J. Membrane Biol. 160, 161–175 (1997)

The Journal of
Membrane
Biology

© Springer-Verlag New York Inc. 1997

Topical Review

The P-Glycoprotein Efflux Pump: How Does it Transport Drugs?

F.J. Sharom
Guelph-Waterloo Centre for Graduate Work in Chemistry and Biochemistry, University of Guelph, Guelph, Ontario Canada N1G 2W1

Received: 3 April 1997/Revised: 11 June 1997

Introduction

The development of multidrug resistance (MDR) is a major obstacle in the chemotherapeutic treatment of many human cancers, including colon, kidney and breast carcinomas, leukemias, multiple myeloma, and pediatric cancers. Resistant tumors are found to be cross-resistant to a broad but well-defined spectrum of structurally unrelated cytotoxic drugs, including the *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, and taxanes (Table 1). Investigation of MDR has been greatly aided by the use of cell lines selected for drug resistance in vitro. MDR cells often show energy-dependent drug efflux, and lower drug accumulation relative to the drug-sensitive parent. Cells may become drug-resistant by several different mechanisms, but one major type of MDR is linked to the overexpression of a 170 kDa plasma membrane glycoprotein, known as the P-glycoprotein (Pgp). This protein, which is proposed to function as an ATP-dependent efflux pump for hydrophobic drugs, is also referred to as the multidrug transporter. Pgp expression in tumors in vivo is often associated with poor overall prognosis and response to chemotherapy [34]. Compounds called chemosensitizers (MDR reversers, or modulators) reverse MDR in vitro, resulting in decreased drug efflux and increased cellular drug accumulation (Table 2). Over the last few years, there has been much interest in combining chemosensitizers with chemotherapeutic agents for the treatment of drug-resistant tumors [29–32], and many Phase I and II clinical trials have been carried out, or are in progress. Pgp is also found in several normal human tissues, including the apical surface of many epithelial cells and the endothelial cells of the blood brain barrier. Its physiological role is not yet clear, although it appears to be involved in protection against toxic natural products. This review will focus on the molecular properties of Pgp with respect to binding and transport of substrates, and how these properties can be related to the possible mechanism of action of the protein. For more general information on Pgp, the reader is referred to several recent comprehensive reviews [13, 33, 35, 52].

Correspondence to: F.J. Sharom

Key words: Multidrug resistance — ABC superfamily — Membrane transport — Chemotherapy drugs — ATPase — Flippase

Pgp is a Member of the ABC Superfamily

Over 200 proteins involved in the transport of substrates across biological membranes are members of the ABC (ATP-binding cassette) superfamily of proteins, also known as the traffic ATPases [18, 40]. Sequence analysis showed that Pgp is a member of the ABC superfamily, suggesting that it might function as an ATP-dependent transporter. A typical ABC transporter protein consists of four units; two membrane-bound domains, each with six transmembrane (TM) segments and two nucleotide-binding domains (NBDs), which bind and hydrolyze ATP. These four modular units can be expressed as separate polypeptides, or they may be fused together in one of several alternative arrangements, with the number of genes varying from one to four. In *E. coli,* the histidine permease is encoded by four separate genes, one for each membrane-bound domain and NBD, whereas there are three genes for the ribose carrier, two encoding membrane-bound domains and one encoding

Table 1. Pgp substrates included in the multidrug resistance spectrum

Anthracyclines	Cytotoxic agents	Steroids
Doxorubicin	Colchicine	Aldosterone
Daunorubicin	Emetine	Dexamethasone
	Actinomycin D	
Vinca alkaloids	Puromycin	Miscellaneous
Vinblastine	Mitoxantrone	Rhodamine 123
Vincristine		Hoechst 33342
	Linear and cyclic peptides	Triton X-100
Epipodophyllotoxins	NAc-Leu-Leu-norLeu-al	Prenyl-Cys methyl esters
Etoposide	NAc-Leu-Leu-Met-al	Calcein acetoxymethylester
Teniposide	Leupeptin	^{99m}Tc-SESTAMIBI
	Pepstatin A	
Taxanes	Gramicidin D	
Paclitaxel	Nonactin	
Docetaxel	Yeast *a*-factor	

Table 2. Chemosensitizing compounds which reverse multidrug resistance

Calcium channel blockers	Steroids	Cyclic peptides
Verapamil	Progesterone	Cyclosporin A
Nifedipine	Tamoxifen	SDZ PSC 833
Azidopine	Cortisol	Valinomycin
Dexniguldipine		
	Detergents and amphiphiles	Miscellaneous
Calmodulin antagonists	Cremophor EL	Quinidine
Trifluoperazine	Solutol HS15	Chloroquine
Chlorpromazine	Tween 80	Reserpine
Trans-flupenthixol		Amiodarone
		Terfenadine
		Dipyridamole
		FK 506

the two fused NBDs [40]. Among the eukaryotic members of the ABC superfamily, the TAP1/2 peptide transporter is encoded by two genes, each giving rise to a membrane-bound domain fused to an NBD, whereas a single gene encodes the two NBDs and two membrane-bound domains of mammalian Pgp [40].

Pgp genes from hamster, mouse, and human have been cloned and sequenced, and Pgp homologues have been identified in several other species. Pgp is encoded by a small multigene family (*mdr* class I, II and III). All three isoforms are present in rodents, while humans express only the Class I and III isoforms. Transfection studies have demonstrated that the Class I and II isoforms can confer MDR, while the Class III isoform is a PC (phosphatidylcholine) translocase, or flippase, responsible for export of this phospholipid into the bile [82, 103, 104]. This review will refer to the MDR-conferring isoforms (Class I and II) as Pgp.

Sequence analysis predicts that Pgp comprises two homologous halves, each consisting of six TM segments and a NBD consensus sequence (Fig. 1). The locations of the C-terminus, N-terminus, NBDs, and several intra- and extracellular loops of Pgp have been verified, and

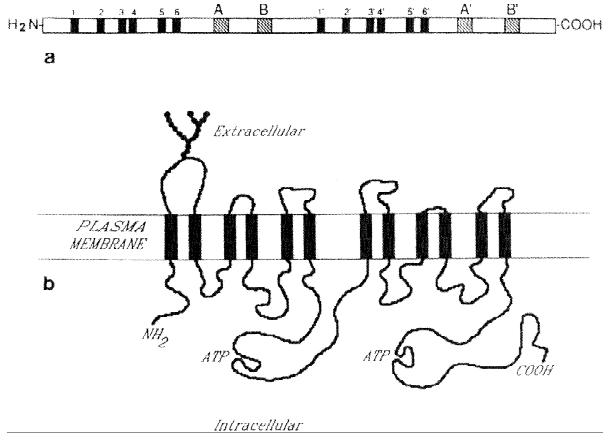

Fig. 1. Structural and topological model of Pgp. (*a*) Linear sequence of the Pgp molecule; the solid boxes represent the 12 putative membrane-spanning segments of the transporter, and the Walker A and B motifs of the two cytosolic nucleotide binding domains are indicated by shaded regions. (*b*) Proposed arrangement of Pgp in the plasma membrane. The site of N-glycosylation is indicated in the first extracellular loop. This orientation is supported by both predictive hydropathy plots, and several independent studies on membrane topology of the full-length protein. Reprinted from Ref. 32 with permission.

two mapping studies on the full-length protein have confirmed the predicted topology [50, 61]. However, this area remains controversial, and alternative topologies have been proposed in which putative cytosolic regions or TM segments are located extracellularly [102, 118].

Pgp is a Promiscuous Transporter

Pgp is an unusual ABC protein in that it appears to be highly promiscuous; hundreds of compounds have been identified as ''substrates'' for the transporter, usually by indirect means. MDR spectrum compounds include a large number of anticancer drugs (anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, taxanes), as well as other cytotoxic agents, linear and cyclic peptides, steroids, fluorescent dyes, and the γ-emitting radiopharmaceutical ^{99m}Tc-SESTAMIBI (Table 1). A ''typical'' compound in the MDR spectrum is large (M_r > 400), hydrophobic, amphipathic, with a planar ring system, and often carries a positive charge at physiological pH [74]. However, not all putative Pgp substrates fall into this category; many are uncharged at physiological pH (e.g., colchicine), and several uncharged cyclic and linear hydrophobic peptides and ionophores have recently been described as Pgp substrates [24, 57, 86, 92, 93, 98].

Pgp in Membrane Vesicles Transports Drugs and Displays Drug-Stimulated ATPase Activity

Many studies have been carried out over the years on drug transport in intact MDR cells. Recently, there has been a move away from whole cells to simpler membrane vesicle systems, where it is possible to characterize better the drug transport process biochemically and kinetically. Several research groups have examined drug transport in plasma membrane vesicles from MDR cultured cell lines [e.g., 19, 39, 45, 75, 88]. In general, transport is saturable, osmotically sensitive, requires ATP hydrolysis, and generates a drug concentration gradient. Other MDR spectrum drugs and chemosensitizers block transport with varying degrees of effectiveness. Drug transport has also been studied in membrane vesicles isolated from epithelial cells expressing naturally occurring Pgp, such as rat intestinal brush border membrane vesicles [46], and rat biliary canalicular membrane vesicles [49, 101]. Heterologous expression of murine Pgp in a yeast *sec*-4 mutant led to the accumulation of secretory vesicles which displayed active, ATP-dependent uptake of vinblastine and colchicine [83].

