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Molecular analysis of the multidrug transporter, P-glycoprotein

Ursula A. Germann[1] & Timothy C. Chambers[2]
[1] *Vertex Pharmaceuticals Incorporated, 130 Waverly Street, Cambridge, MA 02139-4242, U.S.A.; E-mail: Germann@macnet.vpharm.com*
[2] *Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR, U.S.A.*

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Abstract

Inherent or acquired resistance of tumor cells to cytotoxic drugs represents a major limitation to the successful chemotherapeutic treatment of cancer. During the past three decades dramatic progress has been made in the understanding of the molecular basis of this phenomenon. Analyses of drug-selected tumor cells which exhibit simultaneous resistance to structurally unrelated anti-cancer drugs have led to the discovery of the human *MDR*1 gene product, P-glycoprotein, as one of the mechanisms responsible for multidrug resistance. Overexpression of this 170 kDa *N*-glycosylated plasma membrane protein in mammalian cells has been associated with ATP-dependent reduced drug accumulation, suggesting that P-glycoprotein may act as an energy-dependent drug efflux pump. P-glycoprotein consists of two highly homologous halves each of which contains a transmembrane domain and an ATP binding fold. This overall architecture is characteristic for members of the ATP-binding cassette or ABC superfamily of transporters. Cell biological, molecular genetic and biochemical approaches have been used for structure-function studies of P-glycoprotein and analysis of its mechanism of action. This review summarizes the current status of knowledge on the domain organization, topology and higher order structure of P-glycoprotein, the location of drug- and ATP binding sites within P-glycoprotein, its ATPase and drug transport activities, its possible functions as an ion channel, ATP channel and lipid transporter, its potential role in cholesterol biosynthesis, and the effects of phosphorylation on P-glycoprotein activity.

Introduction

The problem of antitumor drug resistance is a serious impediment to successful chemotherapy of patients with cancer. Much effort has been expended for more than 25 years to understand the genetic and biochemical alterations responsible for the multidrug resistance (MDR) phenotype of cancer cells with the ultimate goal to develop novel strategies and therapeutics to overcome the problem of drug resistance in the clinic. Enormous progress has been made in identifying proteins associated with the MDR phenotype and characterizing their structure and function. Progress was possible largely due to the availability of many *in vitro* MDR models (reviewed in Beck and Danks, 1991; Sugimoto and Tsuruo, 1991). The development of such models is based on the early work by Kessel *et al*. (1968) and by Biedler and Riehm (1970) who discovered that exposure of cultured cells to a single cytotoxic agent may lead to selection of clones which are resistant to multiple, structurally and functionally unrelated drugs. During the past three decades, cell culture models to study this MDR phenotype have been established from many different tissue origins and different species by selecting drug-sensitive cells in stepwise increasing, sublethal concentrations of a cytotoxic drug. Most often doxorubicin, vincristine, or etoposide have been applied as selective agents and the acquired drug resistance phenotype typically exhibited cross-resistance to *Vinca* alkaloids, anthracyclines, taxanes, epipodophyllotoxins, and other cytotoxics associated with so-called 'classical' MDR (Table 1).

Three principal differences have been determined between MDR cells and their drug-sensitive par-

Table 1. Cytotoxic agents associated and not associated with 'classical' MDR

Agents associated with MDR		Agents not associated with MDR
Anti-cancer drugs	**Other cytotoxics**	**Anti-cancer drugs**
Anthracyclines	Antibiotics	Alkylating agents
Daunorubicin	Actinomycin D	Cyclophosphamide
Doxorubicin		Mechlorethamine
Idarubicin	Antimicrotubule agents	(nitrogen mustard)
Mitoxantrone	Colchicine	Carmustine (BCNU)
	Podophyllotoxin	Sermustine (CCNU)
Vinca alkaloids		Ifosphamide
Vincristine	Protein synthesis inhibitors	
Vinblastine	Puromycin	Antimetabolites
	Emetine	Fluorouracil
Epipodophyllotoxins		Cytarabine (ara-C)
Etoposide (VP-16)	DNA interchalators	Methothrexate
Teniposide (VM-24)	Ethidium bromide	
Taxanes	Toxic peptides	Miscellaneous
Paclitaxel	Valinomycin	Cisplatin
Docetaxel	Gramicidin D	Carboplatin
Taxotere	N-Acetyl-leucyl-leucyl-norleulinal (ALLN)	Bleomycin
		Hydroxyurea
Miscellaneous		
Dactinomycin		
Mithramycin		
Trimetrexate		
Mitomycin C		
Topotecan		

ents. These include (1) a drug accumulation deficiency due to decreased drug influx and/or increased drug efflux; (2) altered expression and/or activity of drug-interacting and/or other cellular proteins; and (3) physiological changes that affect the intracellular milieu (e.g. pH). Proteins which are overexpressed in MDR cells include the multidrug resistance *MDR*1 gene product P-glycoprotein associated with the classical MDR phenotype (this review), and the multidrug resistance-associated protein MRP (see Center, this issue), and the lung resistance protein LRP (see Scheper and Izquierdo, this issue), which originally have been identified in non-P-glycoprotein expressing MDR cells. Each of these proteins has been associated with a unique drug resistance profile, but patterns of cross-resistance are partially overlapping. Overexpression of these three proteins, however, is not mutually exclusive and in a few tumor cell lines enhanced expression of P-glycoprotein and/or MRP and/or LRP has been detected (Brock *et al*., 1995; Hasegawa *et al*., 1995; Huang *et al*., 1997; Izquierdo *et al*., 1996; Slapak *et al*., 1994). MDR is clearly a complex problem and this review is limited to a discussion of P-glycoprotein, the most ubiquitous MDR marker expressed in drug-selected cultured cells and a well-characterized mechanism used by cancer cells to evade cell death conferred by cytotoxics.

P-glycoprotein was discovered over twenty years ago by Juliano and Ling (1976) as a highly abundant high molecular weight, integral plasma membrane glycoprotein in drug-selected Chinese hamster ovary cells. P-glycoprotein was initially thought to play a role in modulating cellular permeability ('P' stands for permeability) to drugs. Since then it has been recognized that P-glycoprotein confers MDR by acting as an energy-dependent drug efflux pump (reviewed in Endicott and Ling, 1989; Gottesman and Pastan, 1993; Roninson, 1991).

Molecular biological studies have revealed the existence of a small *MDR* gene family which consists of two members in human and three in rodents. All encoded P-glycoproteins share a high degree of amino acid sequence identity (>70%), however, *mdr* gene products belong to two functionally different categories. In gene transfer experiments it has been observed that overexpression of a class 1 P-glycoprotein (Devault and Gros, 1990; Gros *et al*., 1986a; Ueda *et al*., 1987) endows drug-sensitive host cells with MDR, while overexpression of a class 2 P-glycoprotein has no direct effect on cellular drug resistance (Buschman *et al*., 1992; Schinkel *et al*., 1991). Hence, only class 1 includes the drug-transporting P-glycoproteins or multidrug transporters which are the focus of this review. The human *MDR*1 (Chen *et al*., 1986), the mouse *mdr*3 (or *mdr*1a) (Devault and Gros, 1990; Hsu *et al*., 1989) and *mdr*1 (or *mdr*1b) (Gros *et al*., 1986b), the hamster *pgp*1 and *pgp*2 (Endicott *et al*., 1987; Gros *et al*., 1986c), and the rat *pgp*1 and *pgp*2 (or *mdr*1b) gene products (Deuchars *et al*., 1992; Silverman *et al*., 1991) all belong to this class, while non drug-transporting P-glycoproteins, such as the human *MDR*2, or an alternative splice form thereof referred to as *MDR*3 (Schinkel *et al*., 1991; Van der Bliek *et al*., 1987), the mouse *mdr*2 (Buschman *et al*., 1992; Gros *et al*., 1988), the hamster *pgp*3 (Endicott *et al*., 1991), and the rat *mdr*2/*mdr*3 (Brown *et al*., 1993; Furuya *et al*., 1994) gene products belong to class 2 (which sometimes is also described as class 3). Class 2 *mdr* gene products are primarily expressed in the liver bile canaliculi (Buschman *et al*., 1992; Cordon-Cardo *et al*., 1990; Smit *et al*., 1994; van der Valk *et al*., 1990) and analyses of *mdr*2 knock-out mice have indicated that class 2 P-glycoproteins represent phosphatidylcholine transporters (Smit *et al*., 1993). Class 2 P-glycoproteins may function by translocating lipids in a taurocholate-dependent manner (Ruetz and Gros, 1994b; Ruetz and Gros, 1995; reviewed in Oude Elferink and Groen, 1995).

Both class 1 and class 2 P-glycoproteins, together with MRP and many other transport proteins, belong to a large superfamily of *A*TP-*b*inding *c*assette (ABC) transporters (Hyde *et al*., 1990) or traffic ATPases (Mimura *et al*., 1991; also see Croop, this issue). ABC transporters occur in many different organisms (archebacteria, bacteria, yeast, plants, insects, animals, and humans; for reviews see Ames *et al*., 1992; Higgins, 1992; Higgins, 1993) and perform transport functions across intracellular and/or plasma membranes, depending on their intracellular location. For example, ABC transporters are responsible for the uptake of nutrients, the transport of ions and peptides, the extrusion of noxious compounds, the secretion of toxins, or cell signaling. ABC transporters share a common bipartite structure, which is composed of a transmembrane domain (containing a set of multiple (most often six) membrane-spanning segments) and a hydrophilic nucleotide binding domain.

The primary structures of the transmembrane domains differ significantly among ABC transporters and have been recognized as the main determinants of the substrate specificity. The nucleotide binding folds on the other hand share an overall sequence identity of approximately 30%. They contain two core consensus motifs, known as 'Walker A motif' and 'Walker B motif' which are essential for ATP binding and are present in many other nucleotide binding proteins (Walker *et al*., 1982). ABC transporters contain two additional characteristic motifs: a dodecapeptide (L-S-G-G-$(X)_3$-R-hydrophobic-X-hydrophobic-A), known as 'linker dodecapeptide', that immediately precedes the Walker B motif, and another short stretch termed the 'center region' that lies approximately midway between the two Walker motifs (Shyamala *et al*., 1991). It has been hypothesized that these two sequences promote contact between transmembrane domains and nucleotide binding folds during ATP hydrolysis (Hyde *et al*., 1990; Mimura *et al*., 1991). The high conservation of the ATP binding cassettes in the rapidly growing list of discovered ABC transporters suggests that these membrane proteins may use similar mechanisms to execute their transport activities, or at least for transducing energy during the transport process. Thus, knowledge on the mechanism of action of P-glycoprotein as a representative of this large group of membrane transport proteins is of great interest and importance. Here we will review recent cell biological, molecular genetic and biochemical analyses that have contributed to our understanding of the domain organization, topology and higher order structure of P-glycoprotein, the location of the drug- and ATP binding sites within P-glycoprotein, its ATPase and drug transport activities, its possible functions as an ion channel, ATP channel and lipid transporter, its potential role in cholesterol biosynthesis, and the effects of phosphorylation on P-glycoprotein activity. The published information on these topics and additional important aspects of P-glycoprotein-mediated MDR is vast, so this review is necessarily selective. For additional information on biochemical, molecular, pharmacological and clinical aspects the reader is

referred to other recent reviews contributed by Gottesman *et al*. (1995; 1996), Kane (1996), Ling (1995), Childs and Ling (1994), Shustik *et al*. (1995), Borst and Schinkel (1997), Arias (1993), Leveille-Webster and Arias (1995), Skovsgaard *et al*. (1994), Lum *et al*. (1993), Bellamy (1996), and recent special issues of the Journal of Bioenergetics and Biomembranes (Volume 27, issue 1, February 1995), and the European Journal of Cancer (Volume 32A, issue 6, June 1996).

Domain Organization, Topology and Higher Order Structure of P-Glycoprotein

Mammalian P-glycoproteins are single chain, integral membrane proteins of an approximate length of 1280 amino acid residues (for reviews see e.g. Endicott and Ling, 1989; Gottesman and Pastan, 1993; Roninson, 1991). P-glycoproteins are composed of two homologous halves each of which consists of an N-terminal, hydrophobic, membrane-associated domain (approximately 250 amino acid residues) and a C-terminal, hydrophilic nucleotide binding fold (approximately 300 amino acid residues). The amino acid sequences deduced from the nucleotide sequences of a human *MDR*1 or a mouse *mdr*1 cDNA together with a hydropathy analysis yielded a working model for the topology of P-glycoprotein that is schematically illustrated in Figure 1 (Chen *et al*., 1986; Gros *et al*., 1986b). According to this model, the plasma membrane-associated domains in the two halves of P-glycoprotein each consist of six predicted TM segments which are connected by alternating extra- and intracellular loops. Both membrane spanning domains are followed by an intracellular nucleotide binding fold (Chen *et al*., 1986; Gros *et al*., 1986b).

