Journal of Bioenergetics and Biomembranes, Vol. 27, No. 1, 1995

Purification and Reconstitution of Functional Human P-glycoprotein

Suresh V. Ambudkar[1]

Received January 17, 1995; accepted January 17, 1995

The overexpression of the P-glycoprotein, the *MDR*1 gene product, has been linked to the development of resistance to multiple cytotoxic natural product anticancer drugs in certain cancers and cell lines derived from tumors. P-glycoprotein, a member of the ATP-binding cassette (ABC) superfamily of transporters, is believed to function as an ATP-dependent drug efflux pump with broad specificity for chemically unrelated hydrophobic compounds. We review here recent studies on the purification and reconstitution of P-glycoprotein to elucidate the mechanism of drug transport. P-glycoprotein from the human carcinoma multidrug resistant cell line, KB-V1, was purified by sequential chromatography on anion exchange followed by a lectin (wheat germ agglutinin) column. Proteoliposomes reconstituted with pure protein exhibited high levels of drug-stimulated ATPase activity as well as ATP-dependent [^{3}H]vinblastine accumulation. Both the ATPase and vinblastine transport activities of the reconstituted P-glycoprotein were inhibited by vanadate. In addition, the vinblastine transport was inhibited by verapamil and daunorubicin. These studies provide strong evidence that the human P-glycoprotein functions as an ATP-dependent drug transporter. The development of the reconstitution system and the availability of recombinant protein in large amounts due to recent advances in overexpression of P-glycoprotein in a heterologous expression system should facilitate a better understanding of the function of this novel protein.

KEY WORDS: Multidrug resistance; P-glycoprotein; multidrug transporter; octyl glucoside; reconstitution; proteoliposomes; ATPase; drug transport; verapamil; vinblastine.

INTRODUCTION

Tumor cells *in vivo* and cultured cells *in vitro* can develop simultaneous resistance to a wide variety of cytotoxic, natural product, anticancer agents such as vinblastine, colchicine, doxorubicin, and actinomycin D (Endicott and Ling, 1989; Gottesman and Pastan, 1988; Roninson, 1991; Gottesman and Pastan, 1993; Ambudkar *et al.*, 1995). These drugs have little in common structurally, except for hydrophobicity and a tendency to be positively charged at neutral pH. Although there are other mechanisms of resistance involving topoisomerase II (Glisson *et al.*, 1986) and glutathione *S*-transferase (Moscow and Cowan, 1988), the cells which are resistant to natural product cytotoxic agents accumulate much less drug than parental drug-sensitive cells. This is most likely due to the presence in these cells of an energy-dependent drug extrusion system (Fojo *et al.*, 1985; Willingham *et al.*, 1986). Cells with a high level of drug resistance express large quantities of a 150–170-kDa membrane phosphoglycoprotein, referred to as the P-glycoprotein (Pgp), or the multidrug transporter (Juliano and Ling, 1976). In many drug-resistant cells including human KB carcinoma cells, the levels of Pgp and its mRNA are directly proportional to the degree of resistance (Ueda *et al.*, 1986; Gottesman and Pastan, 1993), suggesting that the expression of this protein is the major means by which these cells develop multidrug resistance. Moreover, transfection experiments

[1] Division of Nephrology, Department of Medicine and Department of Physiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland.

0145-479X/95/0200-0023$07.50/0

have demonstrated that expression of the human or the mouse *mdr*1 cDNAs in drug-sensitive cells is sufficient to confer the drug resistance phenotype (Gottesman and Pastan, 1993; Childs and Ling, 1994).