Drug-stimulated ATPase activity of Pgp can be observed in plasma membrane vesicles from MDR cells, as long as the background ATPase activity in the cell line employed is not too high (e.g. [2, 20, 96]), and in plasma membrane from Sf9 insect cells overexpressing Pgp [77, 85].

Pgp Has Been Purified and Functionally Reconstituted into Lipid Bilayers

The high Pgp expression level in MDR cell lines makes them ideal sources for purification and characterization of the protein. There have been several reports of partial purification of Pgp from highly drug-resistant cell lines, and its reconstitution into lipid bilayer membranes [4, 24, 25, 71, 95]. More recently, three research groups independently isolated highly purified Pgp (>90%) from MDR Chinese hamster ovary cells and reconstituted it into defined lipids [54, 89, 90, 97, 111]. Pgp from several tumor cell lines has also been purified and reconstituted [22]. In all cases, high levels of constitutive ATPase activity were retained, up to 3 μmol/min/mg, which is in the same range as the activity of other membrane-transporting ATPases. Pgp is an atypical ATP-dependent transporter in that it exhibits a very high basal ATPase activity, which appears to be partially uncoupled from substrate binding and transport (*see* later for more discussion on this point).

To date, ATP-dependent transport in reconstituted proteoliposomes has been characterized for colchicine [95], the fluorescent dye Hoechst 33342 [90], vincristine [71], the synthetic tripeptide NAc-LLY-amide [98], and the peptide ionophores valinomycin and gramicidin D [24, 25]. It should be noted that the majority of these substrates are uncharged at physiological pH (colchicine, NAc-LLY-amide, valinomycin, gramicidin D). These studies established that transport by Pgp in proteoliposomes is ATP-dependent, saturable, and osmotically sensitive. Pgp-mediated transport generates a substrate concentration gradient, and is inhibited by other MDR spectrum drugs and chemosensitizers. The availability of highly purified Pgp, and the development of simple in vitro membrane systems, will allow further detailed investigation of the function of the transporter at the molecular level.

Hydrophobic Compounds Interact Directly with Pgp

There is now an overwhelming amount of evidence that Pgp directly binds MDR spectrum drugs and chemosensitizers. Pgp can be labeled by photoaffinity analogues of many of these compounds, e.g., azidopine, colchicine, vinblastine, forskolin, prazosin (reviewed in [9, 84]), and the ability of a particular compound to inhibit such photolabeling has frequently been used as an indicator that it is a Pgp ''substrate''. Direct binding of radiolabeled drugs to plasma membrane from MDR cells has also

Table 3. Binding affinity of Pgp substrates determined by fluorescence quenching

Ligand	K_d (μM)	Ligand	K_d (μM)
Nonpeptide drugs and chemosensitizers		Peptide-based drugs and chemosensitizers	
Colchicine	158	NAc-Leu-Leu-norLeu-al	138
Daunorubicin	10.5	NAc-Leu-Leu-Met-al	83.1
Trifluoperazine	7.7	Leupeptin	77.6
Doxorubicin	4.4	Chymostatin	38.2
Verapamil	2.4	Pepstatin A	35.8
Vinblastine	0.77	NAc-LLY-amide	28.4
Reserpine	0.73	NAc-FnorLRF-amide	10.3
Triton X-100	0.37	Valinomycin	0.78
Corticosterone	0.064	Cyclosporin A	0.20

been characterized [27–28]. Chemosensitizers and drugs are able to stimulate the ATPase activity of Pgp, both in a membrane environment and in detergent solution, and specific site-directed mutations in certain TM regions and cytoplasmic loops of Pgp alter both drug resistance (*see*, for example, [17, 38, 48, 58] and drug-stimulated ATP hydrolysis profiles [59, 62, 69, 77] (reviewed in [33]). In the presence of drugs and chemosensitizers, certain mutant Pgps can be induced to traffic normally to the membrane surface, where they are functional [65]. Evidently, occupation of the drug binding site early in the biosynthetic process can facilitate correct folding, and ''rescue'' otherwise misfolded proteins.

Biophysical techniques have provided unequivocal evidence for substrate-induced conformational changes in Pgp. Fluorescence experiments using highly purified Pgp have established the existence of a conformational change induced by binding of several drugs and chemosensitizers [54], and an infrared spectroscopic study has reported a change in the tertiary structure of purified Pgp following binding of the combination of verapamil and ATP [105].

We have recently developed a fluorescence quenching technique that can directly determine the K_d for equilibrium binding of drugs and chemosensitizers to highly purified Pgp [54]. This advance has allowed us to demonstrate unambiguously, at the molecular level, that a particular compound binds directly to Pgp. It is clear from Table 3 that Pgp interacts with many different compounds, including drugs, chemosensitizers, peptides of different classes, and even the amphiphile Triton X-100 [56], with a range of affinities covering several orders of magnitude. To date, binding affinities have been determined for over 35 compounds, in widely different structural classes. The magnitude of K_d is very highly correlated ($r = 0.96$) with the IC_{50} for blocking of drug transport via Pgp in membrane vesicles (R. Liu and F.J. Sharom, *unpublished data*).

How does Pgp interact with such a diverse collection of hydrophobic compounds? What is the molecular nature of the drug binding site(s)? Site-directed mutagenesis experiments and protein mapping studies following photoaffinity labelling point to the TM segments, especially TM5, TM6, TM11 and TM12, as the location where drugs bind (reviewed in [33]). Both the N- and C-terminal halves of Pgp appear to contribute to formation of the drug binding site(s). While N- and C-terminal half molecules of Pgp each showed basal ATPase activity, coupling of drug binding to increased ATPase activity was only observed when both half molecules were co-expressed [60]. These findings indicate that coupling of ATPase activity to drug binding requires interaction between both halves of P-glycoprotein. Gottesman et al. [36] proposed a model for interaction of the substrate and ATP-binding sites which involves proximity of TM5, TM6, TM11 and TM12, and the two NBDs. This model is supported by recent work performed by Loo and Clarke [64], who introduced pairs of cysteine residues into TM6 and TM12 within a cysteine-less Pgp molecule. Cys-332 and Cys-975 were the only residues that could be oxidatively crosslinked, and this crosslinking could be blocked by verapamil and vinblastine. These results suggest that TM6 and TM12 are close to each other in the tertiary structure of Pgp, and that Cys-332 and Cys-975 within these helices face each other, with a maximum separation of 7 Å. Drugs might physically block the interaction of the two cysteines, if this region contains the drug binding site, or they may induce a conformational change that moves the two residues into a spatial arrangement where crosslinking is not possible.

Many attempts have been made to determine the number and nature of the drug binding site(s) by biochemical means, but contradictory conclusions have been reached. Work in this area has been hampered by the lack of methodology for directly measuring drug binding to Pgp and quantitating binding affinities, using a simple in vitro system. When using plasma membrane preparations containing Pgp, it is important to establish unequivocally that the parameter being measured is, in fact, binding. If ATP is supplied in the assay buffer (e.g. [70, 108]) transport may take place; examination of the stoichiometry of drug ''binding'' will indicate if this is indeed the case. Some researchers have used indirect measures of drug binding, such as stimulation of ATPase

activity, or nonequilibrium methods, such as inhibition of photolabeling. ''Kinetic'' analysis has frequently been applied to such data inappropriately, in an attempt to identify compounds that are ''competitive'', ''noncompetitive'', or ''uncompetitive''. The results of some studies have suggested that there are at least two separate (possibly overlapping or allosterically coupled) sites for binding of drugs (for example, [6, 11, 27, 28, 72, 108]), whereas others have proposed that there is a single common binding site or pharmacophore (e.g. [12]). In addition, it has been reported that more than one molecule of drug can bind to Pgp at the same time [11]. No clear consensus on the number, nature, and interrelationships of the drug and chemosensitizer binding sites has emerged from the many studies carried out in this area. Our laboratory favors the hypothesis that drugs and chemosensitizers interact with different overlapping regions of a single flexible drug binding site that is large enough to accommodate more than one compound.

Drug Transport by Pgp is Coupled to ATP-Hydrolysis

The NBDs of all members of the ABC superfamily possess certain characteristic features, which allow them to be identified by sequence analysis. Five highly conserved regions include the Walker A and B motifs, a ''Signature'' or C motif, a ''Center region'', and a region downstream of the Walker B motif [107]. There are several well-characterized ATP-driven membrane transporters for which we have a relatively good understanding of the way in which ATP hydrolysis is coupled to substrate translocation, for example, the Na^+K^+- and Ca^{2+}-ATPases. The mechanism of hydrolysis of ATP by these P-type ATPases involves an aspartyl-γ-phosphate intermediate. However, Pgp does not contain the highly conserved aspartate residue common to the P-type ATPases, and there is no evidence to date for the existence of such a phosphorylated intermediate.