As indicated in Figure 1, the primary structure of human P-glycoproteins also predicted three *N*-linked glycosylation sites to be located in the N-terminal half within the first extracellular loop (Chen *et al*., 1986). Two, three, or four such sites were also predicted at similar positions in other P-glycoproteins (Devault and Gros, 1990; Endicott *et al*., 1991; Gros *et al*., 1986b; Silverman *et al*., 1991; Van der Bliek *et al*., 1988). Using site-directed mutagenesis and deletion analysis, Schinkel *et al*. (1993) have confirmed Asn-91, Asn-94, and Asn-99 as glycosylation sites within the human*MDR*1 gene product. *In vitro* translation/microsomal translocation experiments with P-glycoprotein fragments and studies in *Xenopus* oocytes have suggested potential glycosylation sites in other regions, including the C-terminal half (Skach *et al*., 1993; Zhang and Ling, 1991; Zhang *et al*., 1993). However, none of these sites has been detected by *N*-glycanase (a mixture of peptide-*N*-glycosidase F and endoglycosidase F) treatment of wild-type P-glycoprotein, or of several mutants with deleted glycosylation sites in the first extracellular loop (Schinkel *et al*., 1993). Additional studies of P-glycoprotein domains expressed as individual polypeptides also have suggested that core-glycosylation only occurs in the N-terminal transmembrane domain (Loo and Clarke, 1995c).

The topology model of P-glycoprotein presented in Figure 1 represents a generally accepted working hypothesis, however, conflicting data have been reported on the number of membrane-spanning (or TM) regions present in each half, and on the orientation of a subset of TMs and their connecting loops relative to the plasma membranes. Early studies involving truncated P-glycoprotein polypetide chains that were translated *in vitro* and translocated into microsomes challenged the (six TM) plus (six TM) distribution of P-glycoprotein (Figure 1) and suggested an alternative topological model of P-glycoprotein containing only four TMs in each half (Zhang and Ling, 1991; Zhang *et al*., 1993). In these alternative topological models the predicted TMs 3, 5, 8, and 10 did not transverse the membranes (Zhang and Ling, 1991; Zhang *et al*., 1993). Another four TM configuration for the C-terminal half, with the predicted TMs 8 and 9 located externally, was proposed on the basis of expression studies in *Xenopus* oocytes involving truncated P-glycoprotein molecules fused to an epitope reporter, while a six TM configuration of the N-terminal half consistent with the model in Figure 1 was observed with this approach (Skach *et al*., 1993; Skach and Lingappa, 1993; Skach and Lingappa, 1994). Investigations of the membrane polarity of bacterially expressed P-glycoprotein-alkaline phosphatase (mdr-phoA) hybrids indicated a six TM configuration of the N-terminal half of P-glycoprotein, however, TM4 was postulated to be composed of amino acids 243-260 rather than 214-232 (Bibi and Béja, 1994). Moreover, the pho A fusion *E. coli* expression system suggested that membrane spanning in the C-terminal half was occurring in a different fashion from the model presented in Figure 1 and that the putative TM 7 may consist of two membrane-spanning regions which would cause asymmetric positioning of the C-terminal and N-terminal halves of P-glycoprotein (Béja and Bibi, 1995). Conversely, TM1 and TM7 were found to

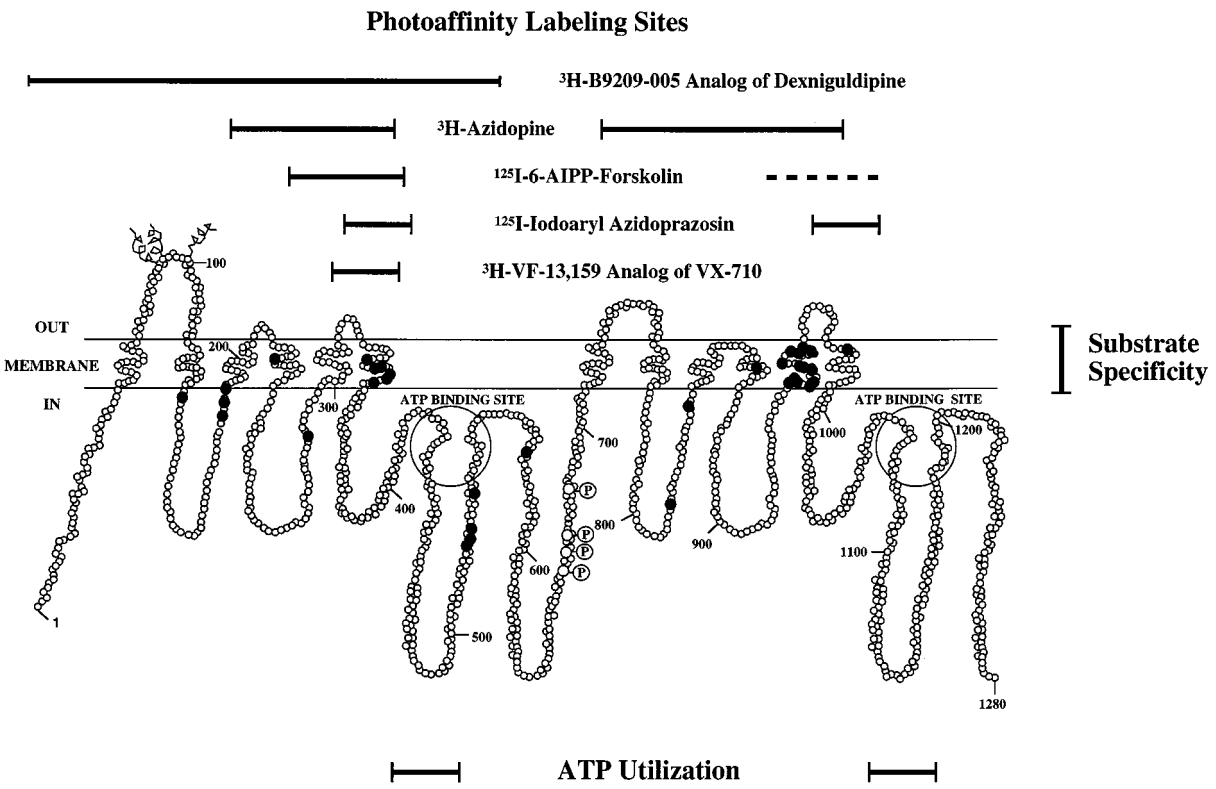

Figure 1. Schematic two-dimensional model of plasma membrane integral human *MDR*1 P-glycoprotein and its functional domains. A twelve-TM domain model was predicted by computer-assisted hydropathy profile analysis and amino acid sequence comparison of P-glycoprotein with bacterial transport proteins (Chen *et al*., 1986; Gros *et al*., 1986b). The N- and C-terminal ATP binding sites are circled and three putative *N*-linked carbohydrates are represented as wiggly lines. Phosphorylation sites are indicated as circled P. Amino acids which upon mutation alter the substrate specificity of the multidrug transporter are colored black. Heavy bars mark regions including photoaffinity labeling sites, membrane-associated segments involved in determining the substrate specificity of P-glycoprotein, and nucleotide binding folds involved in ATP utilization. Adapted from reference (Germann, 1993).

have identical topologies and similar structural and functional roles by an analysis of TM7/TM7 and TM1/TM1 chimeras (Loo and Clarke, 1996c). A study performed in intact mammalian cells using full-length, functional P-glycoprotein mutants with site-specific cysteine residues as reporters for externally and internally located regions (Loo and Clarke, 1995b) fully corroborated the twelve TM model presented in Figure 1. Similarly, extensive analysis of epitope-tagged, functional P-glycoprotein variants expressed in mammalian cells have provided strong support for the same (six TM) plus (six TM) distribution in P-glycoprotein (Kast *et al*., 1995; Kast *et al*., 1996).

Taken together, in all investigations of P-glycoprotein topology TMs 1, 2, 6, 7, 11 and 12 appear to be as predicted by the original computer-assisted hydropathy analysis, whereas discrepancies center around TMs 3–5 and 8–10. The reason for these discrepancies is unknown, but may be linked to the fact that truncated rather than full-length P-glycoprotein constructs were studied in the *in vitro* translation/translocation and heterologous expression systems, perhaps producing an incomplete picture since proper membrane insertion of native P-glycoprotein may depend on signals provided by both halves of the molecule. It has to be noted, however, that the alternate topologies observed in the *in vitro* translation/translocation, bacterial expression and *Xenopus* expression systems were detected only for a fraction of the expressed P-glycoprotein molecules. Another fraction proved to be consistent with (6 TM) plus (6 TM) model, thus, the data generated by these somewhat artificial systems are not entirely inconsistent with the topology observed for full-length P-glycoprotein expressed in mammalian cells.

Limited data from a subset of sequence-specific antibodies are in agreement with the predicted 12 TM model of P-glycoprotein shown in Figure 1. Antibod-

ies directed against the intracellular N- and C-terminus of P-glycoprotein, the intracellular N- and C-terminal nucleotide binding sites, and the cytoplasmic linker region which connects the two halves of P-glycoprotein, as well as antibodies recognizing the extracellular loops connecting TM 1 and TM 2, and TM 7 and TM 8, respectively, have corroborated the orientation of these regions relative to the plasma membranes (Cianfriglia *et al.*, 1994; Georges *et al*., 1990; Georges *et al*., 1993; Kartner *et al*., 1985; Yoshimura *et al*., 1989). Surprisingly, however, recent epitope mapping for three different monoclonal antibodies raised against viable MDR cells provided results that were consistent with an alternative 10 TM topology model for P-glycoprotein (Cianfriglia *et al*., 1996; Poloni *et al*., 1995). Moreover, polyclonal antibodies recognizing the predicted intracellular loops between TM 4 and TM 5, or TM 8 and TM 9, respectively, appeared to interact with extracellular binding sites, also challenging the 12 TM concept of the membrane topology for P-glycoprotein (Zhang *et al*., 1996).

Zhang *et al.* (1993; 1996) speculated that alternate topological forms of P-glycoprotein may be expressed in different cell types and that different topological forms of P-glycoprotein may exhibit different biological functions. As will be discussed later, P-glycoprotein has indeed been associated with multiple functional phenotypes in mammalian cells, but no clear topological structure-function relationship has been established. Further detailed analyses of P-glycoproteins in mammalian cells are required to evaluate whether P-glycoprotein exhibits more than one topological orientation in the endoplasmic reticulum, and if so, whether this is influenced by high levels of expression. Additional investigations also need to address whether P-glycoprotein molecules with different topological structures may be targeted to different cell compartments, and whether alteration of the topology may result in a change of functional activity. Also the factors responsible for P-glycoprotein folding will have to be identified.

As reviewed by Levy (1996), membrane protein folding patterns are assigned to several different classes, depending on the topogenic sequences which regulate the insertion of membrane protein into the endoplasmic reticulum, and depending on the orientation of the membrane protein. Signal recognition particle (SRP) docking protein-dependent signal sequences have been detected in both halves of P-glycoprotein, and it appears that not only the first inserted TM segment is critical, but internal TMs also provide independent topogenic information (Skach *et al*., 1993; Skach and Lingappa, 1993; Skach and Lingappa, 1994; Zhang and Ling, 1991; Zhang *et al*., 1995a). Charged amino acids surrounding the predicted TM4 have also been found to be involved in regulating the membrane orientation of the N-terminal half of P-glycoprotein (Zhang *et al*., 1995a). Besides intragenic signal sequences, cytoplasmic factors have been suggested to modulate the regulation of topological P-glycoprotein structures, although these factors remain to be identified in various cell types and/or expression systems (Zhang and Ling, 1995).

The higher order structure of P-glycoprotein has also not been determined unequivocally. Initially assumed to function as a monomer, several studies have suggested the existence of P-glycoprotein dimers (and possibly oligomers) in MDR cells. Indirect evidence for P-glycoprotein dimers in the plasma membranes has been obtained by radiation inactivation and freeze fracture experiments (Arsenault *et al*., 1988; Boscoboinik *et al*., 1990; Weinstein *et al*., 1990; Wright *et al*., 1985), and in chemical crosslinking studies (Naito and Tsuruo, 1992). More recently, a velocity sedimentation analysis of radiolabeled P-glycoprotein isolated from postnuclear lysates has suggested that a large fraction of P-glycoprotein exists in dimeric and oligomeric complexes in the endoplasmic reticulum (Poruchynsky and Ling, 1994).