The mammalian *mdr* gene family is composed of two members in humans and three members in rodents (Ng *et al.*, 1989). In humans only one gene (*MDR*1) confers drug resistance while in rodents two genes (*mdr*1a and *mdr*1b; also called *mdr*3 and *mdr*1, respectively; Ueda *et al.*, 1987; Gros *et al.*, 1986) confer resistance. Recent work with *mdr*2 knock-out mice indicates that Pgp-encoded by *mdr*2 gene mediates phosphatidylcholine secretion into bile (Smit *et al.*, 1993). In addition, the function of *mdr*2-encoded Pgp as the ATP-dependent phosphatidylcholine transporter has been demonstrated by its expression in yeast secretory vesicles (Ruetz and Gros, 1994). The human *MDR*1 cDNA encodes a 1,280 amino acid protein, and hydropathy profiling of the deduced amino acid sequences has led to a secondary structure model of Pgp which contains 12 putative transmembrane segments with two ATP (nucleotide) binding domains on the cytoplasmic surface of the membrane (Chen *et al.*, 1986; Gottesman and Pastan, 1988). Each half of the protein contains a hydrophobic region with six transmembrane helices and a hydrophilic region with one nucleotide-binding fold; the N-terminal half of the molecule exhibits 43% identity with the C-terminal half, suggesting that the two halves of the molecule might have evolved independently or undergone a major rearrangement of introns after a duplication event (Chen *et al.*, 1990).

The regions of greatest identity in the two halves of the Pgp molecules are also homologous to similar sequences in the ATP-binding domains of the ABC (ATP-binding cassette) superfamily of transporters (Hyde *et al.*, 1990; Higgins, 1992, Childs and Ling, 1994). This superfamily, also known as the "solute ATPases" or "traffic ATPases" (Shyamala *et al.*, 1991; Doige and Ames, 1993), or "M-type ATPases" (Pedersen and Amzel, 1993) now includes over 50 members (Higgins, 1992; Childs and Ling, 1994). The eukaryotic members of this family include Pgp (also a protozoan PfMDR—which is linked with chloroquine resistance in *Plasmodium falciparum* [Foote *et al.*, 1989]), *MRP*, multidrug resistance-associated protein (Cole *et al.*, 1992), CFTR—the cystic fibrosis transmembrane conductance regulator (Riordan *et al.*, 1989), STE-6, the yeast **a** pheromone transporter (McGrath and Varshavsky, 1989), TAP1/TAP2 (also known as RING-4, HAM1, and HAM2)—the human and mouse MHC-linked transporters (Trowsdale *et al.*, 1990; Monaco *et al.*, 1990), and peroxisomal proteins such as PMP70 (Gartner *et al.*, 1992) and adrenoleukodystrophy protein (ALDP; Mosser *et al.*, 1993). The prokaryotic members include binding protein-dependent permeases for amino acids, e.g., histidine permease (Bishop *et al.*, 1989; Doige and Ames, 1993), ions, and sugars, etc., and others involved in the export of toxins (*Hyl*B, *Lkt*B) and glycans (*Chv*A, *Ndv*A). The substrates transported by this superfamily members include ions, solutes, peptides, proteins, phospholipids, and cytotoxic natural product drugs. However, Pgp alone is known to interact with and, in many cases transport, a broad range of structurally heterogenous substrates (e.g., anticancer drugs, reversing agents, hydrophobic peptides, steroids, and detergents (reviewed in Gottesman and Pastan, 1993). Among ABC transporters only CFTR has been shown to function as a channel (Bear *et al.*, 1992).

Currently available data strongly suggest that the *MDR*1 gene product-Pgp catalyzes the ATP-dependent extrusion of natural cytotoxic drugs from multidrug-resistant cells. Recently, Pgp has also been proposed to function as volume-regulated chloride channel (Valverde *et al.*, 1992; Gill *et al.*, 1992), an ATP channel (Abraham *et al.*, 1993), and as modulator of membrane potential/pH gradient (Roepe *et al.*, 1993; Luz *et al.*, 1994). However, neither the drug transport function nor the channel activities have yet been conclusively established, and several basic questions are as yet unanswered. For example, what defines the broad substrate specificity of the Pgp and how is energy transduced by the transporter for active efflux? Similarly, very little is known about the mechanism underlying the drug efflux mediated by Pgp. To address these questions and to prove conclusively that the *MDR*1 protein is the multidrug transporter, the protein will have to be purified to homogeneity in a functional form and reconstituted into proteoliposomes. The availability of purified biologically active protein and the development of an *in vitro* artificial membrane system will also help to elucidate the regulatory role of kinase-mediated phosphorylation of Pgp (see Germann *et al.*, this issue) and to assess various other functions ascribed to this protein. Such studies will contribute toward understanding the function of Pgp and provide an insight into its role in the development of multidrug resistance in cancers. In addition, since Pgp is a prototype of the ABC superfamily of transporter, these data will