In membrane pumps such as the Na^+K^+- or Ca^{2+}-ATPase, significant hydrolysis of ATP occurs only when the substrate is presented to the protein, and concurrently transported across the membrane. Pgp is an unusual translocating ATPase in that the purified protein exhibits a high level of constitutive ATPase activity in the apparent absence of substrates [89, 97, 111]. The basal ATPase activity of highly purified Pgp also seems to vary among different laboratories, depending on the cell species of origin and the purification protocol. There are two possible explanations for these observations. One interpretation is that Pgp ATPase activity may be partially uncoupled from substrate binding and transport. Alternatively, since large amounts of natural membrane lipid mixtures are often added to preserve Pgp function during purification, it is possible that the ATPase activity in these preparations is activated by an endogenous substrate, perhaps a naturally occurring minor lipid, or lipid-soluble compound. However, we have measured very high constitutive ATPase activity for Pgp purified in the absence of exogenous lipids, where only ~53 phospholipids remain tightly associated with each molecule of the protein [94, 97]. ATPase activity is retained following reconstitution into bilayers of defined synthetic phosphatidylcholines [81, 94], which should not contain molecules that can act as endogenous substrates. These results suggest that if there is an activating endogenous substrate, it remains tightly associated with Pgp during purification (in a similar fashion to the bound phospholipids) or, alternatively, the observed constitutive ATPase activity is an intrinsic property of the transporter. In this respect, constitutive ATPase activity has recently been reported for purified CFTR, another member of the ABC superfamily [53]. Although the K_M for ATP hydrolysis by CFTR was high, similar to that of purified Pgp, the V_{max} was 50-fold lower. The high K_M reported for Pgp (0.4–0.8 mM) is another unusual feature, and indicates that the transporter has a very low affinity for ATP when compared to other translocating ATPases. Constitutive ATPase activity does not appear to be an artifact of detergent solubilization of Pgp, since it also a property of native plasma membrane vesicles from MDR cells [2, 19, 96].

Pgp ATPase activity can be further stimulated by the addition of certain MDR spectrum drugs and chemosensitizers. The ATPase activity profiles are often biphasic, with stimulation at low drug concentrations, and inhibition at higher concentrations. One puzzling observation is that not all substrates stimulate activity; several transported substrates actually inhibit activity in a concentration-dependent manner. At the moment, there is no satisfactory explanation for this behavior; it has been suggested that overlapping stimulatory and inhibitory drug binding sites exist within Pgp [37]. In addition, conflicting results have been obtained in different systems; the transport substrate vinblastine inhibited ATPase activity in Chinese hamster Pgp [89, 97, 111], whereas it stimulated the activity of Pgp from human KB cells [4]. It appears that drug modulation of Pgp ATPase can be greatly affected by the local lipid environment and the presence of detergents [21, 95, 110]. Pgp mutants displaying altered substrate specificity often show changes in ATPase stimulation by drugs [62, 69, 76, 77, 116]; however, for some mutants the ATPase stimulation profile does not correlate with the drug resistance phenotype. Based on current data, it seems dubious at best to assume that drug-stimulated ATPase activity is representative of the transport function of Pgp. Fluorescence quenching studies have shown that ATP and drug or chemosensitizer substrates bind to Pgp independently of

each other [54], indicating that substrate binding to Pgp is not ordered.

Both NBDs of full-length Pgp can apparently bind and hydrolyze ATP with similar efficiency [63, 112]. A β-galactosidase fusion protein with the N-terminal half of Pgp displayed constitutive ATPase activity [99], and both N- and C-terminal half-molecules of Pgp showed similar catalytic activity [60]. A variety of inhibitors of Pgp ATPase have been identified [87], including sulfhydryl-modifying agents and orthovanadate. Vanadate inhibition results in trapping of vanadate and ADP in the active site after one catalytic turnover has occurred. Significantly, trapping of vanadate takes place at only one NBD, and this is sufficient to completely block all ATPase activity [113]. Senior [87] has suggested that the two NBDs interact during the catalytic cycle, so that when one site enters the transition state, the other is unable to do so. Each NBD is proposed carry out ATP hydrolysis in turn, in an alternating fashion.

Mutations were introduced into highly conserved amino acid residues within the core consensus sequence for nucleotide binding, GXGKST, within NBD1 and NBD2 of murine Pgp. Mutation of Lys → Arg or Gly → Ala within either NBD1 or NBD2 eliminated drug resistance [7], indicating the importance of these residues to Pgp function. Loo and Clarke [62] introduced several different mutations into the NBDs of human Pgp, expressed and purified the histidine-tagged proteins, and tested them for drug-stimulated ATPase activity. Mutation of the conserved lysine and glycine residues in either or both NBDs eliminated basal and drug-stimulated ATPase activity. Mutation to alanine of the conserved cysteine residues within the NBDs (Cys-431 and Cys-1074), as well as all other cysteines, resulted in a protein that retained 70% of its ATPase activity, implying that these residues are not essential for catalytic activity. However, covalent modification by N-ethylmaleimide of either Cys-431 or Cys-1074 was sufficient to completely abolish ATPase activity. Taken together, these reports indicate that both NBDs must be functional for ATP hydrolysis by full-length Pgp, and suggest that they somehow cooperate in powering drug pumping. Although expression of half-molecules of Pgp showed that each NBD can hydrolyse ATP independently, the activity could no longer be stimulated by drugs [60]. Co-expression of the two half-molecules restored drug stimulation of ATPase activity. Two different effects may be contributing to the outcome of these experiments. First, since the drug binding site is likely made up of contributions from both halves of Pgp, co-expression of the two halves will be necessary to restore drug binding, which is clearly essential for drug-stimulated ATPase activity. Second, it seems likely that the two NBDs, which can evidently operate independently of each other in terms of constitutive ATP hydrolysis when expressed separately, somehow switch to an alternating mode of operation when they interact with each other.

The NBDs of Pgp Have Been Partially Characterized at the Molecular Level

To date, there is no three-dimensional structure available for the NBDs of any ABC transporter. Hyde and co-workers [47] presented a structural model for the ATP-binding cassette of Pgp, CFTR, and the nucleotide-binding subunits of bacterial permeases, based on the known structure of adenylate kinase, and the predicted secondary structures of ABC proteins. A slightly different model for the tertiary structure of the NBD was proposed by Mimura et al. [68], who used sequence alignment of 17 bacterial ABC proteins, secondary structural predictions, and the known tertiary structures of adenylate kinase, p^{21}ras, and elongation factor Tu.

In the absence of 3-dimensional structural information, techniques such as fluorescence spectroscopy can provide valuable details about the architecture of the catalytic and nucleotide binding regions within the NBDs of Pgp. The purified overexpressed NBD2 from Pgp displayed high affinity binding of TNP (2′,3′-(2,4,6-trinitrophenyl)) derivatives of nucleotides [8]. The single tryptophan residue within NBD2 gave a spectrum characteristic of a hydrophobic environment, and was highly quenched on nucleotide binding. Purified overexpressed murine NBD1 also showed high affinity TNP nucleotide binding, and modification of the single cysteine residue (Cys-430) within the sequence GNSGCGKST in the Walker A motif altered interactions with nucleotides [16].

MIANS probes covalently linked to the two conserved cysteine residues in the Walker A motif of NBD1 and NBD2 showed a large blue shift, again indicating that the interior environment of the ATP-binding site is relatively nonpolar [54]. This may indicate the existence of a hydrophobic pocket that can accommodate the aromatic rings of the adenine base. Collisional quenchers were used to assess both the aqueous accessibility of the bound MIANS groups, and provide information on the polarity and charge of the region surround them [55]. The MIANS probes appear to be buried deeply within the protein structure, and the local environment of the two NBDs is virtually identical. The region in which the probe is located was also found to be positively charged, likely reflecting the existence of the nearby lysine residue, which is believed to interact directly with ATP. This idea is in keeping with the observation that quenching studies indicate partially shielding of the positive charge following ATP binding. Fluorescence resonance energy transfer is observed between cysteine-bound MIANS and TNP-ATP, which confirms that the two groups

are located close to each other within the catalytic site [55]. Energy transfer approaches can measure the distances between fluorescent probes, and should be able to provide important information on the spatial organization of the NBDs relative to each other, and the site(s) where transport substrates bind.

Pgp Undergoes Conformational Changes on Binding Substrates and ATP

Several recent studies have employed biophysical techniques to explore the changes that take place in Pgp following binding of ATP and drugs. Liu and Sharom covalently labeled highly purified hamster Pgp on two conserved cysteines (Cys-428 and Cys-1071), one within each NBD, using the fluorescence probe MIANS [54]. These two residues appear to be located close to the catalytic site, since MIANS labelling can be blocked by ATP, and cysteine modification abolishes ATPase activity. Binding of ATP led to concentration-dependent quenching of MIANS fluorescence; fitting of the data to a binding equation was used to determine the K_d for ATP binding (0.46 mM). Quenching of intrinsic tryptophan fluorescence of Pgp has also provided evidence for a conformational change in Pgp following binding of ATP, but not ADP [105].

Binding of a variety of drugs and chemosensitizers also leads to quenching of the fluorescence of the probe within the NBDs [54], providing the first definitive evidence of conformational coupling between the drug binding site(s) and the NBDs. In other words, occupation of the drug binding site induces a conformational change which is relayed to the catalytic site within the NBD. Presumably such communication between the two binding sites is critical for coupling of the energy of ATP hydrolysis to drug transport, and is also responsible for modulation of ATPase activity by drugs and chemosensitizers. The accessibility of Cys-428/1071 to MIANS labeling was also substantially reduced in the presence of drugs and chemosensitizers, providing further evidence for a long-range conformational change. Fluorescence quenching experiments indicated that the effects of drugs and ATP were independent and additive.