Taken together, these studies raise the possibility that P-glycoprotein monomers, dimers and homooligomeric complexes may exist. However, it is unknown whether there are functional differences between monomers, dimers, and oligomeric complexes. Also the dynamics of the oligomerization process remain to be elucidated. *In vitro* studies with bacterially expressed P-glycoprotein fragments have suggested that the cytoplasmic linker region of mouse *mdr*1b P-glycoprotein may serve as potential dimerization domain (Juvvadi *et al*., 1997), however, no support for this hypothesis has thus far been provided by studies of full-length P-glycoprotein expressed in mammalian cells. Co-expression and co-purification studies performed with two different epitope-tagged P-glycoprotein variants in HEK 293 cells revealed no association between *MDR*1 polypetides, and it was concluded that a monomer represents the minimal functional unit of P-glycoprotein (Loo and Clarke, 1996b).

Table 2. Partial list of agents which reverse P-glycoprotein-mediated MDR

Calcium channel blockers	*Vinca* alkaloid analogs
Verapamil	Vindoline
Nifedipine	Thaliblastine
Diltiazem	
Azidopine	Anthracycline analogs
Dexniguldipine	*N*-Acetyl daunorubicin
Gallopamil	
Ro11-2933	Antibiotics
PAK-200	Cephalosporins
	Bafilomycin
Calmodulin antagonists	Erythromycin
Trifluoperazine	
Chlorpromazine	Antiarrhytmics
Fluphenazine	Quinidine
Trans-Flupenthixol	Amiodarone
Protein kinase C inhibitors	Antihypertensives
Calphostine C	Reserpine
Staurosporine	
Safingol	Antihistamines
CPG 41251	Terfenadine
NPC 15437	
GF109203X	Antimalarials
	Quinine
Steroidal agents	Chloroquine
Steroid hormones	
Progesterone	Antidepressants
Cortisol	Tioperidone
Modified steroids	Miscellaneous
Torimefene	VX-710
Tamoxifen	VX-853
Megestrol acetate	S 9788
	GF120918
Cyclic peptides	MS-209
Immunosuppressants	PAK-104P
Rapamycin	Ly335979
FK506	BIBW 22
Cyclosporin A	
Others	
SDZ PSC 833	

Characterization of Drug Binding Sites in P-Glycoprotein

Overexpression of class 1 P-glycoproteins causes cancer cells to become resistant to a great variety of structurally and functionally dissimilar anti-cancer drugs (e.g. vinblastine, vincristine, doxorubicin, daunorubicin, etoposide, teniposide, and paclitaxel), as well as many other cytotoxic agents (Table 1). This points out the most intriguing feature of P-glycoprotein: its apparent promiscuity as a transporter. Binding and transport of a particular drug substrate by P-glycoprotein can be inhibited by another drug substrate that is part of the MDR phenotype, and by agents that reverse MDR in cultured cells. During the past two decades, an enormous number of compounds have been identified which interact with P-glycoprotein directly and block its multidrug transporter activity (Table 2) (Ford, 1996; Tsuruo *et al*., 1981; Tsuruo *et al*., 1982). Like anti-cancer drug substrates of P-glycoprotein, these MDR modulators only share broad structural similarities, but all are amphiphilic, and many are heterocyclic and positively charged. Most chemosensitizers described to date can be classified into several broadly defined groups. These include (1) calcium channel blockers, (2) calmodulin antagonists, (3) protein kinase C inhibitors, (4) steroids and hormonal analogs, (5) cyclic peptides, including immunosuppressants, (6) *Vinca* alkaloid analogs, (7) anthracycline analogs, and (8) other agents including antibiotics, antiarrhytmics, antihypertensives, antihistamines, antimalarials, antidepressants, as well as several novel MDR reversing compounds (Ford, 1996). All these agents antagonize MDR by altering the drug accumulation defect in MDR cells, and they show little or no effects in drug-sensitive cells. Some chemosensitizers (e.g. verapamil, cyclosporin A, azidopine, diltiazem, and FK-506) are substrates for P-glycoprotein-mediated transport (Naito *et al*., 1993; Saeki *et al*., 1993a; Tamai and Safa, 1991; Yusa and Tsuruo, 1989), whereas others act as non-substrate inhibitors (e.g. progesterone and nitrendipine), possibly by interfering with drug substrate binding directly, or via allosteric changes (Saeki *et al*., 1993b; Ueda *et al*., 1992). While only a few of the MDR reversing agents listed in Table 2 possess appropriate pharmacological properties for use as potential modulators of clinical MDR, all these compounds (as well as photoaffinity analogs thereof) have been valuable tools for *in vitro* structure-function analyses of the multidrug transporter. MDR

modulators have contributed tremendously to our understanding of drug interactions by P-glycoprotein.

The identification of protein domains and/or amino acid residues that play a role in drug binding and drug transport by P-glycoprotein is a necessary prerequisite to elucidate the structure, function, and mechanisms of action of the multidrug transporter. In particular, it is important to define the number, location, and nature of the drug interaction sites, and to investigate whether drug interaction sites are present in both halves of P-glycoprotein, and if so, whether the two halves of P-glycoprotein functionally cooperate, or whether they act independently of each other. We will discuss two major approaches that have been used to define drug interaction sites in order to address these questions: (1) the use of drugs and modulators in binding and photoaffinity labeling studies and (2) the analysis of mutants of P-glycoprotein. Additional approaches include structure-activity relationship analyses of drug and/or modulator analogs that may eventually lead to the definition of a common 'MDR pharmacophore' (Ford, 1996).

Cornwell *et al.* were the first to observe that radioactively labeled cytotoxic drugs (e.g. vinblastine) and MDR reversing agents (e.g. verapamil) specifically interacted with P-glycoprotein expressed at the cell surface of MDR, but not drug-sensitive cells (Cornwell *et al.*, 1986a). Subsequently, specific crosslinking of a radioactively labeled, photoactive vinblastine analog (^{125}I-NASV) to P-glycoprotein upon UV-exposure was demonstrated (Cornwell *et al.*, 1986b; Safa *et al.*, 1986). Since then, many different photoaffinity reagents that label P-glycoprotein specifically have been found (reviewed in Beck and Qian, 1992; Safa, 1993). These include derivatives of the cytotoxics vinblastine (Cornwell *et al.*, 1986b; Safa *et al.*, 1986), colchicine (Safa *et al.*, 1989; Safa *et al.*, 1990), daunomycin (Busche *et al.*, 1989; Demmer *et al.*, 1996), paclitaxel (Ojima *et al.*, 1995), as well as several MDR modulators including azidopine (Akiyama *et al.*, 1988; Bruggemann *et al.*, 1992; Hu *et al.*, 1996; Bruggemann *et al.*, 1989; Greenberger *et al.*, 1990; Safa *et al.*, 1987; Yoshimura *et al.*, 1989), derivatives of verapamil (Safa, 1988), BZDC-dihydropyridine (Boer *et al.*, 1996a), forskolin (Morris *et al.*, 1991), prazosin (Greenberger *et al.*, 1991), tamoxifen (Safa *et al.*, 1994), dexniguldipine (Boer *et al.*, 1996b; Borchers *et al.*, 1995), cyclosporin A (Demeule *et al.*, 1997), and VX-710 (Germann *et al.*, 1997).

A common strategy to evaluate cytotoxic drugs and/or MDR modulators for their ability to interact with P-glycoprotein directly involves assessment of their ability to inhibit P-glycoprotein labeling with a radioactive photoaffinity analog (reviewed by Safa, 1993). Most often the commercially available phototoprobes ^{3}H-azidopine or ^{125}I-iodoarylazido prazosin have been used in these types of experiments, although, in principle, all of the photoaffinity analogs mentioned above can be applied. Generally, inhibition of P-glycoprotein photoaffinity labeling has proven to be a rapid and reliable approach for demonstrating that a compound interacts with P-glycoprotein directly. However, photoaffinity labeling experiments are not generally useful for determining the potency of MDR reversing agents, or for measuring their affinity to P-glycoprotein. Studies in which the relative efficiency of a set of compounds for inhibiting P-glycoprotein labeling was correlated with their relative potency as MDR reversing agent *in vitro* (e.g. in proliferation or drug accumulation assays) revealed discrepancies between these methodologies (Hu *et al.*, 1996). Moreover, it was found that some MDR cytotoxics, e.g. colchicine, were ineffective at inhibiting P-glycoprotein photoaffinity labeling, even at very high concentrations. Based on these data it was proposed that more than one drug binding site was present in P-glycoprotein (reviewed by Safa, 1993). It was hypothesized that different cytotoxic drugs, for example vinblastine and colchicine, may bind to separate, possibly overlapping or allosterically coupled sites. Alternatively, it was speculated that P-glycoprotein contains a unique drug-acceptor site common for all agents, but displaying altered affinity for different molecules.

Results of several studies in which drug interactions with P-glycoprotein were quantitated tend to favor a multisite binding model. Ferry *et al.* described equilibrium binding and dissociation kinetic experiments which indicated the presence of a binding site within P-glycoprotein that is selective for 1,4-dihydropyridines (including the MDR modulators azidopine, nifedipine, dexniguldipine), but is allosterically coupled to a distinct, non-equivalent binding site that is selective for *Vinca* alkaloids (Ferry *et al.*, 1995; Ferry *et al.*, 1992). Additional studies on the kinetics of ^{3}H-vinblastine dissociation from P-glycoprotein revealed that doxorubicin, etoposide and cyclosporin A also bind to the same drug acceptor site as *Vinca* alkaloids (Malkhandi *et al.*, 1994). Cyclic peptides and the MDR modulator S9788 bind to a site separate from the 1,4-dihydropyridine binding site based on

photoincorporation experiments (Boer *et al*., 1996b). A recent study investigating the effects of individual and pairs of MDR cycotoxics and/or modulators on daunomycin accumulation in MDR cells also suggested the presence of at least two binding/modulatory sites within P-glycoprotein: one of them preferred by vinblastine, tamoxifen, and mefloquine, another preferred by verapamil, dipyridamole, trifluoroperazine and quinidine, whereas cyclosporin A appeared to interact with both (Ayesh *et al*., 1996). Evidence for multiple receptor sites was also provided indirectly by P-glycoprotein ATPase assays. Modulation of the P-glycoprotein ATPase by vinblastine and verapamil was found to be mutually exclusive, whereas ATPase modulation by vinblastine and progesterone, or verapamil and progesterone was non-competitive (Garrigos *et al*., 1997; Orlowski *et al*., 1996). These results were interpreted with the existence of distinct, but overlapping or interacting binding sites for vinblastine and verapamil, which are separate from progesterone binding sites. Another study investigating the effects of various cytotoxic drug substrates (vinblastine, valinomycin, dolestatin 10, emetine), chemosensitizers (verapamil, progesterone), and peptides on gramicidin D inhibition of the P-glycoprotein ATPase activity, however, provided conflicting data and suggested that all these agents interact with a common binding site in P-glycoprotein (Borgnia *et al*., 1996).