also be potentially applicable to understanding the function of other members of this class of proteins, such as CFTR (Riordan, 1993), peroxisomal proteins, PMP 70 (Gartner *et al.*, 1992) and ALDP (Mosser *et al.*, 1993), and MHC-linked transporters TAP1 and TAP2 (Monaco *et al.*, 1990) which have been associated with manifestation of a disease in humans.

PURIFICATION AND RECONSTITUTION OF HUMAN P-GLYCOPROTEIN

For purification studies we used the multidrug-resistant human carcinoma cell line, KB-V1, as a source of Pgp. These cells have been shown to overexpress the *MDR*1 gene, but not the *MDR*2 gene (Schoenlein, P.V., and Gottesman, M.M., unpublished data). In humans, two closely related *MDR*1 and *MDR2* genes encode proteins which are about 76% identical in their amino acid sequence, but only the *MDR*1 gene has been shown to confer drug resistance (Ueda *et al.*, 1987; Gottesman and Pastan, 1993). In addition, the *MDR*1-*MDR*2 chimeric protein containing *MDR*2 ATP-binding folds is functional as a ATP-dependent drug efflux pump (Dhir and Gros, 1992; Germann, U. A., Wu, P., Currier, S. J., Aksentijevich, I., Pastan, I., and Gottesman, M. M., unpublished data), strongly indicating that the *MDR*2 protein can function as an ATP-dependent transporter. Thus, with the use of KB-V1 cells as the starting material we are certain that the purified preparation will not be contaminated with *MDR*2 gene product.

The degree of success during solubilization and reconstitution of membrane transport proteins very much depends on the steps taken to preserve the normal activity of the protein as it is moved from its natural membrane to the artificial one. Toward that goal, we have developed a protocol, by combining traditional methods (Racker *et al.*, 1979; Newman and Wilson, 1980) with the use of a class of protein-stabilizing agents, osmolytes, that allows the rescue of functional protein (Ambudkar and Maloney, 1986a,b; Maloney and Ambudkar, 1989). Several transport proteins such as bacterial anti- and symporters (Maloney and Ambudkar, 1989; Ambudkar *et al.*, 1990; Ruan *et al.*, 1992), F-, P-, V-, and M-type ATPases (Ambudkar *et al.*, 1986; D'Souza *et al.*, 1987; Bishop *et al.*, 1989; Davidson and Nikaido, 1990), and water [aquaporin-CHIP-28] (Zeidel *et al.*, 1992) and calcium channels (Lockwich *et al.*, 1995) have been reconstituted by using this protocol.