Sonveaux et al., recently investigated the secondary and tertiary structure of purified reconstituted Pgp using Fourier transform attenuated total reflection infrared spectroscopy [105]. The secondary structure of Pgp was found to consist of 32% α-helix, 26% β-sheet, 29% turns, and 13% random coil; this remained unchanged following binding of ATP or the chemosensitizer verapamil, either alone, or in combination. Modifications in Pgp tertiary structure on substrate binding were explored by measuring the kinetics of $^2H/H$ amide exchange in D_2O. A population of slowly exchanging amino acids became more accessible after binding of ATP, whereas addition of both ATP and verapamil led to a protection from the solvent of a population of rapidly exchanging residues. No change was noted on binding of verapamil alone, indicating that the observed conformational change is different from that seen in the fluorescence experiments with MIANS-labelled Pgp. It appears that there is an initial change in Pgp conformation on binding of drug, which is perhaps local and reflected only within the NBDs, and a larger global change in tertiary structure after subsequent binding of ATP, at which point the transporter can undergo a full catalytic cycle. Now that biophysical techniques can be applied to purified functional Pgp, we can expect further progress in this area to be made rapidly.

Pgp Pumps Drugs, Rather than Altering Drug Distribution Indirectly

The distribution of hydrophobic weak bases can be dramatically altered by changes in ΔpH or Δψ. For example, a doxorubicin concentration gradient of up to 20-fold can be generated in large unilamellar vesicles using a transmembrane Δψ of −100 or −130 mV (negative inside) [66], and a transmembrane ΔpH of 2.9 (acid inside) generates a 500-fold gradient [67]. The fact that many Pgp substrates are positively charged at physiological pH led to the suggestion that Pgp is not a drug transporter *per se*, but alters drug distribution across membranes indirectly [80]. This hypothesis also provides an alternative explanation for the unusually broad substrate specificity of Pgp. The altered partitioning model proposes that Pgp modifies the pH or membrane potential across the plasma membrane of MDR cells. These perturbations are believed to have multiple effects on the diffusion and retention of chemotherapeutic drugs, which result in decreased drug accumulation inside MDR cells. In support of this proposal, increased intracellular pH and altered membrane potential, relative to the drug sensitive parent, have been observed in several series of MDR cell lines (reviewed in [80]).

It has been suggested that Pgp functions as both a Cl^- channel [114] and an ATP channel [1]. To perturb ΔpH or Δψ, Pgp could alter the conductance of Cl^-, or it could create an electrochemical ATP gradient. However, it is now generally accepted that while Pgp may regulate the activity of other Cl^- channels in certain cell types, it does not itself possess channel activity [41]. Similarly, arguments that Pgp is an ATP channel have also been refuted [14].

Recent results obtained with membrane vesicle and reconstituted systems do not support the indirect model for Pgp action. A number of uncharged compounds are good substrates for Pgp, including colchicine, and many cyclic and linear hydrophobic peptides and ionophores.

Transport of the uncharged compounds colchicine [95], N-Ac-LLY-amide [98], gramicidin D and valinomycin [24, 25] has been characterized in reconstituted proteoliposomes containing Pgp, and is indistinguishable from transport of other, positively charged, MDR substrates. Vinblastine transport into Pgp-containing secretory vesicles in yeast was unchanged following collapse of both ΔpH and $\Delta\psi$, indicating that $\Delta\mu_H$+ does not influence distribution of this drug across the membrane [83]. This study also demonstrated that the lipophilic cation TPP^+ (tetraphenylphosphonium) could be transported by Pgp against a steep H^+ gradient. Transport of Hoechst 33342 by reconstituted Pgp also occurred in the absence of either a membrane potential or a chloride gradient [90]. Pgp might act as an ATP-driven H^+ pump, or be involved in H^+ movement by a symport or antiport type of mechanism. However, transport of Hoechst 33342 by reconstituted Pgp did not result in acidification of the liposome interior [90], and drug uptake in yeast secretory vesicles was found to be independent of H^+ movements [83]. Finally, as pointed out earlier, the evidence that Pgp interacts directly with its substrates is now overwhelming, and includes demonstrations of drug-mediated changes in Pgp conformation. Although cytosolic alkalinization and alterations in $\Delta\psi$ have been measured in some MDR cells, these observations are not universal, and are likely to be epiphenomena associated with MDR. In some cell lines, they may contribute to MDR, but they are not the basis of Pgp action.

The Classical Pump Model for Transporters May Need to be Modified for Pgp

Our current ideas concerning the mechanism of action of membrane transporters are based on the classical pump model, which includes the following features. The membrane protein is thought to alternate between an inward-facing conformation (with the substrate binding site accessible on the cytosolic side) and an outward-facing conformation (with the substrate binding site accessible on the extracellular side). Binding of substrate is assumed to take place via a specific site in the transporter, which can usually accommodate only a few closely related compounds. Initial interaction of substrate with this site is envisaged as occurring from the aqueous phase (the cytosol, for an exporter), and substrate is released into the aqueous phase on the other side of the membrane (the extracellular medium). A conformational change in the transporter to the alternate conformation, induced by either substrate binding or hydrolysis of ATP, leads to release of the substrate on the other side of the membrane. The transmembrane helices of the transporter are believed to form a hydrophilic transport path through the membrane, so that the substrate does not come into contact with membrane lipids.

There is a large amount of evidence supporting various aspect of the classical pump model for transporters of hydrophilic substrates, and there is every reason to believe that ABC proteins which transport polar molecules also function in a similar way. The past success of this model suggests that we should not discard it as a basis for describing an unusual ABC transporter such as Pgp. However, if this model is to explain the mechanism by which Pgp pumps drugs, it is clear that several modifications need to be made. First, since the substrates for Pgp are largely (although not exclusively) hydrophobic, access to the substrate binding site on the protein may be from the lipid bilayer, rather than the aqueous phase. As discussed further below, both binding and release of the substrate may take place within the membrane environment, rather than the aqueous phase. Second, given the remarkably broad substrate specificity of Pgp, the nature of the binding site may be rather different from the ''enzyme-like'' site usually envisaged. Finally, the lipophilic nature of the substrates must be taken into account when considering features such as the transport path through the protein, the kinetics of transport, substrate concentration gradients, catlytic turnover rates, and the stoichiometry of ATP hydrolysis. We would expect such a transporter to be sensitive to changes in its lipid environment, since these would affect substrate presentation as well as protein function. Other ABC proteins (MRP, *ste*6) share some of the features of Pgp, such as hydrophobic substrates and broad substrate specificity, so it seems likely that aspects of Pgp function will also apply to these other transport systems.

Substrates Gain Access to Pgp from the Lipid Bilayer

Pgp is an atypical membrane transporter, in that most of its substrates are hydrophobic and would, therefore, be expected to show greater solubility in the lipid bilayer than in the external aqueous phase. This led Higgins and Gottesman [42] to suggest that Pgp functions as a ''hydrophobic vacuum cleaner'', removing drugs from the plasma membrane rather than the aqueous phase. Drugs are thought to first partition into the membrane, then interact with the transporter within the lipid phase. The vacuum cleaner model proposes a two-tier recognition process; the primary determinant of substrate specificity is the lipid solubility of a particular compound, and interactions with a relatively nonselective drug binding site within Pgp are of secondary importance. The actual drug concentration seen by the transporter would be substantially higher than the aqueous concentration, and would depend on the partition coefficient of the drug between the aqueous phase and the bilayer. However, we have recently demonstrated that well-defined binding curves can be obtained for interaction of a variety of different

substrates with highly purified Pgp [54]. The K_d values for Pgp substrates cover a 1,000-fold range (Table 3), and this suggests that the protein does, in fact, effectively discriminate between different compounds, resulting in a specific, measurable binding affinity. Further studies with purified protein are necessary to determine whether the two-stage recognition proposal describes the mechanism of Pgp action satisfactorily.

Substantial experimental evidence supports the view that substrates interact with Pgp within the membrane. The fluorescence emission spectrum of rhodamine 123 was indicative of a hydrophilic, aqueous environment in drug-resistant cells, whereas the environment was hydrophobic in drug-sensitive cells, or in resistant cells treated with chemosensitizer [51]. These results suggest that Pgp very efficiently expels the substrate from the membrane. The labile lipophilic probe INA (5-iodonaphthalene-1-azide) can be photoactivated by fluorescence resonance energy transfer from doxorubicin or rhodamine 123, as a consequence of which it labels membrane proteins. In drug-sensitive cells, nonspecific labeling of many membrane proteins was observed, whereas in MDR cells, Pgp was specifically labeled to a high level [78], indicating that an interaction between the drug and Pgp takes place within the bilayer. Pgp appears to intercept drugs in the bilayer before they gain entry to the cytosol. Pgp-expressing cells can efflux the hydrophobic acetoxymethyl (AM) derivatives of several fluorescent indicator dyes [44]. If the nonfluorescent AM derivative gains access to the cytosol, it is rapidly hydrolyzed by cytosolic esterases to the highly fluorescent free acid form, which is trapped in the cytosol since it is not a Pgp substrate. However, in MDR cells, the free acid forms do not accumulate, implying that the AM compound is expelled from the membrane by Pgp before it reaches the cytosol [44].