Attempts have been made to directly map putative drug binding sites within the primary structure of P-glycoprotein using radioactive photoaffinity labeling probes. Generally, P-glycoprotein at the surface of viable cells or in plasma membrane preparations was photolabeled, purified, and subjected to enzymatic or chemical degradation. Subsequently, the radioactive, photolabeled fragments were identified by use of P-glycoprotein-specific antibodies with known epitopes. Such studies have been performed for P-glycoprotein labeled with ^{3}H-azidopine (Bruggemann *et al*., 1989; Yoshimura *et al*., 1989), ^{125}I-iodoaryl azidoprazosin (Greenberger, 1993; Greenberger *et al*., 1991; Greenberger *et al*., 1990), 6-O-[[2-[3-(4-azido-3-^{125}I-iodophenyl)propionam ido]ethyl]carbamyl]-forskolin (^{125}I-6-AIPP-forskolin) (Morris *et al*., 1994), an azido derivative of dexniguldipine, ^{3}H-B9209-005 (Borchers *et al*., 1995), and an azido derivative of VX-710, ^{3}H-VF-13,159 (Germann *et al*., 1997; and UA Germann, manuscript in preparation). Some of these studies have suggested that both halves of P-glycoprotein may participate in drug binding. For ^{125}I-iodoaryl azidoprazosin, a major labeling site has been identified within each half of the mouse *mdr*1b P-glycoprotein: one in the N-terminal half in a segment near or within TM6, and the other at an analogous position (near or within TM12) in the C-terminal half of P-glycoprotein (Greenberger, 1993) (Figure 1). Interestingly, ^{3}H-azidopine and ^{125}I-6-AIPP-forskolin photolabeled related and overlapping, if not identical, regions within both halves of human P-glycoprotein (Bruggemann *et al*., 1989; Morris *et al*., 1994) (Figure 1). Two different interpretations of these results are possible: (1) there are two different drug binding sites, one per half of P-glycoprotein, or (2) regions from both halves, in particular TM6 and TM12, come together to form a single drug binding site. The latter hypothesis appears more likely since two other photoaffinity reagents, ^{3}H-B9209-005 (Borchers *et al*., 1995) and ^{3}H-VF-13,159 (UA Germann, manuscript in preparation), label only a single major site in the N-terminal half of P-glycoprotein, although they act as modulators for the whole spectrum of MDR cytotoxics. For ^{3}H-VF-13,159 this major labeling site is present in a region encompassing TM5 and TM6, and the extracellular loop in between, thus, it is confined to a region which is similar to one of the ^{125}I-iodoaryl azidoprazosin, ^{3}H-azidopine and ^{125}I-6-AIPP-forskolin labeling sites (UA Germann, manuscript in preparation). Additionally, ^{3}H-azidopine has been demonstrated to equally label both halves of human P-glycoprotein, and vinblastine was found to equally decrease labeling of the two ^{3}H-azidopine sites within P-glycoprotein (Bruggemann *et al*., 1992), supporting the hypothesis that amino acid residues from both the N- and C-terminal halves of P-glycoprotein may interact and cooperate to form one major drug interaction pore. The findings that both halves are necessary for drug stimulation of the ATPase activity of P-glycoprotein (Loo and Clarke, 1994c), and that associations between the two membrane spanning domains contribute to interactions between the two halves of P-glycoprotein (Loo and Clarke, 1995c), are also consistent with this hypothesis. Moreover, an oxidative cross-linking analysis of 42 mutants derived from a Cys-less P-glycoprotein variant by reintroducing a pair of Cys residues, one Cys within TM6 (residues 332–3380 and one Cys within TM12 (residues 975–980), suggested that residues Cys332 and Cys975 are close to each other in the tertiary structure of P-glycoprotein (Loo and Clarke, 1996a).

Although these results suggest that a common drug interaction pore, formed in part by TM6 and TM12,

may be present within P-glycoprotein, no evidence has thus far been provided that such a large drug pore would reflect a single drug binding site. Rather, this region may constitute a large three-dimensional structural pocket within P-glycoprotein, with multiple sites for initial binding of drugs and perhaps also multiple sites from which drugs are released again. In such a model a large drug interaction pore would represent a passageway for drugs during the transport process which would allow different classes of drugs to bind to different, possibly allosterically coupled regions within P-glycoprotein, in agreement with drug binding studies summarized above. As will be discussed later, a low resolution structural analysis of P-glycoprotein suggested indeed the presence of large pore with 5 and 2.5 nm openings at the extracellular and cytoplasmic side of the plasma membranes, respectively (Rosenberg *et al*., 1997).

The idea that both halves of P-glycoprotein contribute to MDR substrate and modulator binding specificity is also supported by structure-function analyses of naturally occurring and designed P-glycoprotein mutants. These studies have indicated the presence of critical determinants for drug substrate specificity within or near TM regions. An initial analysis of insertion and deletion mutants of P-glycoprotein suggested that large alterations in the primary structure either reduced expression of P-glycoprotein or abrogated its ability to confer MDR, and that both halves of P-glycoprotein need to be intact for proper functional activity (Currier *et al*., 1989). Further information has been gained from the analysis of hybrids between different *mdr* gene products. Hybrid strategies were devised when it became evident that only class 1, but not class 2, P-glycoproteins are able to confer the MDR phenotype to drug-sensitive cells (Buschman *et al*., 1992; Schinkel *et al*., 1991), and when different P-glycoprotein isoforms, for example the human *MDR*1, the mouse *mdr*1a, and the mouse *mdr*1b gene products, were found to confer overlapping but distinct patterns of resistance to anti-cancer drugs (Devault and Gros, 1990; Tang-Wai *et al*., 1995). Studies with hybrids between mouse *mdr*1 and *mdr*2 (Buschman and Gros, 1991), mouse *mdr*1 and *mdr*3 (Dhir and Gros, 1992), and human *MDR*1 and *MDR*2 (Currier *et al*., 1992; Zhang *et al*., 1995b) have revealed that drug binding sites reside within or in close proximity of membrane-associated regions, and that drug substrate specificity is achieved by complex interactions of structural elements in both homologous halves. Additional insight has been gained from the functional analysis of a multitude of P-glycoprotein mutants containing single or multiple amino acid alterations, some of which will be discussed in the following.

In agreement with the photoaffinity labeling data described above, molecular genetic studies have also underscored the importance of the TM5-TM6 and TM11-TM12 regions for drug interactions. Chinese hamster ovary (CHO) cells that appeared preferentially resistant to actinomycin D expressed a P-glycoprotein double mutant carrying Gly338→Ala and Ala339→Pro substitutions in TM6 (Devine *et al*., 1992). In a subsequent study, these mutations were also found to impair the ability of P-glycoprotein to confer colchicine and daunorubicin resistance (Devine and Melera, 1994). The substrate specificity of human P-glycoprotein was affected by Phe335→Ala or Phe335→Ser substitutions in TM6 which decreased its ability to confer resistance to vinblastine or actinomycin D, while the ability to confer resistance to adriamycin and colchicine was retained (Loo and Clarke, 1993a). Similar results were obtained by a ΔPhe335 deletion mutant that was selected by growing human sarcoma cells in the presence of doxorubicin and PSC 833 (Chen *et al*., 1997). This mutant protein conferred reduced resistance to *Vinca* alkaloids and no resistance to dactinomycin, whereas its ability to confer doxorubicin resistance and paclitaxel resistance was unchanged. This ΔPhe335 mutant also exhibited decreased susceptibility to modulation by PSC 833 and cyclosporin A and transported cyclosporin A less effectively. A human P-glycoprotein mutant carrying an Val338→Ala mutation in TM6 also conferred decreased resistance to vinblastine, but additionally conferred increased resistance to colchicine (Loo and Clarke, 1994b). In contrast, a Gly341→Val mutation in TM6 reduced the ability to confer colchicine and doxorubicin resistance (Loo and Clarke, 1994b). An Ala342→Leu mutation in TM6 caused a more general effect, impairing the ability of human P-glycoprotein to confer resistance to multiple cytotoxics including doxorubicin, vinblastine, colchicine, and actinomycin D (Loo and Clarke, 1994b). Even more dramatically, Ser344→Ala, Ser344→Thr or Ser344→Cys mutations in TM6 completely abrogated the ability of human P-glycoprotein to confer drug resistance and all the Ser344 mutants exhibited reduced drug-stimulated ATPase activity when compared with wild-type enzyme (Loo and Clarke, 1994b).

In the C-terminal half, a TM11 Ser941→Phe mutation in mouse *mdr*1 P-glycoprotein and a TM11 Ser939→Phe mutation in mouse *mdr*3 P-glycoprotein

decreased their ability to confer colchicine and adriamycin resistance (Gros *et al*., 1991) and also reduced their susceptibility to modulation by several MDR reversing agents including verapamil, progesterone, and cyclosporin A (Kajiji *et al*., 1993). Moreover, these TM11 mutants exhibited reduced binding capacity for ^{3}H-azidopine and ^{125}I-iodoaryl azidoprazosin when compared with wild-type P-glycoprotein (Kajiji *et al*., 1994). A human P-glycoprotein mutant carrying a Phe978→Ala or Phe978→Ser mutation in TM12 conferred decreased resistance to vinblastine and actinomycin D, and no detectable resistance to colchicine and adriamycin (Loo and Clarke, 1993a). Another *MDR*1 mutant containing *MDR*2 substitutions in the TM12 segment was only poorly labeled with ^{125}I-iodoaryl azidoprazosin and conferred decreased resistance to vincristine, doxorubicin, and actinomycin D, but not colchicine (Zhang *et al*., 1995b). Moreover, an *MDR*1 mutant in which the intervening loop between TM11 and TM12 was replaced with appropriate *MDR*2 sequences conferred enhanced resistance to doxorubicin, actinomycin D, and colchicine, but not to vincristine (Zhang *et al*., 1995b).

Taken together, these studies suggest that several key amino acid residues located within the TM5-TM6 and TM11-TM12 segments contribute to the drug substrate specificity of P-glycoprotein. Recently, a more systematic analysis of mouse *mdr*3 P-glycoprotein has been carried out in which each of the 21 amino acid residues in TM11 was independently mutated to Ala, or to Gly in the case of endogenous Ala residues (Hanna *et al*., 1996). This study revealed that 13 out of 21 TM11 mutants conferred decreased resistance to at least one of the four drugs vinblastine, adriamycin, colchicine or actinomycin D that were analyzed. The aromatic residues Tyr949 and Tyr953, which are highly conserved in TM5 as well, were found to be the most mutation-sensitive sites (Hanna *et al*., 1996). Clusters of deleterious mutations and neutral mutations appeared to alternate in the primary structure. Projection of TM11 in a alpha-helical configuration together with an analysis of the distribution of the deleterious mutations indicated that the more hydrophilic face of the amphipathic helix was mutation-sensitive, whereas the more hydrophobic face seemed mutation-insensitive. Thus, it was concluded that the more hydrophilic face of TM11 may be involved in drug recognition, binding, and transport (Hanna *et al*., 1996).

Amino acids residues in membrane-spanning segments other than TM5-TM6 and TM11-TM12, or in close proximity thereof, may also play an important structural or functional role in drug recognition and transport by P-glycoprotein. Site-directed mutagenesis of thirteen proline residues, five of which are confined to TM regions according to the model presented in Figure 1, implicated two of these, Pro223 in TM4 and Pro866 in TM10, as critical determinants for the drug resistance profile conferred by human P-glycoprotein (Loo and Clarke, 1993b). A Pro223→Ala and a Pro866→Ala mutation each impaired the ability of human P-glycoprotein to confer resistance to doxorubicin, colchicine, and actinomycin D, whereas the ability to confer vinblastine resistance was enhanced in the Pro223→Ala mutant and remained unchanged in the Pro866→Ala mutant (Loo and Clarke, 1993b).

Another functionally significant and well-characterized mutation is located near TM3 in human P-glycoprotein and involves a Gly185→Val amino acid change. This Gly185→Val mutant has been associated with decreased resistance to vinblastine, vincristine, actinomycin D, doxorubicin, and taxol, and increased resistance to colchicine and etoposide (Choi *et al*., 1989; Kioka *et al*., 1989; Safa *et al*., 1990). Photoaffinity labeling experiments using analogs of *Vinca* alkaloids and colchicine indicated increased vinblastine and decreased colchicine labeling. Furthermore, it was suggested that a decrease in the rate of drug dissociation rather than an alteration of the initial binding reaction was the cause for reduced vinblastine transport by this mutant protein (Safa *et al*., 1990). Further characterization of this mutant indicated that the Gly185→Val substitution conferred pleiotropic alterations on P-glycoprotein, including an altered basal ATPase activity and altered interactions with drug substrates and MDR modulators (Cardarelli *et al*., 1995; Müller *et al*., 1996; Ramachandra *et al*., 1996; Rao, 1995). Interestingly, an additional Asn183→Ser mutation near the Gly185→Val mutation resulted in recovery of the decreased resistance to actinomycin D, vinblastine, and doxorubicin, whereas resistance to colchicine was still increased (Currier *et al*., 1992). In another study, Gly→Val mutations were also introduced at position 141 near TM2, at position 187 near TM3, at position 288 near TM5, at position 812 between TM8 and TM9, and at position 830 near TM 9, and all the resulting P-glycoprotein mutants exhibited enhanced resistance to colchicine and doxorubicin (Loo and Clarke, 1994a). The Gly→Val mutations at positions 187, 288, and 830 also lowered resistance to actinomycin D (Loo and Clarke, 1994a).