Purified plasma membrane vesicles of KB-V1 cells, which exhibit ATP-dependent [^{3}H]vinblastine transport (Horio *et al.*, 1988), were solubilized with the nonionic detergent, octylglucoside, in the presence of lipid and an osmolyte protectant (20% glycerol). With these extraction conditions almost all (>90%) of the Pgp content of plasma membranes was recovered in the detergent solution (Ambudkar *et al.*, 1992). A similar protocol has subsequently been used successfully for solubilization of Pgp from Chinese Hamster ovary cells (Urbatsch *et al.*, 1994) and rat liver (Eytan *et al.*, 1994) and also of PDR5 (pleiotropic drug resistance) protein (a homolog of mammalian Pgp) of yeast (Decottignies *et al.*, 1994). The detergent extract was chromatographed on anion exchange (DEAE Sepharose CL-6B) resin, and the Pgp enriched 0.1 M NaCl eluate was subsequently subjected to lectin-affinity chromatography. Thus, with just two steps, homogeneously (>90%) pure Pgp was obtained and two proteolytic fragments of Pgp were the major contaminants in the purified fraction (Ambudkar, S. V., Lelong, I. H., Zhang, J., Cardarelli, C. O., and Park, G., unpublished data). Urbatsch *et al.* (1994) have purified Pgp by single-step chromatography on Reactive Red 120 agarose of an octylglucoside extract of membranes of highly colchicine-resistant Chinese Hamster ovary cells that contain 20–30% (w/w) Pgp as a percent of total membrane protein. However, the above procedure was not useful for the purification of Pgp from bacculovirus-*MDR*1-infected insect (Sf9) cell membranes (Ambudkar, S. V., Chen, D., Pastan, I., and Gottesman, M. M., unpublished data). This anomalous behavior may either be due to the alteration in posttranslational modification(s) of recombinant Pgp or the lipid composition of insect call membranes, or both (Germann *et al.*, 1990). A combination of anion exchange and immunoaffinity chromatography has also been used for the isolation of Pgp from Chinese Hamster ovary cells (Shapiro and Ling, 1994). Purified human Pgp was reconstituted into proteoliposomes prepared from a lipid mixture of *Escherichia coli* bulk phospholipid, phosphatidylcholine, phosphatidylserine, and cholesterol (60:17.5:10:12.5), either by the detergent-dilution method (Ambudkar *et al.*, 1992) or by the detergent dialysis followed by Sephadex-G50 chromatography. In proteoliposomes, prepared by either method, >90% of Pgp is reconstituted with an inside-out orientation (i.e., ATP-binding and cytoplasmic domains exposed to extravesicular medium). Thus, these proteoliposomes are most suitable for the

characterization of drug-stimulatable ATPase as well as ATP-dependent drug transport activities of Pgp.

P-GLYCOPROTEIN-ASSOCIATED DRUG-STIMULATABLE ATPase ACTIVITY

Earlier studies, following the identification of the multidrug resistance phenomenon, indicated that treatment with azide resulted in accumulation of drugs in multidrug-resistant cells, suggesting the involvement of an energy-dependent process (Dano, 1973). The molecular biological studies including the mutational analysis of conserved residues in the nucleotide-binding domains further demonstrated that the drug efflux is mediated by an ATP-driven pump. In recent years the Pgp-associated ATPase activity has been biochemically characterized. Crude membranes of Sf9 (insect) cells infected with baculovirus-*MDR*1 (Germann *et al.*, 1990) exhibit high levels of vinblastine- or verapamil-stimulated ATPase activity (3–5 μmol/min/mg Pgp) (Sarkadi *et al.*, 1992). The ATPase activity of Pgp in plasma membranes from highly drug-resistant Chinese hamster ovary cell lines, which overexpress Pgp so that it constitutes 5–30% by weight of total plasma membrane protein, has been characterized (Doige *et al.*, 1992; Al-Shawi and Senior, 1993). These cells were selected for resistance to colchicine at very high concentrations (5–30 μg/ml) and this might result in the overexpression of various *mdr* genes (i.e., *mdr*1, *mdr*3, and *mdr*2). Thus, the observed ATPase activities may be due to Pgps encoded by one or more *mdr* genes.

