes, and many failures. However, there is still no consensus on the useful of MDR modulators in treating human cancers, and the controversy is likely to continue.[198,199] Four contributing factors make the results of many clinical trials with modulators uninterpretable. First, there is a need to establish whether the patients' tumor contains Pgp and whether the level is clinically significant. Second, many modulator clinical trials have used first and second generation compounds that are poorly effective at the clinically achievable dose. This limitation will hopefully be overcome by new, more potent and specific third generation Pgp modulators. A third factor is that modulators affect the disposition of other drugs, either by decreasing drug elimination via Pgp, or by inhibiting drug metabolism via cytochrome P450. Cancer patients treated with both chemotherapy drugs and a modulator are thus exposed to higher levels of anti-cancer drug, which confounds interpretation of the results.

In some trials, the dose of anti-cancer drug was lowered to avoid toxicity and allow direct comparison of results from the two study arms. Finally, tumors have multiple, often redundant mechanisms of cellular resistance to drugs.[200] Not only do tumour cells have other defence mechanisms at their disposal, they can also express other multidrug efflux pumps. Thus the potential contribution of Pgp to drug resistance in a tumour is very difficult to assess. Modulation of Pgp in tumors is likely to be accompanied by altered Pgp function in normal tissues.[201] However, in some trials, tumor regression was obtained without apparent increases in normal tissue toxicity. There have been suggestions that MDR modulation may delay the emergence of clinical drug resistance. Thus, administration of modulators in the earlier stages of cancer may prevent drug resistance.

Regulation of P-glycoprotein expression

Cells adapt to the presence of toxic xenobiotics in their environment by upregulation of drug efflux pumps, such as Pgp, which provides them with a long-term survival advantage. The MDR1 gene is activated, and a stable MDR phenotype induced, after short-term exposure of cells to a variety of environmental insults. This response is of fundamental importance in the case of emergence of MDR in tumor cells exposed to anticancer drugs. MDR1 expression may be upregulated by two mechanisms; an increase in the amount of MDR1 message by transcriptional regulation, and stabilization of the mRNA. A considerable amount is now known about the transcriptional regulation of ABC proteins, including the MDR1 gene.[202-204] Transcription of a particular gene is determined by various response elements present within the promoter sequence, their accessibility, and the transcription factors available to interact with them, which depend on both the intracellular milieu and extracellular signals. The multi-protein complexes that assemble on the promoter sequence are also dynamic in nature, and influenced by chromatin structure. There appear to be multiple interacting pathways for activation of MDR1. A redundant network of MDR1 regulation ensures the rapid emergence of resistance in cells subjected to chemical

stress. By more fully understanding the molecular mechanisms by which the MDR1 gene is activated, it may be possible to intervene clinically to prevent its transcriptional activation.

Most MDR1 transcripts arise from downstream promoter sequences located in the middle of exon 1,[205] which lacks a TATA box. An inverted CCAAT box interacts with the trimeric transcription factor NF-Y, and the Sp family transcription factors, Sp1 and Sp3. In general, MDR1 transcription is upregulated as part of a general cellular "stress" response to stimuli such as heat shock, exposure to anticancer drugs and carcinogens, serum deprivation, inflammation, hypoxia, and ionizing radiation. The activation of several signalling pathways, including the protein kinase C and mitogen-activated protein kinase cascades, an increase in intracellular Ca^{2+}, and induction of NF-κB, can upregulate MDR1 expression.[204] Chemical modification of chromatin may affect gene expression, and the MDR1 promoter is negatively regulated by methylation. Post-transcriptional mechanisms also appear to play a role in regulating MDR1 expression, and the stability of MDR1 mRNA is increased in cells subjected to various stresses.

P-glycoprotein gene polymorphisms and their implications in drug therapy and disease

Changes in Pgp expression and function would be expected to alter the absorption, plasma concentration, tissue distribution and excretion of its drug substrates. Pgp polymorphisms might thus influence the outcome of drug treatment. Variations in the nucleotide sequence of the Pgp gene can affect both expression and function of the transporter. The first polymorphism to be reported in the human MDR1 gene was the G2677T variant, which results in the amino acid change A893S. Since then, about 30 single nucleotide polymorphisms (SNPs) have been discovered by sequencing the MDR1 gene in large numbers of individuals of different ethnic origin.[206-209] The most common variants have probably been identified, although it is possible that some rare polymorphisms still remain to be detected. There are considerable differences in the frequency of these variant alleles in different populations of Caucasian, African and Asian origin.[210] Distinct haplotypes exist, with considerable heterogeneity found within a single ethnic

group, however, all ethnic groups appear to possess the three most common haplotypes, which were found in >70% of the total population. Some SNPs result in a change in the amino acid coding sequence of the protein (nonsynonymous), whereas others do not (synonymous).