Mutations in the ATP binding fold near the

'Walker B motif' in the N-terminal half also alter the cross resistance profile conferred by P-glycoprotein. A Lys536→Gln substitution within human *MDR*1 yielded a P-glycoprotein mutant conferring slightly reduced resistance to vinblastine and significantly lowered resistance to doxorubicin and colchicine (Hoof *et al*., 1994). A P-glycoprotein mutant with a Lys536→Arg substitution, however, conferred unchanged vinblastine resistance, but increased resistance to doxorubicin and colchicine (Hoof *et al*., 1994). The replacement of discrete segments within the N-terminal nucleotide binding fold of mouse *mdr*3 P-glycoprotein by homologous regions of the C-terminal nucleotide binding fold indicated Glu522, Arg523, Ala525 and Thr578 to be mutation-sensitive (Beaudet and Gros, 1995). Glu522→Asp, Arg523→Lys, Ala525→Thr and/or Thr578→Cys substitutions yielded P-glycoprotein mutants with profoundly decreased ability to confer resistance to colchicine, doxorubicin, and actinomycin D, whereas the ability to confer vinblastine resistance was retained (Beaudet and Gros, 1995). Interestingly, most of these mutation sites are confined to the 'linker peptide' which is believed to promote contact between the nucleotide binding fold and the membrane spanning domain(s) during the drug transport process (Hyde *et al*., 1990; Mimura *et al*., 1991). Hence, it has been suggested that amino acid residues Glu522, Arg523, Ala525 and Thr578 may be directly involved in interactions between the ATP binding fold(s) and discrete TM domains upon drug and/or ATP binding, or upon ATP hydrolysis (Beaudet and Gros, 1995). Observations that associations between the N-terminal ATP binding fold and the C-terminal membrane spanning domain, between the C-terminal ATP binding fold and the N-terminal membrane spanning domain, between the two ATP binding folds themselves, and between the two membrane spanning domains themselves contribute to interactions between the two halves of P-glycoprotein are in agreement with this hypothesis (Loo and Clarke, 1995c).

Taken together, as summarized in Figure 1, molecular genetic data are generally consistent with the physical mapping of drug binding sites to both halves of P-glycoprotein. Mutations in each half of P-glycoprotein can alter the drug resistance profile and TM5, TM6, TM11, and TM12 appear to be the most critical regions for substrate interactions. Similar to the biochemical data, the molecular genetic data also suggest that different drugs interact with different sites within P-glycoprotein. Often mutations affect the relative resistance against vinblastine and colchicine in an opposite manner, i.e. one is increased and the other decreased (sometimes unchanged), and *vice versa*. Although the amino acid residues which have been identified as important determinants of the drug substrate specificity are scattered throughout the primary structure of P-glycoprotein, they usually reside within or near membrane-spanning segments. However, it is unknown at present, which of these amino acid residues are functionally important and are in direct contact with drug and/or modulator substrate(s), and which ones are structurally important and act indirectly, for example by contributing to conformational changes upon binding of drug and/or ATP, or upon ATP hydrolysis.

Characterization of ATP Binding Sites in P-Glycoprotein

Early drug uptake experiments performed with MDR cells revealed that P-glycoprotein requires energy in the form of ATP to execute its drug transport function. Energy depletion of drug-selected cells by poisons of mitochondrial respiration (e.g. azide) lead to an increase in drug accumulation, and restoration of ATP levels decreased drug accumulation levels again (Danø, 1973). By use of radioactive, photoactivatable analogs of ATP, ^{32}P-8-azido-ATP (Cornwell *et al*., 1991; Cornwell *et al*., 1987; Sarkadi *et al*., 1992; Schurr *et al*., 1989) and ^{32}P-2-azido-ATP (Al-Shawi and Senior, 1993) P-glycoprotein was identified as an ATP-binding protein. An excess of ATP, GTP, AMP-PNP, but not ADP, ribose-5-phosphate, nor drug substrates inhibited ^{32}P-8-azido-ATP-labeling of P-glycoprotein (Cornwell *et al*., 1987), suggesting that P-glycoprotein contains specific nucleotide binding region(s) that are different from the drug binding sites.

As detailed in a previous section, P-glycoprotein, as a member of the ABC superfamily of transporters, harbors two nucleotide binding folds which contain a 'Walker A motif', a 'Walker B motif' (Walker *et al*., 1982) , a 'center region', and a 'linker dodecapeptide' (Shyamala *et al*., 1991). Discrete mutations of highly conserved residues in the 'Walker A motif' in either the N- or C-terminal half of the mouse *mdr*1b gene product completely abrogated its ability to confer MDR, implying that both nucleotide binding folds may interact and/or cooperate for the P-glycoprotein drug transport function (Azzaria *et al*., 1989). These Gly431→Ala, Gly1073→Ala,

Lys432→Arg or Ly1074→Arg substitutions, even when combined and introduced into the same molecule, did not interfere with ^{32}P-8-azido-ATP labeling, suggesting that a step subsequent to ATP binding in the drug transport process was impaired (Azzaria *et al*., 1989). Consistent with this idea, similar P-glycoprotein mutants containing Lys433→Met and/or Lys1076→Met substitutions partially retained ^{32}P-8-azido-ATP binding ability, but lacked drug-stimulated ATPase activity (Müller *et al*., 1996).

Bacterial overexpression, purification and subsequent biochemical characterization has been applied to both the N- and C-terminal ATP binding domains. Both ATP binding folds of P-glycoprotein were shown to efficiently bind ATP and ATP derivatives (Baubichon-Cortay *et al.*, 1994; Dayan *et al.*, 1996; Sharma and Rose, 1995). The precise amino acids that are involved in ATP-binding, however, have not yet been identified by biochemical means. Recent fluorescence studies suggest that the two highly conserved Cys residues in the Walker A motifs are located close to each other within the catalytic site of P-glycoprotein (Liu and Sharom, 1996; Liu and Sharom, 1997) and, as will be discussed in the following sections, both the N- and C-terminal ATP binding domains are essential for the ATPase and drug efflux activity of P-glycoprotein (Azzaria *et al*., 1989; Loo and Clarke, 1995b).

Characterization of ATPase Activity of P-Glycoprotein

Studies with plasma membrane vesicles from MDR-expressing cell lines demonstrated that P-glycoprotein-mediated drug transport required a constant source of energy in the form of ATP (or GTP; Lelong *et al*., 1992), that non-hydrolyzable analogs could not support transport, and that the ATPase inhibitor vanadate inhibited drug transport (Horio *et al*., 1988). These results suggested that drug transport required an ATPase activity, and the first biochemical evidence supporting this idea was provided by Hamada and Tsuruo (Hamada and Tsuruo, 1988a; Hamada and Tsuruo, 1988b). They partially purified P-glycoprotein from K562/ADM cells by membrane extraction with the detergent CHAPS and immunoaffinity chromatography and demonstrated an intrinsic ATPase activity. The low specific activity of their preparation (1–3 nmol ATP hydrolyzed min^{-1} mg^{-1} of protein) was probably due in part to the presence of inhibitory amounts of CHAPS. They nevertheless established several enzymatic properties of the ATPase activity to set the stage for further investigation. Subsequently, Sarkadi *et al*. (1992) expressed P-glycoprotein in Sf9 insect cells via a recombinant baculovirus and characterized its ATPase activity in a light membrane fraction where P-glycoprotein was estimated to represent about 3% of the protein. Significantly, the ATPase activity was stimulated 2- to 5-fold by P-glycoprotein substrates (vinblastine, vincristine, colchicine, daunomycin) or P-glycoprotein transport inhibitors (verapamil, trifluoperazine), with a maximal drug-stimulated, P-glycoprotein-specific ATPase activity of 3–5 μmol min^{-1} mg^{-1}. This relatively high specific activity is comparable to that of other transport ATPases, and could account for the large consumption of ATP characteristic of MDR cell lines. P-glycoprotein-specific ATPase activity has also been demonstrated in membranes from the highly multidrug-resistant CR1R12 CHO cell line, in which P-glycoprotein was estimated to represent up to 32% by weight of the membrane protein (Al-Shawi and Senior, 1993). The K_m for ATP was reported as 1.4 mM and the ATPase specific activity was in the range 1-2 μmol min^{-1} mg^{-1}, depending on specific assay conditions, with a 5-fold increase to about 9 μmol min^{-1} mg^{-1} in the presence of verapamil.

The development of methods for the purification of P-glycoprotein from mammalian MDR cell lines, together with methods for functional reconstitution, has enabled more detailed characterization of its ATPase activity. Table 3 shows a summary of the methods used to purify P-glycoprotein from different MDR cell lines and the ATPase characteristics of these preparations. The highly purified P-glycoprotein preparations exhibit a high K_m for ATP and a high capacity for ATP hydrolysis. One of the features of P-glycoprotein ATPase activity is stimulation by MDR drugs and chemosensitizers. For example, after reconstitution in a mixture of phospholipids, the ATPase activity of the preparation from KB-V1 cells was stimulated 3- to 4-fold by verapamil, vinblastine or doxorubicin (Ambudkar *et al*., 1992). After reconstitution into phospholipid bilayers formed from L-α-phosphatidylethanolamine, Shapiro and Ling (1994) reported a 10-fold stimulation of P-glycoprotein ATPase by verapamil, and strong stimulation (4.8 to 6.6-fold) was also observed with vinblastine, trifluoperazine and amiodarone. However, the substrates colchicine, daunomycin and actinomycin D showed only poor stimulation of P-glycoprotein

Table 3. ATPase Characteristics of Purified P-glycoprotein Preparations[a]

Method	Cell line	Purity	ATPase activity (μmol min^{-1} mg^{-1})	K_m for ATP (mM)	Reference
1. Octyl β-D-glucopyranoside extraction/DEAE-cellulose chromatography	KB-V1	25–30%	1.3	0.285	Ambudkar *et al*., 1992
2. Two-step CHAPS extraction	CH^{R}C5 CHO	30–40%	0.54	0.88	Doige *et al*., 1992
3. Extraction with Zwittergent 3-12/DEAE-cellulose chromatography/immunoaffinity column	CH^{R}B30, CHO	90%	0.32	0.94	Shapiro and Ling, 1994
4. Octylglucoside extraction/Reactive Red 120 agarose chromatography	CR1R12	>90%	2.1	0.8	Urbatsch *et al*., 1994
5. Two-step CHAPS extraction/lentil lectin affinity chromatography	CH^{R}C5 CHO	~90%	1.65	0.4	Sharom *et al*., 1993

[a] In each case the starting material was plasma membranes.

ATPase, of 1.1 to 1.5-fold, with the same preparation (Shapiro and Ling, 1994). Another apparent anomaly is cyclosporin A, one of the most potent MDR modulator substrates, which fails to significantly stimulate P-glycoprotein ATPase, but interestingly does interfere with stimulation by other agents including verapamil and VX-710 (Germann *et al*., 1997; Rao and Scarborough, 1994). In general, only poor drug stimulation of P-glycoprotein ATPase is observed with purified preparations lacking lipids, but significant drug stimulation occurs with isolated membrane preparations or with purified protein after lipid reconstitution. This underscores the need for proper lipid-P-glycoprotein interaction, and may partly explain the variation in the biochemical properties of P-glycoprotein ATPase reported in different studies. The effects of lipids have been investigated by Doige *et al*. (1993) who found that membrane lipids, especially phosphatidylethanolamines, activate the ATPase activity of P-glycoprotein, and are able to restore activity after delipidation. Urbatsch and Senior (1995) showed that the kinetic parameters (V_{max} and K_m) of P-glycoprotein ATPase differed depending on the source of lipid (*E. coli*, sheep brain or bovine liver) used in reconstitution of the purified protein.

Several general characteristics of the intrinsic ATPase activity of P-glycoprotein have emerged from recent studies. Inhibitors include detergents such as CHAPS and Triton X-100, excess Mg^{2+}, N-ethylmaleimide (NEM) and sodium vanadate (summarized in Shapiro and Ling, 1994). Inhibition by CHAPS is variable, however, and for reconstituted P-glycoprotein, CHAPS appears to stimulate ATPase (Shapiro and Ling, 1995). Sensitivity to NEM and vanadate and insensitivity to ouabain and sodium azide, together with the relatively high K_m for ATP, emphasize the unique properties of P-glycoprotein ATPase, clearly distinguishable from P-, V-, and F-type ATPases. 8-azido-ATP covalently binds and inactivates P-glycoprotein coincident with the incorporation of 2 mol 8-azido-ATP/mol protein (Al-Shawi *et al*., 1994). NBD-Cl is another covalent inhibitor of P-glycoprotein ATPase, with 100% inactivation at an incorporation level of 1 mol NBD-Cl/mol protein. The mechanism most likely involves reaction with a single lysine residue (Al-Shawi and Senior, 1993). The P-glycoprotein-specific monoclonal antibody C219 also inhibits ATPase activity (Kokubu *et al*., 1997).