The vacuum cleaner model also accounts for the observation that many kinetic studies on intact MDR cells have shown both increased efflux rates and decreased influx rates for most drugs. Stein et al. [106] examined the rates of influx and efflux of different drugs in intact MDR cells expressing both wild-type and mutant (Gly-185 $\rightarrow$ Val) Pgp, in the absence and presence of a chemosensitizer. Their results suggested that drugs within the inner and outer leaflets of the membrane may follow different paths when effluxed by Pgp.

There may be more than one route for substrates to gain access to Pgp. Altenberg et al. [3] found that the unidirectional influx of rhodamine 123 in MDR cells was independent of Pgp expression levels and insensitive to the presence of chemosensitizers. They argued that Pgp extracts this compound from the aqueous compartment, rather than the membrane. Colchicine is a relatively hydrophilic compound, which is transported very well by Pgp in membrane vesicle systems in vitro (e.g. [19, 83, 95]). Despite the fact that it is a good transport substrate, there have been many reports that colchicine is unable to compete with other more hydrophobic drugs in photoaffinity labeling experiments. One possible explanation for these observations is that drugs with a high degree of polarity, or high charge, may gain access to the transporter from the aqueous phase, whereas more hydrophobic compounds may interact with the protein from within the bilayer. Indeed, even membrane proteins which transport very hydrophilic physiological substrates (lactose permease, glucose transporter) are able to transport hydrophobic sugar derivatives quite well, and may have lipid-accessible binding sites.

Pgp Generates a Substrate Concentration Gradient

Transport studies in simple membrane systems have clearly shown that Pgp is an active transporter, generating a drug concentration gradient. In plasma membrane vesicles from MDR Chinese hamster ovary cells, ATP-dependent concentration gradients were measured for colchicine (18-fold) and vinblastine (10-fold) [19]. Pgp expressed in yeast secretory vesicles generated a 7-fold gradient of colchicine [83]. Pgp reconstituted into proteoliposomes can produce substrate concentration gradients of a similar magnitude. Partially purified Pgp gave rise to a 5.6-fold colchicine gradient [95], and highly purified protein generated a 5.2-fold gradient of NAc-LLY-amide [98]. Proteoliposomes containing partially purified Pgp were able to accumulate Rb^+ ions in their interior to a concentration of 8 mM, which was driven by the ATP-dependent transport of the K^+-specific ionophore, valinomycin [25].

Measurement of concentration gradients for MDR substrates involves consideration of the lipid solubility of this group of compounds. In the case of ABC proteins that transport hydrophilic substrates, the substrate can accumulate in the vesicle interior to much higher concentrations, since it is membrane impermeable. Very large substrate gradients have been reported for such systems (e.g., 100-fold for reconstituted histidine permease [10]). In the case of Pgp substrates, accumulation in the vesicle interior reaches an equilibrium level after several minutes. This equilibrium represents a balance between inward-pumping by Pgp up a concentration gradient, and outward passive diffusion down the concentration gradient (for more discussion on this point, *see* [93]). The observed drug gradient for hydrophobic compounds will, therefore, necessarily be smaller than that achievable for highly polar substrates. The gradient is maintained by the energy provided by ATP hydrolysis. In plasma membrane vesicle systems, depletion of extravesicular ATP by other membrane-found ATPases leads to collapse of the drug gradient at extended times, so that inclusion of an ATP-regenerating system is often neces-

sary (e.g. [19]). ATP depletion is generally not a problem in purified reconstituted systems, which can maintain the drug gradient for a considerable time without an ATP-regenerating system.

In the absence of ATP, drug associates with the vesicle system in two ways; it partitions into the lipid bilayer itself, and it also equilibrates with the internal aqueous compartment. Colchicine accumulation by Pgp-containing reconstituted proteoliposomes in the absence of ATP was about one sixth of the maximal accumulation measured in the presence of ATP, and calculations using the included volume of the vesicles indicated that the interior drug concentration was the same as the external concentration. In other words, drug ''uptake'' in the absence of ATP represents diffusional equilibration with the vesicle lumen [95]. In the case of a relatively hydrophilic Pgp substrate, such as colchicine, the amount of drug present within the bilayer is very small compared to the amount that equilibrates inside the lumen. A more complex picture emerged for the peptide NAc-LLY-amide which is considerably more hydrophobic [98]. In this case, substantial partitioning of the peptide into the lipid bilayer takes place, generating an apparent 25-fold concentration gradient (assuming that all associated peptide is intraluminal) in the absence of ATP. In the presence of ATP, the amount of peptide associated with the vesicles increased 5.2-fold, indicating the generation of a concentration gradient. A similar situation was observed previously for uptake of the very hydrophobic drug vinblastine into MDR plasma membrane vesicles [19]. These examples illustrate the importance of accounting for the contribution of drug equilibration into the vesicle interior and partitioning into the membrane bilayer, by comparing drug uptake in the absence and presence of ATP.

Aromatic Amino Acid Residues May be Important in Substrate Binding and Transport

In general, membrane proteins that transport polar substrates, such as sugars or ions, are believed to contain a hydrophilic passageway through the lipid bilayer. Such a passageway is necessary to generate favorable interactions between the polar substrate and amino acid side chains of the transporter. The role of these interactions is to reduce the energy barrier associated with removal of hydration from the substrate prior to translocation, and also to ''guide'' the substrate during its passage through the protein. For example, in the erythrocyte Band 3 anion exchanger, the transport path is made up of the hydrophilic surfaces of several protein α-helices, which contain charged amino residues and face the interior of the helix bundle [79].

In the case of Pgp, where the transport substrates are hydrophobic, a different type of transport path must be envisaged. Many Pgp substrates contain aromatic rings, and aromatic amino acid residues are known to provide binding sites for molecules of this type. Pawagi and coworkers [73] have presented convincing arguments for the involvement of aromatic amino acid side chains in binding and transport of drugs by Pgp. Pgp has a high content of aromatic residues within the TM regions compared to other ABC transporters with polar substrates, and these residues are highly conserved. Using molecular modeling, these researchers demonstrated that the Pgp substrate rhodamine 123 can readily intercalate between several phenylalanine side chains in the TM helices. They proposed that the transport path followed by Pgp substrates may involve either an internal ''channel'' lined by aromatic residues facing the inside of a bundle of 5 or 6 α-helices, or drugs may interact with the protein via gaps between externally oriented aromatic side chains at the interface between TM helices and surrounding lipid.

The involvement of several aromatic-rich TM helices in drug binding and transport would provide the conformational flexibility needed to accommodate many hydrophobic substrates of diverse size, shape, and structure. Different sets of aromatic residues may be brought into play to bind different substrates. This is consistent with the idea that there is a single flexible location for drug binding within the protein structure, and may also explain why attempts to locate the site precisely have not been successful to date.

Pgp May Be a Drug Flippase

The unusual nature of the Pgp transporter led to the suggestion that it acts as a translocase or flippase, moving substrates from the inner leaflet to the outer leaflet of the membrane [42]. Since the drug in each leaflet is in equilibrium with the aqueous phase, the presence of different amounts of drug within each leaflet (i.e., an *intra*-membrane gradient) would generate a drug concentration gradient across the membrane. The net effect of drug translocation within the bilayer would thus be the same as that expected for a classical membrane pump. It is also possible that Pgp may move substrates from the inner leaflet directly to the aqueous extracellular space, since this mode of action would produce the same net effect as inter-leaflet flipping. Distinguishing between these two related modes of action experimentally will undoubtedly prove challenging. Support for the flippase model of Pgp action has been growing in recent years. Studies of transgenic knockout mice lacking Class III Pgp, which does not confer MDR, showed that the protein is involved in export of PC from the apical surface of the hepatocyte canalicular membrane into the bile [104]. In vitro studies indicated that Class III Pgp was able to translocate PC, but not PE (phosphatidylethanol-

amine), from the inner to the outer leaflet of fibroblasts [104] and yeast secretory vesicles [82]. PC translocation was ATP-dependent, and was inhibited by vanadate, a known inhibitor of ATP hydrolysis by Class I Pgp isoforms. Since the Class I and III Pgp isoforms have sequence identity of over 75%, it seems likely that they would also share important aspects of their mode of action (reviewed in [43]). The lipid specificity of the Class III Pgp flippase activity is distinct from that of the phospholipid flippase of the human erythrocyte membrane, which translocates PE and PS (phosphatidylserine) from the outer to the inner leaflet in an ATP-dependent fashion [5]. The erythrocyte phospholipid flippase may be an excellent model for the mode of action of both the Class I and Class III Pgps; unfortunately, little is currently known of its structure and translocation mechanism.

van Helvoort and coworkers have recently shown that Class I Pgp is able to translocate short chain derivatives of various membrane lipids from the inner to the outer leaflet of epithelial cells [115]. These derivatives included fluorescent analogues of PC, PE, sphingomyelin, and glucosylceramide carrying C6 fatty acid chains in place of a long chain fatty acid, and di-C8 derivatives of PC and PE. Class III Pgp was apparently unable to translocate any of these short chain lipid derivatives. Since it is already known that Class I Pgp cannot translocate full-length phospholipids [83], it appears that the change in structure from a full-length phospholipid to a more amphiphilic molecule is sufficient to allow it to be a Class I Pgp substrate. These data provide convincing evidence to support the proposal that Class I Pgp acts as a drug flippase.