Polymorphisms have been reported to alter both the expression and the function of the transporter. For example, the synonymous C3435T variant (exon 26) appears to result in reduced Pgp expression levels, leading to increased oral absorption of digoxin and higher plasma drug levels. These results, however, were later contradicted by those of other groups. A recent meta-analysis suggested that the C3435T SNP has no effect on the expression of MDR1 mRNA or the pharmacokinetics of digoxin.[211] Conflicting data have been reported on the effects of other alleles using various drug substrates, and the controversy seems likely to continue. The differential effects of Pgp polymorphisms on Pgp expression and drug disposition will not likely be resolved until progress is made in standardizing parameters such as sample size and makeup, environmental factors, and the assays used for Pgp protein and mRNA quantification. MDR1 haplotypes, rather than individual SNPs, are also more likely to affect the pharmacokinetics of MDR1 substrates. Two common Pgp polymorphisms (G2677T/A and C3435T) may play a role in the differential response to the cholesterol-lowering statin drugs.[212] When haplotypes were also considered, a subgroup of female patients was identified that showed a remarkable response to treatment, which was not linked to a single polymorphism.

Pgp variants carrying spontaneous mutations have been found in cultured cell lines. The first to be reported was the G195V substitution, which confers increased resistance to colchicine, but has little effect on resistance to several other drugs.[213] Deletion of F335 was reported in another cell line,[214] which also showed altered resistance to a variety of drugs. The effect of several polymorphic sequence variations common in human populations on Pgp drug transport function has been investigated in transfected mammalian cells *in vitro*. Little difference in cell surface expression and transport function was noted between any of the variants and the wild-

type protein.[215,216] On the other hand, the G1199A polymorphism, which results in a S400N change, changed the efflux and trans-epithelial flux of a fluorescent substrate, and altered cellular resistance to some drugs, but not others.[217] Thus it seems likely that a number of Pgp polymorphisms may influence the disposition and therapeutic efficacy of selected drugs.

Given the role played by Pgp in protecting tissues and organs from toxicants, it would not be surprising to find that polymorphisms play a role in the susceptibility of individuals to various disease states. mdr1 knockout mice spontaneously develop a form of colitis that can be prevented by antibiotic treatment,[218] suggesting that Pgp functions as a defence against bacteria or toxins in the intestine. Confirming this idea, inflammatory bowel diseases (Crohn's disease and ulcerative colitis) are linked to the missense variant A893S/T,[219] and patients with ulcerative colitis (but not Crohn's disease) have a higher frequency of the C3435T genotype, which results in lowered Pgp expression in the intestine.[220] Anti-HIV drugs are known to be Pgp substrates, so a link between treatment efficacy and Pgp polymorphisms would not be unexpected. Although several common polymorphisms had no apparent effect on susceptibility to infection,[221] they were reported to influence drug treatment,[222,223] however, this was contradicted by another study.[224]

Variant Pgp alleles can also affect cancer susceptibility. The genotypic frequency of the C3435T SNP was not altered in colorectal tumor cells from a total patient population as compared to controls,[225] however, when an under-50 patient population was examined, carriers of the 3435TT genotype or 3435T allele were at substantially higher risk of developing the disease.[226] Evidence also suggests that Pgp polymorphisms influence the risk of developing renal epithelial tumors; C3435T and C3435TT carriers are again at higher risk.[227]

An association was reported between response of epilepsy patients to drug treatment and the C3435T polymorphism in the *MDR1* gene.[228] Patients with seizures that were not controlled by drugs were more likely to be homozygous for the C-variant allele, which is associated with higher Pgp transport function, suggesting that the drugs have a lower efficiency of penetration

across the blood-brain barrier in this group. However, two later studies failed to confirm these results.[229,230] The anti-Parkinson drug budipine is exported actively out of the brain by Pgp in mice,[231] and Parkinson's disease susceptibility has been linked to Pgp polymorphisms in Chinese populations, where a MDR1 haplotype containing the SNPs 2677T and 3435T was found to protect against the disease.[232]