P-glycoprotein has two predicted nucleotide binding folds, one in each half of the molecule. Each ATP-binding domain has been separately expressed and each can independently catalyze ATP hydrolysis, but with low specific activity of 180 and 24 nmol min^{-1} mg^{-1}, respectively (Sharma and Rose, 1995; Shimabuku *et al*., 1992). Each half of P-glycoprotein has basal ATPase activity when independently expressed in a baculovirus system, but the half molecules do not exhibit drug-stimulated ATPase activity (Loo and Clarke, 1994c). However, when the half molecules are expressed together, stimulation of ATPase by verapamil, vinblastine and colchicine is restored to levels approaching that of wild-type. These results demonstrate that drug-stimulated ATPase activity requires interaction or cooperation between both

halves of P-glycoprotein, and this concept has received support from other studies (see also previous sections). For example, NEM has no effect on the drug-stimulated ATPase activity of a cysteine-less mutant P-glycoprotein, but reintroduction of a single cysteine into either nucleotide binding fold restores NEM sensitivity and abolishes drug-stimulated ATPase activity (Loo and Clarke, 1995a). The technique of vanadate trapping has been used to demonstrate that both nucleotide-binding sites are catalytically active and that catalysis may alternate between the two sites (Urbatsch *et al*., 1995). Mutagenesis data have also provided insight into the role of each ATP binding domain in basal and drug-stimulated ATPase. For example, when Lys433 in one binding fold or Lys1076 in the other was replaced with Met, drug-stimulated ATPase activity was abolished (Müller *et al*., 1996). Loo and Clarke (1995d) similarly demonstrated that mutations in either ATP binding domain abolished drug stimulated ATPase activity. Several studies have also compared the drug-stimulated ATPase activity of P-glycoprotein mutants with the pattern of resistance to the same drugs. Often, the two parameters are related. For example, the stimulation of ATPase by colchicine and etoposide is significantly increased in the Gly185→Ala mutant of the human *MDR*1 gene product, and cells expressing this mutant exhibit increased resistance to the same drugs (Müller *et al*., 1996; Rao, 1995). However, there are exceptions; a Phe335→Ala mutant has a significantly higher vinblastine-stimulated ATPase compared to wild-type P-glycoprotein, but only confers a low level of resistance to vinblastine when expressed in cells (Loo and Clarke, 1995d). Nonetheless, these results emphasize the close interdependence between ATP hydrolysis and transport, and suggest that, in general, the efficiency of transport for a given P-glycoprotein substrate is related to its ability to stimulate the intrinsic ATPase activity.

In summary, P-glycoprotein exhibits an intrinsic, high capacity, vanadate-sensitive, drug-stimulated ATPase activity with characteristics unique among transport ATPases. ATP hydrolysis at both nucleotide binding sites is required for the substrate-stimulated ATPase activity critical for substrate transport.

Characterization of Drug Transport Activity of P-Glycoprotein

Characterization of P-glycoprotein-mediated drug transport has been performed in intact MDR cell lines, with plasma membrane vesicles from MDR cell lines, and with the purified protein after reconstitution in artificial membrane systems. We will describe studies performed with each of these approaches, in turn, to provide an overview of what has been learned about the drug transport characteristics of P-glycoprotein.

The first suggestions that P-glycoprotein was capable of drug export came from studies of drug accumulation in MDR cell lines overexpressing P-glycoprotein (reviewed in Bradley *et al*., 1988). Fojo *et al.* (1985) examined drug uptake in a series of MDR human KB carcinoma cell lines that exhibited increasing resistance to drugs in the MDR spectrum, and a relationship was found between decreased drug accumulation and increased drug resistance for colchicine, vinblastine, vincristine and daunomycin. Later studies showed that the degree of drug resistance in these cell lines correlated with levels of P-glycoprotein (Shen *et al*., 1986).

Plasma membrane vesicles from the MDR KB-V1 cell line have been used to demonstrate ^{3}H-vinblastine transport into inside-out vesicles (Horio *et al*., 1988; Lelong *et al*., 1992). Transport was found to be osmotically sensitive and required energy in the form of ATP, GTP or an ATP regenerating system, and was not observed in membrane vesicles prepared from drug-sensitive cells lacking P-glycoprotein. MDR drugs (vincristine, actinomycin D, daunomycin, colchicine) and modulators (verapamil, quinidine) inhibited ^{3}H-vinblastine transport. P-glycoprotein-mediated drug transport has also been studied in membranes from liver and kidney cells which normally express P-glycoprotein. Kamimoto *et al.* (1989) prepared canalicular membrane vesicles from rat liver which contain endogenous P-glycoprotein and demonstrated transport of daunorubicin into inside-out vesicles. Transport was energy-dependent, osmotically sensitive and was inhibited by MDR drugs and MDR modulators. Horio *et al.* (Horio *et al*., 1989) used Madin-Darby canine kidney (MDCK) cells in order to further characterize P-glycoprotein-mediated drug transport and demonstrate directional transport. These cells form a highly polarized epithelium with localization of P-glycoprotein in the apical membrane. Basal-to-apical transepithelial transport of ^{3}H-vinblastine was about six times

higher than apical-to-basal transport in wild type cells, consistent with the hypothesis that P-glycoprotein acts as an extrusion pump at the apical membrane. Studies were also performed with *MDR*1-transfected MDCK cells where the overexpressed P-glycoprotein was found localized on the apical surface of the epithelium. Basal-to-apical transport of ^{3}H-vinblastine was 20-fold higher than apical-to-basal transport. Basal-to-apical transport in both wild-type and *MDR*1-transfected MDCK cells was inhibited by excess vinblastine, by other MDR drugs, and by verapamil.

Several interesting features of P-glycoprotein-mediated ^{3}H-vinblastine transport were defined in a study using inside-out plasma membrane vesicles from *MDR*3-overexpressing murine erythroleukemia cells (Schlemmer and Sirotnak, 1994). Uptake of ^{3}H-vinblastine was observed at 37 °C but not at 4 °C and only by inside-out vesicles, not by rightside-out vesicles, and not by vesicles from drug-sensitive cells lacking P-glycoprotein. Drug uptake occurred in two distinct phases, a rapid initial phase of <1 min which was osmotically insensitive and ATP-independent, and a second slower phase of >1 min which was osmotically sensitive and strictly ATP-dependent. Measurement of the rate of uptake in the second phase with respect to ATP concentration gave a K_m for ATP of 0.37 mM, similar to the K_m for ATP of P-glycoprotein ATPase activity. The authors concluded that the two steps represented drug binding and drug transport, respectively. Interestingly, both binding and transport were inhibited by the MDR modulators verapamil, reserpine and quinidine, whereas only transport was inhibited by C219 antibody and vanadate. Measurement of ^{3}H-vinblastine uptake at different vinblastine concentrations gave evidence of intravesicular drug accumulation against a concentration gradient, indicating that drug transport was an active, saturatable process consistent with earlier studies (Horio *et al*., 1988; Lelong *et al*., 1992).

The membrane systems described above have yielded a good deal of information on the characteristics of drug transport by P-glycoprotein but, because they contain multiple components, still leave open the question of whether P-glycoprotein, acting alone, can mediate drug transport. Therefore, methods for the purification and reconstitution of P-glycoprotein have been developed. The first successful demonstration of the functional reconstitution of drug transport by purified P-glycoprotein was reported by Sharom *et al*. (1993). They purified P-glycoprotein from the $CH^{R}C5$ cell line and prepared proteoliposomes by reconstitution in a mixture of phosphatidylcholine and dipalmitoylphosphatidylethanolamine. It was estimated that the orientation of the P-glycoprotein molecules was such that 55% faced inward with their ATP binding domains exposed. The other 45%, with their ATP binding domains inaccessible, would not contribute to transport. Colchicine was chosen as substrate because it is relatively hydrophilic compared to other MDR spectrum drugs, and partitioning into the lipid bilayer is less of a problem, thus reducing background binding. The proteoliposomes showed a time- and ATP-dependent uptake of ^{3}H-colchicine that was sensitive to inhibition by P-glycoprotein substrates and modulators, and that occurred against a drug concentration gradient. Drug accumulation in the vesicle lumen was osmotically sensitive and abolished by detergents, demonstrating that accumulation was the result of transport, not simply binding. This system has proven useful in the characterization of non-conventional substrates of P-glycoprotein and the structural requirements for transport, for example synthetic hydrophobic peptides (Sharom *et al*., 1996).

Shapiro and Ling (1995) also reported the successful reconstitution of drug transport using their highly purified P-glycoprotein preparation. Reconstitution was performed with L-α-phosphatidylcholine and the P-glycoprotein molecules in the reconstituted liposomes were uniformly in the inside-out orientation. An elegant fluorescence-based transport assay was developed using the substrate Hoechst 33342. It was found that the fluorescence of Hoechst 33342 was greatly increased on binding to the liposomes and decreased in the transition from the lipid to aqueous phase. Therefore, P-glycoprotein-mediated substrate transport from the liposome membrane into the interior aqueous space could be monitored by fluorescence reduction. Transport of Hoechst 33342 required hydrolysis of ATP, was blocked by vanadate and NEM, and was inhibited by the MDR chemosensitizers verapamil and amiodarone, but only at low substrate concentration. The rate of transport was slow, about 1 molecule of Hoechst 33342 per molecule of P-glycoprotein per minute, apparently due to futile recycling of the dye back from the aqueous to the lipid phase, with about 50 molecules of ATP hydrolyzed per molecule transported. Although some technical difficulties need to be overcome to assess the true transport rate and energy requirements of the transport process, the system developed clearly has much potential for answering many outstanding questions on the mechanism of transport (see below).

A general procedure for the purification and reconstitution of P-glycoprotein from different MDR cell lines was reported recently (Dong *et al*., 1996). P-glycoprotein, estimated to be 99% pure, was prepared by solubilization of plasma membranes with SDS followed by ceramic hydroxyapatite HPLC. After SDS removal, P-glycoprotein was reconstituted in a mixture of L-α-phosphatidylcholine and L-α-phosphatidic acid, and ATP-dependent ^{3}H-vinblastine uptake by the vesicles demonstrated. However, the kinetics of drug uptake was significantly slower compared to other systems, drug leakage was observed and drug-stimulated ATPase activity was only 2-fold or less. It is possible, therefore, that some irreversible damage may occur with this procedure. Other P-glycoprotein proteoliposome reconstitution systems have been developed and the transport of vinblastine (Ambudkar, 1995) and polypeptide ionophores (Eytan *et al*., 1994) described.

Models for the Mechanism of Drug Transport by P-Glycoprotein

One of the most interesting and controversial issues surrounding P-glycoprotein concerns the mechanism whereby a single transporter molecule can reduce the intracellular concentration of a large number of structurally heterogeneous substrates. Two main hypotheses for the mechanism of drug transport by P-glycoprotein have been suggested, one in which P-glycoprotein interacts with substrates directly to mediate their transport, and one in which an indirect mechanism is responsible. The latter hypothesis, postulated to explain the broad substrate specificity of P-glycoprotein, proposes that P-glycoprotein reduces drug concentration indirectly by altering the cell's electrical membrane potential or by elevating intracellular pH (reviewed in Wadkins and Roepe, 1997). These changes in turn would alter drug partitioning. For example, most of the drug substrates are weakly basic and positively charged at neutral pH, and an increase in intracellular pH would reduce net charge and thus reduce intracellular retention of such compounds. In support of this model, alterations in ion transport characteristics, cytosolic pH and electrical plasma membrane potential have been observed in some cell lines that express P-glycoprotein (reviewed in Roepe, 1995). However, these changes are not found in all *MDR*1-expressing cells (reviewed in Gottesman and Pastan, 1993). An alternate indirect model postulates that P-glycoprotein acts as an outwardly directed ATP channel, transporting drug molecules out of the cell via an ATP electrochemical gradient (Abraham *et al*., 1993).

Although indirect mechanisms may contribute to reduced drug accumulation in some cases, such mechanisms are unlikely to explain the high level of resistance observed in MDR cell lines, and most of the experimental data support a direct mechanism of drug transport by P-glycoprotein. Firstly, P-glycoprotein has been shown to directly bind drug analogs; specific regions of the transporter involved in substrate binding have been identified; and mutation of residues in these regions affects substrate binding and substrate specificity (also reviewed in Gottesman *et al*., 1995). Secondly, as summarized above, highly purified preparations of P-glycoprotein upon reconstitution are able to transport drugs and other substrates, with characteristics reflecting active transport against a concentration gradient. Thirdly, active transport across the membrane of vinblastine and colchicine has been demonstrated by functional expression of mouse P-glycoproteins in yeast secretory vesicles (Ruetz and Gros, 1994a). Substrate transport in the vesicle system was found to occur independently of a proton gradient and was not affected by changes in membrane potential (Ruetz and Gros, 1994a).