Due to the very high level of P-type ATPases such as Na^+/K^+-ATPase and alkaline phosphatase in human carcinoma KB-V1 cells, the Pgp-associated ATPase activity could not be measured in the plasma membrane vesicles. Thus, we initially separated Pgp from other ATPases and phosphatases by anion exchange chromatography (Ambudkar *et al.*, 1992) in order to characterize the ATPase activity. The partially purified Pgp was reconstituted into proteoliposomes prepared from a lipid mixture of *E. coli* bulk phospholipid, phosphatidylcholine, phosphatidylserine, and cholesterol (Ambudkar *et al.*, 1992). Pgp-associated ATPase activity was stimulated only by drugs which are known to be its substrates. Purified Pgp when reconstituted into proteoliposomes exhibits verapamil- or vinblastine-stimulated high levels of ATPase activity ranging from 5 to 25 μmol/min/mg of protein. The stimulation of ATP hydrolysis by the substrate vinblastine is due to an increase in the maximal velocity of ATP hydrolytic activity without affecting the apparent K_m for ATP. These studies demonstrate that Pgp can be purified without significant loss of activity and that it exhibits a high level of ATP hydrolytic activity similar to other ion-translocating ATPases. One of the unexpected findings is that unlike many ion-translocating ATPases, the stimulation by substrates (cytotoxic drugs and reversing agents) of ATPase activity of Pgp is not observed when the protein is in soluble form in the detergent solution. Thus, even though the basal ATPase activity of Pgp is preserved in the detergent-solution, the stimulation by reversing agent, verapamil, is not (Fig. 1). This is consistent with the observations that the Pgp-ATPase activity in native membranes is inactivated by low concentrations of detergents (Sarkadi *et al.*, 1992; Al-Shawi and Senior, 1993). The lack of effect of the hydrophobic agents may be due to an interaction of hydrophobic detergent with the drug-substrate binding site(s) on Pgp. Alternatively, the conformation of soluble Pgp may not be suitable for substrate-induced activation. The requirement of a membrane environment for the substrate-induced stimulation has also been reported for Pgp purified from Chinese Hamster ovary cells (Shapiro and Ling, 1994; Urbatsch *et al.*, 1994) and other members of the superfamily of ABC transporters such as histidine and maltose permeases (Bishop *et al.*, 1989;

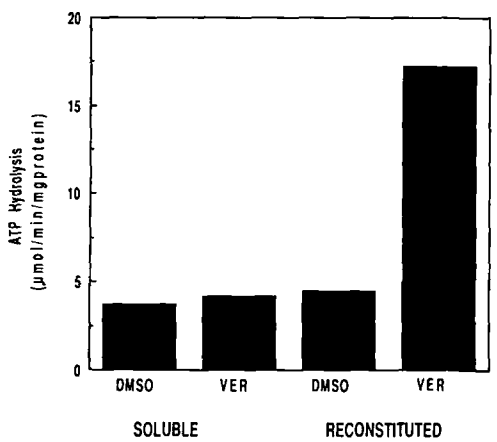

Fig. 1. Drug-stimulated ATPase activity of soluble and reconstituted purified P-glycoprotein. Purified human P-glycoprotein was reconstituted into proteoliposomes by the detergent-dilution method. P-glycoprotein-ATPase activity in detergent solution and proteoliposomes, in the presence and absence of 0.1 mM vanadate, was measured (Ambudkar *et al.*, 1992). DMSO, dimethylsulfoxide, vehicle control; VER, 100 μM verapamil. Only the vanadate-sensitive activities are shown.

Davidson *et al.*, 1992). It is tempting to postulate that this property may turn out to be the diagnostic feature of these transporters.

Several cytotoxic drugs, such as vinblastine, vincristine, and daunorubicin, and reversing agents such as verapamil, and quinidine, stimulate the ATPase activity of purified Pgp in a dose-dependent manner. However, colchicine is only marginally effective as a stimulator of Pgp-ATPase activity even in Chinese Hamster cells, which are selected in high concentrations of this drug (Ambudkar *et al.*, 1992; Sharom *et al.*, 1993; Shapiro and Ling, 1994). The reason for this discrepancy is not presently known. There is a wide variation in the concentrations of drugs required for half-maximal stimulation of ATPase activity of human or rodent Pgp (Table 1 in Shapiro and Ling, 1994). As most of the drugs are highly lipid soluble and due to the high lipid-to-protein ratio (300–1500) of proteoliposomes, a significant portion of drugs may be bound to the lipid. Thus, the concentration required for half-maximal stimulation may be much lower than the observed value. The half-maximal stimulation of human Pgp ATPase by verapamil in native membranes of NIH3T3 transfectants and in proteoliposomes with lipid-to-protein ratios of 400 : 1 and 1400 : 1 is observed at 1, 15, and 35 μM, respectively (Ambudkar, unpublished data). In addition, the properties of Pgp-ATPase may be influenced by the nature of lipid composition as well as its association with lipid, i.e., whether it is associated with membrane fragments or integrated in "transport-competent" sealed vesicles.