Conclusions and future perspectives

P-Glycoprotein is a drug transporter of the ABC superfamily which functions as an ATP-powered drug efflux pump. Rapid progress has been made in recent years in understanding the three-dimensional structure and ATP hydrolysis cycle of this protein, and many tools are now available for its study at the molecular level. Although the transporter can interact with hundreds of nonpolar, weakly amphipathic compounds with no apparent structural similarity, progress is being made in developing a pharmacophore model to describe its binding regions. The protein appears to interact with its multiple substrates via a large flexible drug binding pocket, to which drugs gain access from the bilayer, leading to the suggestion that it is a "vacuum cleaner" for hydrophobic compounds that concentrate within the membrane. The drug transport mechanism of P-glycoprotein remains ill-defined, and may involve "flipping" of substrates from the inner to the outer membrane leaflet. The primary physiological role of the protein appears to be protection of sensitive organs and tissues from xenobiotic toxicity. Many drugs used in clinical therapy are P-glycoprotein substrates, and the transporter is now increasingly recognized to play a central role in the absorption and disposition of many drugs, including chemotherapeutic agents. Other compounds, known as modulators, that block the drug efflux function of P-glycoprotein are under development, and may have clinical applications in the future. Nucleotide polymorphisms in the P-glycoprotein gene that may affect its regulation and expression have been identified in human populations. The effect of these variants on the drug response and

disease susceptibility of individuals is an important focus of future research.

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Table 10-1 Pgp Substrates and Modulators

SUBSTRATES

Vinca alkaloids
vinblastine
vincristine

Anthracyclines
doxorubicin
daunorubicin

Taxanes
paclitaxel
docetaxel

Epipodophyllotoxins
etoposide
teniposide

Steroids
aldosterone
dexamethasone

HIV protease inhibitors
indinavir
saquinavir
nelfinavir
ritonavir

Analgesics
morphine

Cardiac glycosides
digoxin

Antihelminthics
ivermectin

Detergents
Triton X-100
nonylphenol ethoxylate

Fluorescent dyes
rhodamine 123
tetramethylrosamine
Hoechst 33342
LDS-751
calcein acetoxymethyl ester

Linear/cyclic peptides
ALLN
NAc-LLY-amide
leupeptin
pepstatin A

Ionophores
gramicidin D
nonactin
beauvericin

Cytotoxic agents
colchicines
actinomycin D
mitoxantrone

Miscellaneous
loperamide
cimetidine

MODULATORS

Ca^{2+} channel blockers
verapamil
nifedipine
azidopine
dexniguldipine

Calmodulin antagonists
trifluoperazine
chloropromazine
trans-flupethixol

Cyclic peptides
cyclosporin A
PSC833

Steroids
progesterone
tamoxifen
cortisol

Miscellaneous
GF120918
LY335979
XR9576
OC144-093
disulfiram
quinidine
chloroquine
reserpine
amiodarone
terfenadine

FIGURE LEGENDS

Figure 10-1

Topology and structure of Pgp. (A) Pgp is proposed to consist of two equivalent halves, each with 6 TM segments and an NB domain on the cytosolic side. Both the N- and C-terminus are cytosolic. (B) Low resolution structural model of Pgp generated using several different FRET measurements of the distances separating key regions of the protein.[158] (C) Medium resolution structural model of Pgp obtained from cryo-EM studies.[45] Top: a side view of the protein is shown with the NB domains at the bottom. The 12 putative TM α-helices are arranged in a pseudo-symmetrical relationship. Bottom: view of Pgp looking down on the TM helices from the extracellular side of the membrane. The dashed lines indicate the putative boundary of a 4.5 nm-thick lipid bilayer (scale bar = 5 nm). Adapted from Rosenberg *et al*.[45] with permission (for more details, refer to this paper).

Figure 10-2

(A) Classical pump, vacuum cleaner and flippase models of Pgp action. Classical pumps, such as lactose permease, transport a polar substrate from the aqueous phase on one side of the membrane to the aqueous phase on the other side. The substrate does not come into contact with the lipid bilayer, and moves through a hydrophilic path formed by the TM regions of the protein. In the vacuum cleaner model, drugs (both substrates and modulators) partition into the lipid bilayer, and interact with Pgp within the membrane. They are subsequently effluxed into the aqueous phase on the extracellular side of the membrane. In the flippase model, drugs partition into the membrane, interact with the drug binding pocket in Pgp (which is located within the cytoplasmic leaflet) and are then translocated, or flipped, to the outer membrane leaflet. Drugs will be present at a higher concentration in the outer leaflet compared to the inner leaflet, and an

experimentally measurable drug concentration gradient is generated when drugs rapidly partition from the two membrane leaflets into the aqueous phase on each side of the membrane. (B) The effect of membrane partitioning on drug binding to Pgp. The binding affinity of Pgp for a particular substrate or modulator (K_d) is related to the lipid-water partition coefficient of the drug (P_{lip}). A drug with a high value of P_{lip} (left side of the figure) will accumulate to a high concentration within the membrane. This will favour binding to Pgp and result in a low apparent K_d. In contrast, a drug with a low value of P_{lip} (right side of the figure), will have a lower membrane concentration, and a high apparent K_d.