While these and other results support a model for the direct transport of substrates by P-glycoprotein, the actual mechanism is not well understood. However, several theories have been forwarded and some significant experimental and conceptual advances made. Because kinetic data implied that P-glycoprotein may be responsible for both increased efflux as well as decreased influx of substrate, the notion arose that P-glycoprotein intercepts its substrates and expels them directly from the membrane before they enter the cytosol. Two of the most intriguing models to account for this property are that P-glycoprotein acts as a 'hydrophobic vacuum cleaner' (Gottesman and Pastan, 1993) or as a 'flippase' (Higgins and Gottesman, 1992). The vacuum cleaner model was proposed following an elegant study by Raviv *et al.* (1990). These authors used the photolabile membrane probe 5-[^{125}I]iodonaphthalene-1-azide to show that a direct and specific interaction occurs between P-glycoprotein and its drug substrates in MDR cell membranes. Consistent with this hypothesis, a study with hydrophobic acetoxymethylester derivatives of fluorescent calcium and pH indicators also provided evidence that these transport substrates of P-glycoprotein are removed directly from the plasma membrane (Ho-

molya *et al*., 1993). The flippase mechanism also argues that substrates gain access to P-glycoprotein directly from the lipid phase. In this model, the role of P-glycoprotein is to transport or 'flip' substrate molecules from the inner to the outer leaflet of the membrane bilayer, thus encouraging their export from the cell (Higgins and Gottesman, 1992). However, evidence has also been presented against the hypothesis that drug extrusion occurs from the plasma membrane. Altenberg *et al*. (1994) found that the unidirectional influx of the P-glycoprotein substrate rhodamine 123 was the same in drug-sensitive and MDR cell lines, suggesting that P-glycoprotein does not intercept the dye but transports it out from the cytosolic compartment.

Both the vacuum cleaner and flippase models envision P-glycoprotein having a pocket or chamber, sufficiently flexible to accommodate substrate structural heterogeneity, in which the molecules are trapped prior to their expulsion. A recent study has provided the first insight into the three-dimensional structure of P-glycoprotein, which appears quite compatible with this suggestion (Rosenberg *et al*., 1997). The structure was determined to 2.5 nm resolution by electron microscopy and by single particle image analysis. The overall shape is one of a cylinder with a diameter of about 10 nm and height of about 8 nm, appearing toroidal when viewed from the extracellular surface. A large pore is present, open at the extracellular surface with a diameter of about 5 nm but closed at the cytoplasmic face of the membrane with a smaller diameter of 2.5 nm, thus forming a conical chamber. The two transmembrane domains of P-glycoprotein most likely make up the protein surrounding this pore. Other essential features of this structure include a second pore which is asymmetric and opens within the plane of the membrane bilayer. The authors speculate that this may represent the route of entry for substrates from the lipid phase. Two 3 nm lobes at the cytoplasmic face, of a size and orientation appropriate for the two nucleotide binding folds, complete the picture.

Another significant advance in our understanding of the mechanism of action of P-glycoprotein has been made with regards to the catalytic cycle and the stoichiometry of substrate transport and ATP hydrolysis. A scheme has been proposed where ATP hydrolysis occurs in a cyclic fashion, alternating between the two ATP binding sites in each half of P-glycoprotein (Senior *et al*., 1995). In this scheme, ATP binding to site 1 allows ATP to be hydrolyzed at site 2. This produces a conformational change, resulting in substrate transport and dissociation of the ADP product from site 2. A new molecule of ATP is postulated to then bind to site 2, causing the ATP molecule on site 1 to be hydrolyzed in turn, and the cycle is repeated, with the roles of site 1 and site 2 reversed. This mechanism predicts that one molecule of substrate is transported per molecule of ATP hydrolyzed.

In order to obtain data in support of the models proposed, efforts have been made to determine the stoichiometric relationship between drug transport and ATP hydrolysis. In the reconstitution system of Shapiro and Ling (1995), a value of 50 molecules of ATP per molecule of Hoechst 33342 transported was estimated. However, the authors conceded and emphasized that this low stoichiometry may be due to futile recycling and rebinding of the substrate, together with a high basal ATPase activity. In another study, proteoliposomes containing partially purified P-glycoprotein were used to estimate the quantitative relationship between transport and ATP hydrolysis (Eytan *et al*., 1996). The rate of valinomycin-dependent $^{86}Rb^{+}$ uptake and ATPase activity were measured under identical conditions, and a value of 0.5-0.8 ionophore molecules transported/ATP hydrolyzed was determined. A more recent study also showed a near stoichiometric relationship between drug transport and ATP molecules hydrolyzed (Ambudkar *et al*., 1997). In this case, the authors were able to compute the maximal rates of vinblastine transport and of vinblastine-stimulated ATPase activity. It was calculated that 2.8 molecules of ATP are hydrolyzed per molecule of vinblastine transported, consistent with the metabolic capacity of the cell, and suggesting that drug transport is indeed coupled to ATP hydrolysis.

Other Functions of P-Glycoprotein

P-glycoprotein is best known as a multidrug transporter of broad specificity. However, other functional properties have been suggested and these will be briefly discussed. In particular, we will review the evidence that P-glycoprotein may act as a chloride channel, as an ATP channel, or as a phospholipid translocase, and discuss evidence for a role in cholesterol biosynthesis.

Chloride channel

Because of the sequence and domain homology between P-glycoprotein and CFTR, the latter a known

chloride channel (Anderson *et al.*, 1991), it was speculated that P-glycoprotein may also exhibit such an activity. To test this hypothesis, Valverde *et al.* (1992) measured whole-cell chloride channel activities in control or *MDR*1-transfected fibroblasts. They found an increase in chloride current upon exposure of the *MDR*1-expressing cells to hypotonic medium which was not detected in the untransfected cells. Similarly, hypotonicity-activated chloride currents were observed in epithelial cells transfected with *MDR*1, but not in untransfected cells. In addition, antisense oligonucleotides to the *MDR*1 gene abolished the volume-activated chloride current. Further characterization revealed that non-hyrolyzable ATP analogs could substitute for ATP in chloride channel activation, suggesting that ATP hydrolysis was unnecessary and ATP binding was sufficient (Gill *et al.*, 1992). This conclusion was supported by data showing that mutant proteins with defective ATPase activity, but intact ATP binding capacity, still exhibited channel activity. Channel activation was prevented by MDR drugs, but these drugs failed to inhibit preactivated channels. The authors proposed a model in which the two activities, a drug transporter on the one hand requiring ATP hydrolysis, and a chloride channel on the other hand requiring only ATP binding, represented distinct, but interconvertible functional states of P-glycoprotein (Gill *et al.*, 1992).

These results predicted that P-glycoprotein expression should lead to the appearance of a volume-regulated chloride channel with properties similar to those described earlier in epithelial cells (Worrel *et al.*, 1989). While several studies subsequently substantiated this model, other reports failed to link P-glycoprotein expression and increased volume-regulated chloride currents (reviewed in Higgins, 1995). In addition, it remained an open question whether P-glycoprotein was the channel itself, or whether P-glycoprotein activated an endogenous channel in some cell types. This debate was resolved to some degree by an important study by Luckie *et al.* (1994). These investigators used untransfected and *MDR*1-transfected fibroblasts to measure chloride conductance as a function of osmotic challenge. They observed a swelling-induced chloride conductance which was present only in the *MDR*1-expressing cell line, but only under conditions representing a change from isotonic conditions (300 mosM) to 30% hypotonic (210 mosM). However, upon increasing the hypotonic challenge, both cell lines responded similarly, and the extent to which they manifested swelling-activated whole cell chloride currents equalized. Thus, the difference between the two cell lines with regard to volume-regulated chloride channel activity was in the sensitivity to osmotic challenge, with the *MDR*1-expressing cells showing a dose-response curve (of chloride current versus hypotonicity) shifted slightly to the left. The observation that large volume-regulated chloride channels are present in cells that do not express detectable P-glycoprotein, together with other experimental data, suggests strongly that P-glycoprotein is not the channel itself (reviewed in Wine and Luckie, 1996). However, the identity of the ubiquitous volume-sensitive chloride channel is still uncertain. It may, at least in part, be composed of I_{Cln}, a protein of 235 residues that may form a membrane pore through dimerization (Paulmichl *et al.*, 1992).

The possibility that P-glycoprotein might act as a regulator of endogenous chloride channels has led to the presentation of several models to explain such a property (Higgins, 1995). These include a direct interaction between P-glycoprotein and the channel protein in the cell membrane, an indirect interaction mediated by a third party, or a mechanism where P-glycoprotein transports out of the cell a molecule which in turn regulates the channel. This latter possibility seems unlikely since channel regulation is still observed in transport-defective mutants. Another possibility is that the presence of P-glycoprotein causes non-specific alterations in membrane architecture to effect chloride conductance upon osmotic challenge in certain cell types. It has also been suggested that the ability of P-glycoprotein to regulate endogenous swelling-induced chloride channels depends on protein kinase C-mediated phosphorylation of sites in the linker region of P-glycoprotein (Hardy *et al.*, 1995). Further studies, for example with *MDR*1 knockout mice, will be required to establish whether the chloride channel activity associated with P-glycoprotein expression is physiologically important.

ATP channel

The rate of release of ATP from MDR CHO cells was found to be 3-fold higher than from drug-sensitive CHO cells (Abraham *et al.*, 1993). In addition, a correlation was found between ATP release and expression level of P-glycoprotein in different cell lines. The rate of ATP efflux from the cells expressing high levels of P-glycoprotein was estimated to be about 10^7 ATP moleculess^{-1} cell^{-1}. These results suggested that P-glycoprotein might act as an ATP channel.

Patch-clamp techniques were employed to support this conclusion. A model was proposed in which the ATP supplied by P-glycoprotein to the cell exterior might be utilized by an ecto-ATPase, creating an ATP gradient linked to substrate transport. How such a gradient might be formed is not clear in view of the high capacity of P-glycoprotein to hydrolyze ATP.

Phospholipid translocase: Based on findings that mouse *mdr2* transported phosphatidylcholine (Ruetz and Gros, 1994b; Smit *et al*., 1993), van Helvoort *et al*. (1996) used a novel system to investigate whether human *MDR*1 exhibited phospholipid translocating activity. They expressed *MDR*1 P-glycoprotein in epithelial LLC-PK1 cells and monitored the transport across the apical membrane of short-chain analogs of membrane lipids. The substrates examined, added as lipid precursors and converted intracellularly, included N-6[7-nitro-2,1,3-benzoxadiazol-4-yl]-aminohexanoyl derivatives of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and glucosylceramide. Vesicular transport to the cell surface of the lipids was blocked by performing the experiments at 15 °C, and translocation of these substrates was clearly demonstrated in *MDR*1-expressing but not in control cells. It was concluded that the ability of P-glycoprotein to act as a lipid translocase of broad specificity may reflect a physiological role in organization of membrane lipids.

Cholesterol biosynthesis

Because free excess cholesterol is toxic to cells, it is esterified by enzymes resident in the endoplasmic reticulum (ER) and stored in this form as lipid droplets in the cytosol (reviewed in Goldstein and Brown, 1990). This requires the intracellular transport of cholesterol from the plasma membrane to the ER, a process inhibited by progesterone (Lange, 1994). In addition, progesterone appears to inhibit the transport from the plasma membranes to the endoplasmic reticulum of sterols required for cholesterol biosynthesis (Metherall *et al*., 1996b). Metherall and coworkers (Debry *et al*., 1997; Metherall *et al*., 1996a; Metherall *et al*., 1996b) reasoned that the inhibitory effects of progesterone were likely due to interaction with either the progesterone receptor or with P-glycoprotein, with which progesterone also interacts (Qian and Beck, 1990; Yang *et al*., 1989). The progesterone receptor was ruled out by a careful series of experiments thus implicating P-glycoprotein in intracellular cholesterol transport. A series of steroid hormones of different hydrophobicity were examined for their ability to inhibit cholesterol biosynthesis/esterification and to inhibit P-glycoprotein activity, measured by vinblastine accumulation (Debry *et al*., 1997; Metherall *et al*., 1996a). A good correlation was found between steroid hydrophobicity and *MDR*1 inhibition, as reported previously (Yang *et al*., 1989), and between steroid hydrophobicity and inhibition of cholesterol biosynthesis/esterification. Furthermore, two known *MDR*1 inhibitors, verapamil and Triton X-100, inhibited cholesterol esterification in several cultured human cell lines. It was proposed that P-glycoprotein functions in the trafficking of cholesterol and cholesterol precursors from the plasma membranes to the endoplasmic reticulum. However, the mechanisms involved in this putative role for *MDR*1 remain unknown. An indirect mechanism seems most likely, given the directionality of transport, and the fact that cholesterol does not require active transport to cross membrane bilayers (Dawidowicz, 1987).