The basal or vinblastine-stimulated ATPase activity of Pgp is not affected by cations such as K^+, Na^+, *N*-methyl-D-glucamine, or anions such as Cl^-, SO_4^{2-}, up to 150 mM concentration. However, the divalent cation, Mg^{2+} is required for the ATPase activity and the maximal activity is observed at an ATP/Mg^{2+} molar ratio of 1 : 2. The ATPase activity and the drug transport by Pgp is inhibited by vanadate (Ambudkar *et al.*, 1992; Urbatsch *et al.*, 1994). The mechanism of vanadate inhibition of Pgp is not clear at present. Sulfhydryl reagents, such as *N*-ethylmaleimide, also inhibit the Pgp-ATPase activity and Mg-ATP protects from such inactivation (Urbatsch *et al.*, 1994). This suggests that the sulfhydryl groups may be located within the catalytic site. The analysis of primary structure indicates that the cysteine residue in the Walker A region of both ATP binding domains is conserved in all mammalian Pgps encoded either by *MDR*1 or *MDR*2 genes and also in some of the other ABC transporters (Chen *et al.*, 1986; Gros *et al.*, 1986; Doige and Ames, 1993; Higgins, 1992; and Hyde *et al.*, 1990). It is not clear at present whether cysteine(s) play a direct role in Pgp function or whether the inhibition by maleimides is due to introduction of bulky groups in the ATP binding pocket. Directed mutagenesis of these cysteines will help to elucidate their role in Pgp function. Although it is known that the ATP-binding domain alone is sufficient for ATP binding, it is not clear whether other parts of the molecule, such as the transmembrane region(s), are also required for ATP hydrolysis. The availability of large quantities of pure ATP-binding domains by overexpression in a heterologous expression system (see Evans *et al.*, this issue) may help resolve some of these issues.

TRANSPORT OF [^{3}H]VINBLASTINE BY PROTEOLIPOSOMES RECONSTITUTED WITH PURIFIED P-GLYCOPROTEIN

During reconstitution, Pgp is incorporated in the artificial membrane with inside-out orientation (see above). Hence, it is convenient to assay transport function by following ATP-dependent accumulation of [^{3}H]vinblastine in the proteoliposomes [this is equivalent to the efflux of vinblastine from cells]. We initially used proteoliposomes prepared by the detergent-dilution method (lipid : protein, 1200–1500 : 1). Although, the drug-stimulatable ATPase activity could be measured, we failed to detect ATP-dependent vinblastine accumulation, most likely due to the high level of nonspecific binding of hydrophobic vinblastine to the phospholipid vesicles. Recently, we have used proteoliposomes prepared by detergent dialysis followed by Sephadex-G50 chromatography. By this method the lipid-to-protein ratio in proteoliposomes is significantly reduced to 300–400 : 1. Proteoliposomes reconstituted with purified Pgp accumulated 4 to 5-fold more [^{3}H]vinblastine in the presence of ATP and this accumulation is totally blocked by vanadate, indicating that the hydrolysis of ATP is required for transport. In addition, omission of 5 mM $MgCl_2$ from the assay medium also gave similar results. The radioactivity associated with liposomes is not affected by ATP. The ATP-dependent drug accumulation reaches a steady-state level within 10 min. Vinblastine accumulation is inhibited significantly by verapamil and daunorubicin and only marginally by cholchicine,

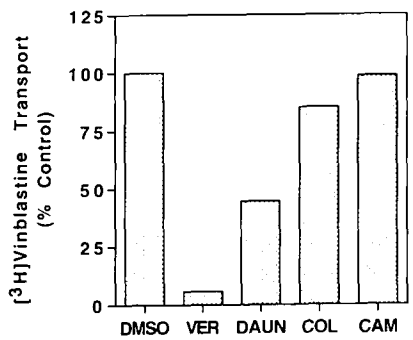

Fig. 2. Effects of selected drugs on vinblastine transport by proteoliposomes. Proteoliposomes containing human pure P-glycoprotein were prepared by detergent-dialysis followed by Sephadex-G50 chromatography. The ATP-dependent [^{3}H]vinblastine accumulation by proteoliposomes in the absence or presence of 5 μM drug was determined. At steady state in control (DMSO treated) proteoliposomes the ATP-dependent vinblastine accumulation level was 120 pmol/mg protein. VER, verapamil; DAUN, daunorubicin; COL, colchicine; CAM, camptothecin.