The flippase aspect of Pgp function introduces a perplexing but very important problem; how to estimate the true transport turnover rate of the protein. Since all of the substrates for Pgp are lipophilic, they are expected to have a finite rate of spontaneous flip-flop across lipid bilayers, with half-times on the order of a few minutes up to an hour [26]. Thus, assuming that Pgp carried out inter-leaflet flip-flop, while the transporter is flipping the drug to the outer leaflet, some will be re-entering the inner leaflet, which will result in ''futile cycling'' of drug. Pgp action will soon result in different steady-state drug concentrations in each leaflet of the bilayer. We might predict that Pgp would be able to generate a larger concentration difference for a drug with a slow flip-flop rate when compared to a drug with a fast flip-flop rate. Experimentally, we usually measure the *net* movement of drug from the aqueous phase on one side of the membrane to the aqueous phase on the other side, which, in this case, will clearly not represent the actual rate of turnover of the transporter. It seems likely that all transport experiments carried out to date have seriously underestimated the true transport turnover of Pgp.

If the mode of action of Pgp involves translocation of substrate from the inner leaflet of the membrane directly to the aqueous external phase, we are faced with a similar problem. Lipophilic substrates, once expelled, would have a finite rate of re-entry from the aqueous phase into the outer leaflet of the membrane, again resulting in futile cycling. Trapping of the substrate following transport by Pgp (*see* below for more on this point) may prevent re-entry into the membrane, and allow estimation of a true turnover rate for the transporter.

Can Pgp Pump Drugs Fast Enough to Cause MDR?

It has been suggested that the rate of drug pumping by Pgp measured in membrane vesicle systems is too slow to account for MDR [80, 100]. However, as pointed out above, because of the nonpolar nature of its substrates, the true activity of Pgp will be underestimated in conventional transport experiments. Drug ''transport'', as measured by the change in drug concentration in the aqueous phase on one side of the membrane barrier, represents the rate of *net* drug accumulation, not the true turnover rate of the transporter. Consideration of this issue leads to an explanation of why the rate of drug transport appears to be many-fold lower than the rate of ATP hydrolysis in the reconstituted systems examined to date. Because of underestimation of the true rate of transport turnover, the question of the stoichiometry of ATP hydrolysis relative to drug transport has been difficult to address. Shapiro & Ling reported that the apparent rate of Hoechst 33342 transport was 50-fold slower than the rate of ATP hydrolysis [90]. They point out that since the fluorescence technique they used to measure transport monitors the membrane-bound drug, this represents a net rate of transport only. An additional complication is the high constitutive ATPase activity displayed by Pgp in the absence of drug. Sharom et al. [95] estimated that an *additional* 4 molecules of ATP were hydrolyzed for each molecule of colchicine transported; however, this was a 15% increase in ATP hydrolysis measured against a high background of constitutive ATPase activity. More recently, it was estimated that 0.5–0.8 molecules of Rb^+-valinomycin were transported into proteoliposomes reconstituted with partially purified Pgp for each ATP molecule hydrolyzed [25].

How can we estimate the true rate of substrate translocation by Pgp? One approach that might circumvent this problem is to use a Pgp substrate which is immediately converted to a hydrophilic nondiffusible form when it gains access to the lumen of membrane vesicles or proteoliposomes. Such a strategy has already been developed for intact cells, using the acetoxymethyl derivatives of certain fluorescent dyes [44], and may be adaptable to vesicle systems. The experimental system employed by Eytan et al. [25] to measure the rate of

transport of valinomycin avoided problems with re-entry of the hydrophobic substrate into the bilayer by examining the accumulation of Rb^+ in the vesicle lumen. Alternatively, it may be possible to estimate the true turnover rate using biophysical techniques, such as measurements of rates of conformational change in Pgp.

Pgp May Handle Chemosensitizers in the Same Way as Drugs

Many pharmacologic agents from diverse structural classes (Table 2) have been identified as Pgp chemosensitizers. One outstanding puzzle in the quest to understand Pgp at the molecular level is the transporter's discrimination between ''substrates'' and chemosensitizers, all of which may interact with the same binding site. This is a key issue; understanding the differences in how Pgp handles substrates and chemosensitizers at the molecular level is clearly of great importance in the search for clinically more effective MDR reversers. Drug ''substrates'' are transported actively, to generate a concentration gradient, and MDR cells display resistance to killing by these compounds. Chemosensitizers ''reverse'' drug resistance, leading to killing of intact MDR cells when combined with drugs. They apparently do this by competing with drug substrates, as shown in transport assays in vitro. Many of them also stimulate the biggest increases in Pgp ATPase activity, much larger than several ''substrates'', which often either stimulate activity weakly, or inhibit it. Some chemosensitizers (verapamil, *trans*-flupenthixol, cyclosporin A) appear to be transported by Pgp (the majority have not been tested), but MDR cells are not resistant to these compounds. If chemosensitizers simply behave as ''alternative substrates'', why are MDR cells not resistant to them?

One attractive solution to these apparently paradoxical observations has been provided recently by Eytan et al. [26]. They propose that, in fact, both drugs and chemosensitizers are handled by Pgp in exactly the same way; they are transported, with hydrolysis of ATP. Compounds that have been ''flipped'' to the outer leaflet by Pgp can ''flop'' back into the inner leaflet (i.e., diffuse across the membrane), before interacting with Pgp once more, and being flipped again. The difference in what we observe experimentally will depend on the rate at which drugs flip-flop across the membrane. ''Substrates'' were found to equilibrate cross lipid bilayers relatively slowly (from minutes to hours) whereas chemosensitizers crossed bilayers too rapidly to measure experimentally (seconds) [26]. For substrates, the rate of transmembrane movement is presumably slow enough that flipping by Pgp can more than keep pace, and a drug gradient is established. For chemosensitizers, the rate of membrane equilibration is so rapid that flipping via Pgp cannot keep pace with it. The transporter essentially operates in a futile cycle; the transport turnover is high, with high rates of ATP hydrolysis, but no gradient is generated, and cells will not, therefore, be resistant to chemosensitizers. One could easily come to the incorrect conclusion that a particular chemosensitizer is not, in fact, a substrate for Pgp, since no net transport will be observed in this situation, even though the compound is being flipped or transported by Pgp. This may explain the report that progesterone, which has been identified as a substrate for Pgp, and binds to the purified protein with high affinity (R. Liu and F.J. Sharom, *unpublished data*), is apparently not effluxed by MDR cells [109]. Eytan et al. [26] found that progesterone crossed membranes at a very high rate. This model for chemosensitizer action also accounts for the observation that chemosensitizers are generally effective at molar concentrations similar to that of the transported substrate, rather than the transporter protein, a fact which was previously used to argue against the efflux pump model for Pgp action [100].

One possible alternative explanation for the mode of action of some chemosensitizers in interfering with the action of Pgp may be that they are ATPase inhibitors. For example, the flavonoid quercetin has been proposed to block Pgp function in intact cells and reconstituted membrane systems by inhibiting the ATPase activity of Pgp, which is required for drug transport [91]. Chemosensitizers in this category would clearly not be transported by Pgp.

If the ideas of Eytan and coworkers are correct, then the criteria for the molecular properties of effective chemosensitizers must undergo radical modification. High affinity binding to Pgp is still essential, but equally important is the ability to spontaneously flip-flop across lipid bilayers rapidly. Since little is known about the movement of drugs across bilayers, it is not obvious how one could predict the flip-flop rate from the structure. Clearly, it is important to test these ideas, since they may have a major impact on the strategy to be used for chemosensitizer development. Preliminary data obtained in our laboratory supports the idea that the rate of transmembrane movement of a drug is important. Using Pgp-proteoliposomes, we observed that both the net rate of colchicine accumulation and the total drug accumulated were much higher in rigid gel phase lipid than in fluid liquid crystalline phase lipid [94]. Therefore, in the case of a rigid lipid vesicle where transmembrane drug movement is slow, Pgp appears to establish a drug concentration gradient and accumulate drug in the lumen more rapidly. In contrast, a concentration gradient is established more slowly, and accumulation is lower, in a highly fluid lipid vesicle, where transmembrane drug movement is fast. Our findings are in agreement with a report that membrane fluidizers reduce the apparent rate of drug transport via Pgp by 4-fold [101]. If a compound

which modifies membrane properties increases the flip-flop rate of a chemotherapeutic drug sufficiently, it may be able to reverse drug resistance to some extent, without the necessity for a specific interaction with Pgp. This suggests that a second class of chemosensitizers may exist, consisting of agents such as detergents, amphiphiles and membrane fluidizers. In fact, several chemosensitizing agents that fall into this category are already known (e.g., Cremophor EL, Solutol HS15, Tween 80) [117]. In addition, alterations in membrane fluidity were able to reverse MDR [15], and several chemosensitizers were observed to alter membrane fluidity and increase membrane permeability [23].

It should be noted that amphiphiles which affect the action of Pgp appear to fall into two classes. Compounds such as Tween 80 do not interact with Pgp directly, as assessed by fluorescence quenching (R. Liu and F.J. Sharom, *unpublished data*), and do not appear to be Pgp ''substrates''. On the other hand, the polyoxyethylene nonionic detergent Triton X-100 stimulates Pgp ATPase activity [21], inhibits azidopine photoaffinity labelling of Pgp [56], and binds to the purified protein with high affinity in a saturable fashion as indicated by fluorescence quenching (K_d = 0.37 μM; *see* Table 3), which suggests that detergents of this class are in fact Pgp substrates.