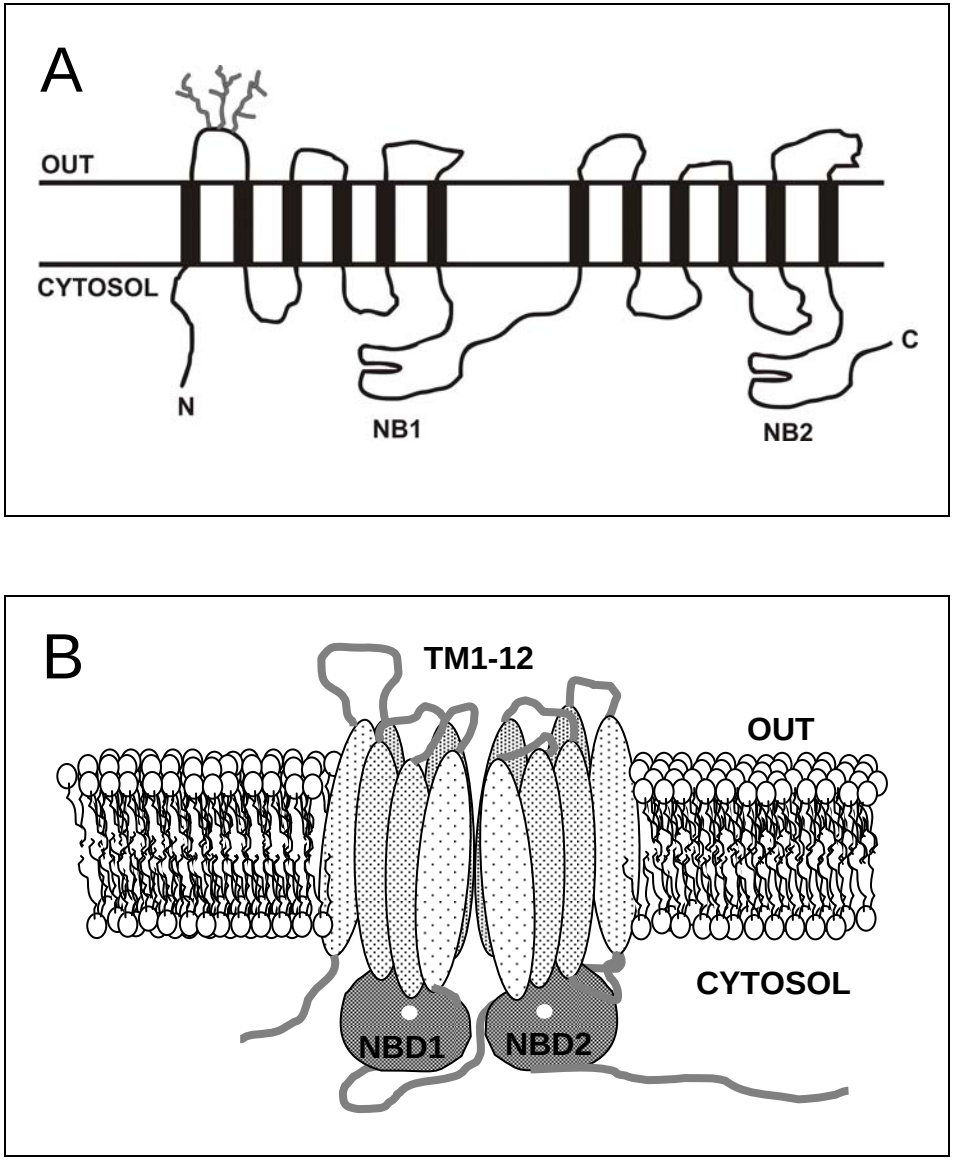
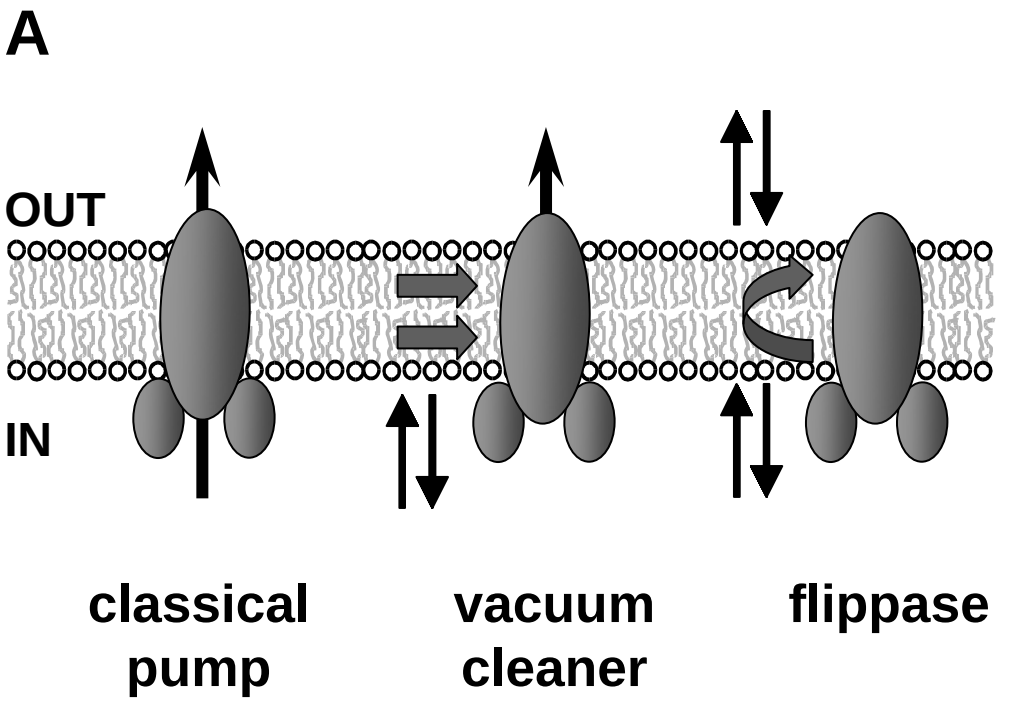
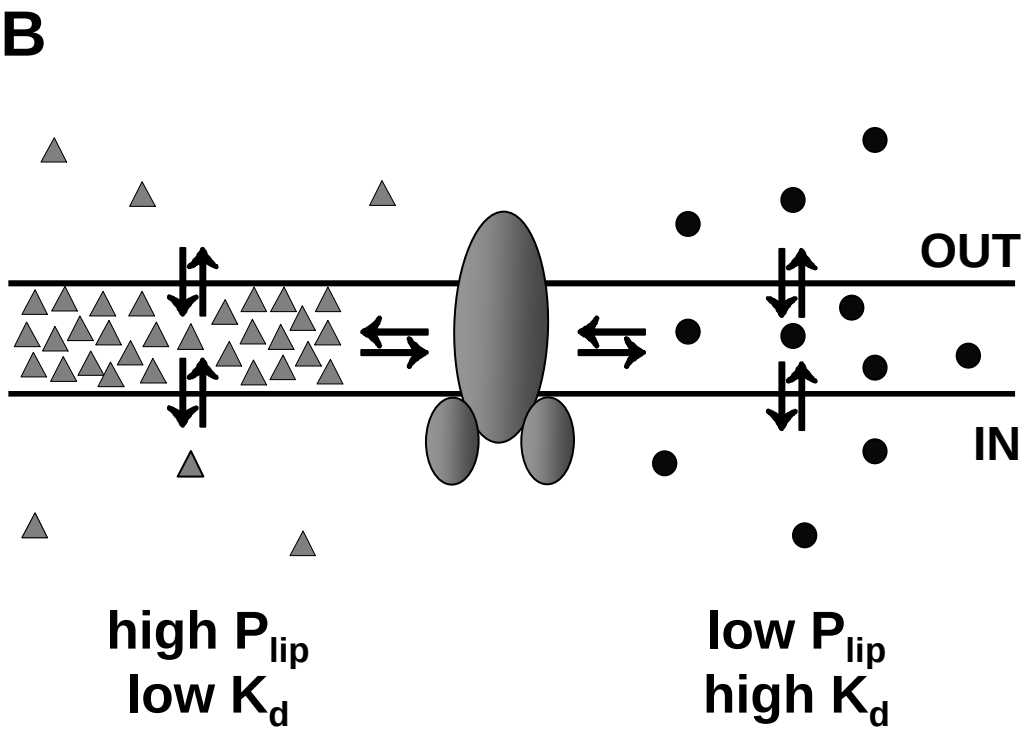

A
OUT
CYTOSOL
N
NB1
NB2
C
B
TM1-12
OUT
CYTOSOL
NBD1
NBD2
X
TMDs
ICDs
X
NBDs
C

Figure 1


A
OUT
IN
classical pump
vacuum cleaner
flippase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OUT
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high P_{lip}
low K_d
low P_{lip}
high K_d

Figure 2