Phosphorylation of P-Glycoprotein

Phosphorylation is a widespread and apparently universal characteristic of P-glycoproteins, occurring in native and recombinant mammalian MDR products as well as in P-glycoprotein homologs, and several reviews dealing with this subject have been published (Chambers, 1996; Fine *et al*., 1996; Germann *et al*., 1995; Germann, 1996). Because phosphorylation is a major regulatory mechanism for many proteins, understanding the role of phosphorylation in P-glycoprotein function has been keenly pursued in the hopes of developing new MDR modulators. One approach to this problem has been to treat MDR cell lines with activators or inhibitors of phosphorylation and measure short-term effects on drug accumulation or long-term effects on drug resistance. A popular and widely used compound is the protein kinase C activator TPA, first reported by Hamada *et al*. (1987) to stimulate P-glycoprotein phosphorylation, and first reported by Fine *et al*. (1988) to influence MDR drug accumulation. Short-term TPA treatment stimulates P-glycoprotein phosphorylation and reduces drug accumulation in a wide variety of MDR cell lines (Aftab *et al*., 1994; Bates *et al*., 1992; Bates *et al*., 1993; Chambers *et al*., 1990; Chambers *et al*., 1992; Yu *et al*., 1991). The effect of TPA on drug accumulation is dose-dependent, with a half-maximal effect at around 20 nM TPA, and is observed in MDR

cells and not in the respective drug-sensitive cell line (Aftab *et al*., 1994; Chambers *et al*., 1992). Consistent with these results, long-term treatment of MDR cells with TPA, which leads to downregulation of TPA-responsive PKC isozymes, results in decreased P-glycoprotein phosphorylation and increased drug accumulation (Bates *et al*., 1993).

The effects of kinase inhibitors on drug accumulation in MDR cells has also been examined. Increased drug accumulation occurs in MDR cell lines treated with staurosporine or calphostin C, both effective inhibitors of P-glycoprotein phosphorylation (Aftab *et al*., 1994; Bates *et al*., 1993; Chambers *et al*., 1992; Ma *et al*., 1991). Sodium butyrate has also been shown to inhibit P-glycoprotein phosphorylation and cause an increase in accumulation of several cytotoxic drugs that are substrate for P-glycoprotein (Bates *et al*., 1992). However, both staurosporine and calphostin C can bind to P-glycoprotein (Gupta *et al*., 1994; Sato *et al*., 1990), and such interaction may be the basis for their ability to interfere with drug transport. Smith and Zilfou (1995) also presented evidence that the pattern of MDR reversal by different phosphorylation modulators did not correlate with their effects on protein kinases. In addition, many agents that affect phosphorylation (kinase inhibitors, sodium butyrate, phorbol esters) also influence P-glycoprotein expression level (reviewed in Germann *et al*., 1995). Because of these difficulties, phosphorylation inhibitors that do not interact with P-glycoprotein have been sought and several identified. These include the lysosphingolipid safingol (Sachs *et al*., 1995), N-myristoylated peptide inhibitors of PKC (Gupta *et al*., 1996), and sphingosine stereoisomers (Sachs *et al*., 1996). All of these agents inhibit P-glycoprotein phosphorylation and increase drug accumulation in MDR cells, without directly interacting with P-glycoprotein or affecting its expression. These results and those with TPA provide compelling evidence that PKC-mediated phosphorylation of P-glycoprotein increases its drug transport capacity. Elegant work by Glazer's group using PKC transfection and PKC antisense strategies supports the conclusion that the drug transport and ATPase properties of P-glycoprotein are modulated by PKC-mediated phosphorylation (Ahmad and Glazer, 1993; Ahmad *et al*., 1994; Yu *et al*., 1991).

In vitro, P-glycoprotein is phosphorylated by PKC (Chambers *et al*., 1990), by PKA (Mellado and Horwitz, 1987), and by a phospholipid-dependent kinase (Staats *et al*., 1990). The PKC sites in human *MDR*1 have been unambiguously identified by amino acid sequence analysis as Ser661, Ser667 and Ser671, clustered in the linker region of the molecule (Chambers *et al*., 1994; Chambers *et al*., 1993). Comparisons of tryptic phosphopeptide maps have indicated that these three sites are the major sites utilized *in vivo* (Chambers *et al*., 1994; Chambers *et al*., 1993; Chambers *et al*., 1992), and this has been confirmed by site-directed mutagenesis (Germann *et al*., 1996; see below). Phosphorylation in mouse *mdr*1b also appears to be confined to the linker region, with the major sites analogous to those in *MDR*1 (Glavy *et al*., 1997; Orr *et al*., 1993).

Despite the fact that the major phosphorylation sites in P-glycoprotein have been identified and these occur within established PKC consensus sites, the identity of the kinase or kinases that catalyze the basal phosphorylation of P-glycoprotein remains elusive. Partial purification from KB-V1 cell membranes of a protein kinase active toward P-glycoprotein has been achieved (see Germann, *et al*., 1995; Chambers, *et al*., 1995). The 'V1 kinase', which has a native apparent molecular mass of 55 kDa, phosphorylates P-glycoprotein but not histone H1, casein, nor peptide substrates for PKC or PKA, and is inhibited by staurosporine but not by other PKC or PKA inhibitors. However, the V1 kinase avidly phosphorylates Ser-661 and Ser-667 in the linker region of P-glycoprotein *MDR*1 (Chambers *et al*., 1995) two of the established *in vivo* sites. Thus, the V1 kinase appears related to PKC family members in its ability to recognize PKC consensus sites in P-glycoprotein, but it is clearly distinguishable from known PKC isoforms in its physical and biochemical properties. Whether the V1 kinase is the only kinase that phosphorylates P-glycoprotein *in vivo* is not known; perhaps it shares this responsibility with one or more of the established PKC isozymes, or with other PKC-like enzymes. Several PKC isozymes, including α, β, γ, ϵ and θ, have been shown by coimmunoprecipitation to be physically associated with P-glycoprotein in MCF-7 cells (Yang *et al*., 1996). In addition, P-glycoprotein is phosphorylated *in vitro* by most of the recognized PKC isozymes including the α, βI, βII, γ, δ, ϵ, ζ, and η subtypes (Fine *et al*., 1996).

Identification of the phosphorylation sites has enabled the generation of P-glycoprotein mutants with alanine or aspartic acid substitutions of the relevant serine residues (Germann *et al*., 1996; Goodfellow *et al*., 1996). The mutant molecules fail to undergo phosphorylation *in vivo*, thus confirming that the sites targeted for substitution likely include all those utilized *in vivo*. The phosphorylation-defective

mutants transport drugs normally and confer MDR when overexpressed, with efficiencies similar to wild-type P-glycoprotein. These studies clearly show that phosphorylation/dephosphorylation reactions are non-essential for P-glycoprotein-mediated drug transport. It is possible that individual phosphorylation sites have opposing effects on transport, so it will also be important to test the activity of single site mutants. In addition, phosphorylation may affect the transport of substrates yet to be identified, or be more important for other roles such as chloride channel regulation (Hardy *et al*., 1995).

Superficially, the pharmacological data suggesting that P-glycoprotein-mediated drug transport is modulated by phosphorylation, and the mutagenesis data indicating that phosphorylation is dispensable, may seem at odds. However, the results with the mutants are not entirely unexpected, because the pharmacological data also suggest that phosphorylation plays a modulatory role, not an essential one. For example, the drug accumulation levels in MDR cells where P-glycoprotein phosphorylation is strongly inhibited do not approach those found in the corresponding drug sensitive cell line. In fact, the data from both pharmacological and molecular approaches show that drug pumping can occur when phosphorylation is shut down. If it is assumed that P-glycoprotein has a basal level of activity, that is improved somehow by increasing its degree of phosphorylation, cells which highly overexpress P-glycoprotein may have sufficient drug transport capacity, regardless of the transporter's phosphorylation status. Thus, overexpression systems may be inappropriate models to test the role of phosphorylation. Phosphorylation may be most important and make a real difference when expression levels are low, and when substrate concentrations fluctuate and rapid changes in pumping efficiency are needed. Low P-glycoprotein expression level and fluctuating substrate concentrations are conditions rarely seen in the laboratory, but may more accurately reflect physiological situations. If phosphorylation is a mechanism to moderately but effectively adjust drug transport capacity in response to environmental conditions, allowing a tumor cell with low P-glycoprotein expression cell to survive for example, what predictions could be made? First, we would expect the kinase to be able to respond rapidly to external signals and be part of an integrated signaling pathway, and the kinase and substrate to be colocalized to allow rapid covalent modification. A membrane-associated kinase responsive to activation by lipid-derived second messengers, such as PKC, would be an excellent candidate for such a role. Indeed, evidence for regulation of P-glycoprotein phosphorylation through activation of a stress-responsive phospholipase C has been reported (Yang *et al*., 1995). Second, we would expect rapid reversibility, so a membrane-associated phosphatase should be present that can dephosphorylate phosphorylated P-glycoprotein, and such an activity has been observed and identified as protein phosphatase 1 (Chambers and Kuo, 1993). Third, we would expect phosphorylation levels to be responsive to external drug concentrations. Although to our knowledge this has not been systematically examined, Lee (1995) has shown that when MDR lymphoma cells are shifted to high vincristine concentrations, P-glycoprotein becomes hyperphosphorylated and more effective as a drug transporter.

The conservation of PKC phosphorylation site motifs in the linker regions of drug transporting P-glycoproteins from human, mouse and hamster origin (Germann *et al*., 1995), a region of the molecule otherwise tolerant of amino acid substitutions, indicates a fundamentally important role for these modifications in P-glycoprotein function. As the main physiological function of P-glycoprotein is to transport toxins (Borst and Schinkel, 1996; Borst and Schinkel, 1997), it follows that phosphorylation is important for this role. A challenge for the future will be to develop models to test the idea that phosphorylation is an important modulator of substrate transport by P-glycoprotein in physiological situations.

Increased PKC activity due to increased expression of specific PKC isozymes, in particular PKCα, has been documented in many MDR cell lines (reviewed in Germann *et al*., 1995), but the significance of this observation is not clear. Because transcriptional activation of the *MDR*1 gene can occur in response to PKC activation (Chaudhary and Roninson, 1992), increased PKC activity may be related to regulation of P-glycoprotein expression. However, the signaling pathway of *MDR*1 induction by PKC activators like TPA has not been established. One potential candidate is the Raf/MEK/ERK MAP kinase pathway, but studies in K562 cells have shown that *MDR*1 induction by TPA occurs independently of ERK activation (Osborn and Chambers, submitted for publication). Recent evidence has suggested that another MAP kinase pathway, mediated by the stress-activated or c-Jun NH_2-terminal protein kinase (JNK), may play an important role in the MDR phenotype (Osborn and Chambers, 1996). Significantly increased JNK activ-

ity was found in two MDR cell lines, KB-V1 and KB-A1, relative to drug sensitive KB-3 cells. In addition, JNK was shown to be strongly activated by several MDR drugs including Adriamycin, vinblastine and etoposide, and drug concentrations optimal for JNK activation were also optimal for *MDR*1 mRNA induction. Interestingly, many other stressful stimuli including thermal, radiant and chemical stress cause both JNK activation (reviewed in Kyriakis and Avruch, 1996) and *MDR*1 induction (reviewed in Cornwell, 1996), suggesting a possible link between these two parameters. Further investigations into the mechanisms of *MDR*1 induction by mitogenic or stressful stimuli may be fruitful in designing strategies to prevent the emergence of MDR cells, and complement methods to block P-glycoprotein function at the level of the membrane-embedded protein itself.

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Address for correspondence: Ursula A. Germann, Vertex Pharmaceuticals Incorporated, 130 Waverly Street, Cambridge, Massachusetts 02139-4242, U.S.A.
E-mail: Germann@macnet.vpharm.com