We can predict that membrane fluidizers and permeabilizers of the Cremophor EL type will enhance the action of chemosensitizing agents in blocking drug pumping by Pgp. This strategy may be useful for clinical application if effective nontoxic compounds of this type can be identified.

Summary

Pgp is an atypical translocating ATPase, with low affinity for ATP and high constitutive ATPase activity. Pgp also has an unusually broad specificity for hydrophobic substrates, including many chemotherapeutic drugs. Transport studies in reconstituted systems indicate that drug transport requires ATP hydrolysis and is active, generating a drug concentration gradient. Binding of drugs and ATP to Pgp induces conformational changes in the protein, and the drug binding site is conformationally coupled to the NBDs. Evidence accumulated to date suggests that the transporter interacts directly with nonpolar substrates within the membrane environment, and may act as a drug flippase, moving drugs from the inner to the outer leaflet of the bilayer. Chemosensitizers that block the action of Pgp are proposed to act as alternative substrates, but their high rate of spontaneous flip-flop across the membrane results in futile cycling of the transporter.

References

1. Abraham, E.H., Prat, A.G., Gerweck, L., Seneveratne, T., Arceci, R.J., Kramer, R., Guidotti, G., Cantiello, H.F. 1993. *Proc. Natl. Acad. Sci. USA* **90:**312–316
2. Al-Shawi, M.K., Senior, A.E. 1993. *J. Biol. Chem.* **268:**4197–4206
3. Altenberg, G.A., Vanoye, C.G., Horton, J.K., Reuss, L. 1994. *Proc. Natl. Acad. Sci. USA* **91:**4654–4657
4. Ambudkar, S.V., Lelong, I.H., Zhang, J., Cardarelli, C.O., Gottesman, M.M., Pastan, I. 1992. *Proc. Natl. Acad. Sci. USA* **89:**8472–8476
5. Auland, M.E., Roufogalis, B.D., Devaux, P.F., Zachowski, A. 1994. *Proc. Natl. Acad. Sci. USA* **91:**10938–10942
6. Ayesh, S., Shao, Y.-M., Stein, W.D. 1996. *Biochim. Biophys. Acta* **316:**8–18
7. Azzaria, M., Schurr, E., Gros, P. 1989. *Mol. Cell. Biol.* **9:**5289–5297
8. Baubichon-Cortay, H., Baggetto, L.G., Dayan, G., Di Pietro, A. 1994. *J. Biol. Chem.* **269:**22983–22989
9. Beck, W.T., Qian, X.-D. 1992. *Biochem. Pharmacol.* **43:**89–93.
10. Bishop, L., Agbayani, R., Ambudkar, S.V., Maloney, P.C., Ames, G.F.-L. 1989. *Proc. Natl. Acad. Sci. USA* **86:**6953–6957
11. Boer, R., Dichtl, M., Borchers, C., Ulrich, W.R., Maracek, J.F., Prestwich, G.D., Glossmann, H., Striessnig, J. 1996. *Biochemistry* **35:**1387–1396
12. Borgnia, M.J., Eytan, G.D., Assaraf, Y.G. 1996. *J. Biol. Chem.* **271:**3163–3171
13. Bosch, I., Croop, J. 1996. *Biochim. Biophys. Acta* **1288:**F37–F54
14. Boyum, R., Guidotti, G. 1997. *Biochem. Biophys. Res. Commun.* **230:**22–26
15. Callaghan, R., Stafford, A., Epand, R.M. 1993. *Biochim. Biophys. Acta* **1175:**277–282
16. Dayan, G., Baubichon-Cortay, H., Jault, J.-M., Cortay, J.-C., Deléage, G., Di Pietro, A. 1996. *J. Biol. Chem.* **271:**11652–11658
17. Devine, S.E., Ling, V., Melera, P.W. 1992. *Proc. Natl. Acad. Sci. USA* **89:**4564–4568
18. Doige, C.A., Ames, G.F. 1993. *Annu. Rev. Microbiol.* **47:**291–319
19. Doige, C.A., Sharom, F.J. 1992. *Biochim. Biophys. Acta* **1109:**161–171
20. Doige, C.A., Yu, X., Sharom, F.J. 1992. *Biochim. Biophys. Acta* **1109:**149–160
21. Doige, C.A., Yu, X., Sharom, F.J. 1993. *Biochim. Biophys. Acta* **1146:**65–72
22. Dong, M., Penin, F., Baggetto, L.G. 1996. *J. Biol. Chem.* **46:**28875–28883
23. Drori, S., Eytan, G.D., Assaraf, Y.G. 1995. *Eur. J. Biochem.* **228:**1020–1029
24. Eytan, G.D., Borgnia, M.J., Regev, R., Assaraf, Y.G. 1994. *J. Biol. Chem.* **269:**26058–26065
25. Eytan, G.D., Regev, R., Assaraf, Y.G. 1996. *J. Biol. Chem.* **271:**3172–3178
26. Eytan, G.D., Regev, R., Oren, G., Assaraf, Y.G. 1996. *J. Biol. Chem.* **271:**12897–12902
27. Ferry, D.R., Malkhandi, P.J., Russell, M.A., Kerr, D.J. 1995. *Biochem. Pharmacol.* **49:**1851–1861
28. Ferry, D.R., Russell, M.A., Cullen, M.H. 1992. *Biochem. Biophys. Res. Commun.* **188:**440–445
29. Ford, J.M. 1995. *In:* Drug Resistance in Clinical Oncology and Hematology. G.A. Fisher and B.I. Sikic, editors. Vol. 9, pp 337–361. Saunders, Philadelphia
30. Ford, J.M. 1996. *Eur. J. Cancer* **32A:**991–1001
31. Ford, J.M., Hait, W.N. 1993. *Cytotechnology* **12:**171–212

32. Georges, E., Sharom, F.J., Ling, V. 1990. *Adv. Pharmacol.* **21:**185–220
33. Germann, U.A. 1996. *Eur. J. Cancer* **32A:**927–944
34. Goldstein, L.J. 1996. *Eur. J. Cancer* **32A:**1039–1050
35. Gottesman, M.M., Pastan, I. 1993. *Annu. Rev. Biochem.* **62:**385–427
36. Gottesman, M.M., Hrycyna, C.A., Schoenlein, P.V., Germann, U.A., Pastan, I. 1995. *Annu. Rev. Genet.* **29:**607–649
37. Gottesman, M.M., Pastan, I., Ambudkar, S.V. 1996. *Current Opinion Genet. Dev.* **6:**610–617
38. Gros, P., Dhir, R., Croop, J., Talbot, F. 1991. *Proc. Natl. Acad. Sci. USA* **88:**7289–7293
39. Guiral, M., Viratelle, O., Westerhoff, H.V., Lankelma, J. 1994. *FEBS Lett.* **346:**141–145
40. Higgins, C.F. 1992. *Annu. Rev. Cell Biol.* **8:**67–113
41. Higgins, C.F. 1995. *J. Bioenerg. Biomembr.* **27:**63–70
42. Higgins, C.F., Gottesman, M.M. 1992. *Trends Biochem. Sci.* **17:**18–21
43. Higgins, C.F. 1994. *Cell* **79:**393–395
44. Homolya, L., Holló, Z., Germann, U.A., Pastan, I., Gottesman, M.M., Sarkadi, B. 1993. *J. Biol. Chem.* **268:**21493–21496
45. Horio, M., Gottesman, M.M., Pastan, I. 1988. *Proc. Natl. Acad. Sci. USA* **85:**3580–3584
46. Hsing, S., Gatmaitan, Z., Arias, I.M. 1992. *Gastroenterology* **102:**879–885
47. Hyde, S.C., Emsley, P., Hartshorn, M.J., Mimmack, M.M., Gileadi, U., Pearce, S.R., Gallagher, M.P., Gill, D.R., Hubbard, R.E., Higgins, C.F. 1990. *Nature* **346:**362–365
48. Kajiji, S., Talbot, F., Grizzuti, K., Van Dyke-Phillips, V., Agresti, M., Safa, A.R., Gros, P. 1993. *J. Biol. Chem.* **32:**4185–4194
49. Kamimoto, Y., Gatmaitan, Z., Hsu, J., Arias, I.M. 1989. *J. Biol. Chem.* **264:**11693–11698
50. Kast, C., Canfield, V., Levenson, R., Gros, P. 1996. *J. Biol. Chem.* **271:**9240–9248
51. Kessel, D. 1989. *Cancer Commun.* **1:**145–149
52. Leveille-Webster, C.R., Arias, I.M. 1995. *J. Membrane Biol.* **143:**89–102
53. Li, C., Ramjeesingh, M., Wang, W., Garami, E., Hewryk, M., Lee, D., Rommens, J.M., Galley, K., Bear, C.E. 1996. *J. Biol. Chem.* **271:**28463–28468
54. Liu, R., Sharom, F.J. 1996. *Biochemistry* **35:**11865–11873
55. Liu, R., Sharom, F.J. 1997. *Biochemistry* **36:**2836–2843
56. Loe, D.W., Sharom, F.J. 1993. *Br. J. Cancer* **68:**342–351
57. Loe, D.W., Sharom, F.J. 1994. *Biochim. Biophys. Acta* **1190:**72–84
58. Loo, T.W., Clarke, D.M. 1993. *J. Biol. Chem.* **268:**19965–19972
59. Loo, T.W., Clarke, D.M. 1994. *Biochemistry* **33:**14049–14057
60. Loo, T.W., Clarke, D.M. 1994. *J. Biol. Chem.* **269:**7750–7755
61. Loo, T.W., Clarke, D.M. 1995. *J. Biol. Chem.* **270:**843–848
62. Loo, T.W., Clarke, D.M. 1995. *J. Biol. Chem.* **270:**21449–21452
63. Loo, T.W., Clarke, D.M. 1995. *J. Biol. Chem.* **270:**22957–22961
64. Loo, T.W., Clarke, D.M. 1996. *J. Biol. Chem.* **271:**27482–27487
65. Loo, T.W., Clarke, D.M. 1997. *J. Biol. Chem.* **272:**709–712
66. Mayer, L.D., Bally, M.B., Hope, M.J., Cullis, P.R. 1985. *Biochim. Biophys. Acta* **816:**294–302
67. Mayer, L.D., Bally, M.B., Cullis, P.R. 1986. *Biochim. Biophys. Acta* **857:**123–126
68. Mimura, C.S., Holbrook, S.R., Ames, G.F. 1991. *Proc. Natl. Acad. Sci. USA* **88:**84–88
69. Müller, M., Bakos, E., Welker, E., Váradi, A., Germann, U.A., Gottesman, M.M., Morse, B.S., Roninson, I.B., Sarkadi, B. 1996. *J. Biol. Chem.* **271:**1877–1883
70. Naito, M., Tsuruo, T. 1989. *Cancer Res.* **49:**1452–1455
71. Naito, M., Tsuruo, T. 1995. *J. Cancer Res. Clin. Oncol.* **121:**582–586
72. Orlowski, S., Mir, L.M., Belehradek, J., Garrigos, M. 1996. *Biochem. J.* **317:**515–522
73. Pawagi, A.B., Wang, J., Silverman, M., Reithmeier, R.A.F., Deber, C.M. 1994. *J. Mol. Biol.* **235:**554–564
74. Pearce, H.L., Safa, A.R., Bach, N.J., Winter, M.A., Cirtain, M.C., Beck, W.T. 1989. *Proc. Natl. Acad. Sci. USA* **86:**5128–5132
75. Piwnica-Worms, D., Rao, V.V., Kronauge, J.F., Croop, J.M. 1995. *Biochemistry* **34:**12210–12220
76. Ramachandra, M., Ambudkar, S.V., Gottesman, M.M., Pastan, I., Hrycyna, C.A. 1996. *Mol. Biol. Cell* **7:**1485–1498
77. Rao, U.S. 1995. *J. Biol. Chem.* **270:**6686–6690
78. Raviv, Y., Pollard, H.B., Bruggemann, E.P., Pastan, I., Gottesman, M.M. 1990. *J. Biol. Chem.* **265:**3975–3980
79. Reithmeier, R.A.F., Chan, S.L., Popov, M. 1996. *In:* Handbook of Biological Physics. W.N. Konings, H.R. Kaback and J.S. Lolkema, editors. Vol. 2, Chapter 13, pp. 281–309. Elsevier Science
80. Roepe, P.D. 1995. *Biochim. Biophys. Acta* **1241:**385–405
81. Romsicki, Y., Sharom, F.J. 1997. *Biochemistry* **36:**9807–9815
82. Ruetz, S., Gros, P. 1994. *Cell* **77:**1071–1081
83. Ruetz, S., Gros, P. 1994. *J. Biol. Chem.* **269:**12277–12284
84. Safa, A.R. 1993. *Cancer Invest.* **11:**46–56
85. Sarkadi, B., Price, E.M., Boucher, R.C., Germann, U.A., Scarborough, G.A. 1992. *J. Biol. Chem.* **267:**4854–4858
86. Sarkadi, B., Müller, M., Homolya, L., Holló, Z., Seprődi, J., Germann, U.A., Gottesman M.M., Price, E.M., Boucher, R.C. 1994. *FASEB J.* **8:**766–770
87. Senior, A.E., Al-Shawi, M.K., Urbatsch, I.L. 1995. *FEBS Lett.* **377:**285–289
88. Schlemmer, S.R., Sirotnak, F.M. 1994. *J. Biol. Chem.* **269:**31059–31066
89. Shapiro, A.B., Ling, V. 1994. *J. Biol. Chem.* **269:**3745–3754
90. Shapiro, A.B., Ling, V. 1995. *J. Biol. Chem.* **270:**16167–16175
91. Shapiro, A.B., Ling, V. 1997. *Biochem. Pharmacol.* **53:**587–596
92. Sharma, R.C., Inoue, S., Roitelman, J., Schimke, R.T., Simoni, R.D. 1992. *J. Biol. Chem.* **267:**5731–5734
93. Sharom, F.J. 1995. *J. Bioenerg. Biomembr.* **27:**15–22
94. Sharom, F.J. 1997. *Biochem. Soc. Trans.* (*in press*)
95. Sharom, F.J., Yu, X., Doige, C.A. 1993. *J. Biol. Chem.* **268:**24197–24202
96. Sharom, F.J., DiDiodato, G., Yu, X., Ashbourne, K.J.D. 1995. *J. Biol. Chem.* **270:**10334–10341
97. Sharom, F.J., Yu, X., Chu, J.W.K., Doige, C.A. 1995. *Biochem. J.* **308:**381–390
98. Sharom, F.J., Yu, X., DiDiodato, G., Chu, J.W.K. 1996. *Biochem. J.* **320:**421–428
99. Shimabuku, A.M., Nishimoto, T., Ueda, K., Komano, T. 1992. *J. Biol. Chem.* **267:**4308–4311
100. Simon, S.M., Schindler, M. 1994. *Proc. Natl. Acad. Sci. USA* **91:**3497–3504
101. Sinicrope, F.A., Dudeja, P.K., Bissonnette, B.M., Safa, A.R., Brasitus, T.A. 1992. *J. Biol. Chem.* **267:**24995–25002
102. Skach, W.R., Calayag, M.C., Lingappa, V.R. 1993. *J. Biol. Chem.* **268:**6903–6908
103. Smit, J.J.M., Schinkel, J.H. Oude Elferink, R.P.J., Groen, A.K., Wagenaar, E., van Deemter, L., Mol, C.A.A.M., Ottenhof, R., van der Lugt, N.M.T., van Roon, M.A., van der Walk, M.A., Offerhaus, G.J.A., Berns, A.J.M., Borst, P. 1993. *Cell* **75:**451–462
104. Smith, A.J., Timmermans-Hereijgers, J.L.P.M., Roelofsen, B.,

Wirtz, K.W.A., van Blitterswijk, W.J., Smit, J.J.M., Schinkel, A.H., Borst, P. 1994. *FEBS Lett.* **354:**263–266
105. Sonveaux, N., Shapiro, A.B., Goormaghtigh, E., Ling, V., Ruysschaert, J.M. 1996. *J. Biol. Chem.* **271:**24617–24624
106. Stein, W.D., Cardarelli, C., Pastan, I., Gottesman, M.M. 1994. *Mol. Pharmacol.* **45:**763–772
107. Taglicht, D., Michaelis, S. 1997. *Methods Enzymol.* (*in press*)
108. Tamai, I., Safa, A.R. 1991. *J. Biol. Chem.* **266:**16796–16800
109. Ueda, K., Okamura, N., Hirai, M., Tanigawara, Y., Saeki, T., Kioka, N., Komano, T., Hori, R. 1992. *J. Biol. Chem.* **267:**24248–24252
110. Urbatsch, I.L., Senior, A.E. 1995. *Arch. Biochem. Biophys.* **316:**135–140
111. Urbatsch, I.L., Al-Shawi, M.K., Senior, A.E. 1994. *Biochemistry* **33:**7069–7076
112. Urbatsch, I.L., Sankaran, B., Bhagat, S., Senior, A.E. 1995. *J. Biol. Chem.* **270:**26956–26961
113. Urbatsch, I.L., Sankaran, B., Weber, J., Senior, A.E. 1995. *J. Biol. Chem.* **270:**19383–19390
114. Valverde, M.A., Diaz, M., Sepulveda, F.V., Gill, D.R., Hyde, S.C., Higgins, C.F. 1992. *Nature* **355:**830–833
115. van Helvoort, A., Smith, A.J., Sprong, H., Fritzsche, I., Schinkel, A.H., Borst, P., van Meer, G. 1996. *Cell* **87:**507–517
116. Welker, E., Szabo, K., Holló, Z., Müller, M., Sarkadi, B., Varadi, A. 1995. *Biochem. Biophys. Res. Commun. 216:*602–609
117. Woodcock, C.M., Linsenmeyer, M.E., Chojnowski, G., Kriegler, A.B., Nink, V., Webster, L.K., Sawyer, W.H. 1992. *Br. J. Cancer* **66:**62–68
118. Zhang, M., Wang, G., Shapiro, A., Zhang, J.-T. 1996. *Biochemistry* **35:**9728–9736