but not by camptothecin, which is not a substrate for Pgp (Fig. 2; Ambudkar, S.V., *et al.*, unpublished data). This pattern of inhibition is similar to that observed with the plasma membrane vesicles of KB-V1 cells (Horio *et al.*, 1988). These proteoliposomes also exhibited vinblastine-stimulated ATPase activity although at a reduced level (3–5 μmol/min/mg of protein). This is probably due to loss of activity due to prolonged (18 h) incubation during dialysis. Thus, these experiments provide compelling evidence that the purified protein retains its biological activity and that Pgp, the *MDR* gene product, functions as a multidrug transporter. Similarly, Sharom and coworkers have demonstrated ATP-dependent [^{3}H]colchicine transport in proteoliposomes reconstituted with partially purified Pgp from Chinese Hamster ovary cells (Sharom *et al.*, 1993). In these proteoliposomes about 50% of Pgp molecules are reconstituted with an inside-out orientation. The ATP-dependent [^{3}H]colchicine transport is osmotically sensitive and also inhibited by vinblastine, verapamil, or daunomycin.

The detailed kinetic analysis of the drug transport process as well as the stoichiometry of ATP hydrolysis and vinblastine transport have not yet been determined. In general, the hydrophobic nature of Pgp substrates leads to increased nonspecific binding to lipid due to the high lipid-to-protein ratio in proteoliposomes. Thus, the drug transport rate in proteoliposomes is significantly underestimated. In addition, the yield of pure human Pgp is quite low at present, which makes it difficult to reduce the lipid-to-protein ratio in proteoliposomes. The availability of a large quantity of pure recombinant Pgp by using a heterologous expression system (see Evans *et al.*, this issue) will greatly help to overcome these obstacles and to assess the mechanism of drug transport by Pgp.

ACKNOWLEDGMENTS

I am grateful to Drs. Michael Gottesman and Ira Pastan for advice and encouragement throughout the course of this work. I thank Dr. Peter Maloney for helpful discussions. This work was supported by NIH research grant 1R55 CA57154-01 and American Cancer Society research grant BE-157.

REFERENCES

Abraham, E. H., Prat, A. G., Gerweck, L., Seneveratne, T., Arceci, R. J., Kramer, R., Guidotti, G., and Cantiello, H. F. (1993). *Proc. Natl. Acad. Sci. USA* **90**, 312–316.

Al-Shawi, M. K., and Senior, A. E. (1993). *J. Biol. Chem.* **268**, 4197–4206.

Al-Shawi, M. K., Urbatsch, I. L., Senior, A. E. (1994). *J. Biol. Chem.* **269**, 8986–8992.

Ambudkar, S. V., and Maloney, P. C. (1986a). *J. Biol. Chem.* **261**, 10079–10086.

Ambudkar, S. V., and Maloney, P. C. (1986b). *Methods Enzymol.* **125**, 558–563.

Ambudkar, S. V., Lynn, A. R., Maloney, P. C., and Rosen, B. P. (1986). *J. Biol. Chem.* **261**, 15596–15600.

Ambudkar, S. V., Anantharam, V., and Maloney, P. C. (1990). *J. Biol. Chem.* **265**, 12287–12292.

Ambudkar, S. V., Lelong, I. H., Zhang, J., Cardarelli, C. O., Gottesman, M. M., and Pastan, I. (1992). *Proc. Natl. Acad. Sci. USA* **89**, 8472–8476.

Ambudkar, S. V., Pastan, I., and Gottesman, M. M. (1995). In *Drug Transport in Antimicrobial and Anticancer Chemotherapy: Cellular and Biochemical Aspects of Multidrug Resistance* (Georgapapadakou, N. H., ed.). Marcel Dekker, New York, 525–547.

Bear, C. E., Li, C., Kartner, N., Bridges, R. J., Jensen, T. J., Ramjeesing, M., and Riordan, J. R. (1992). *Cell* **68**, 809–818.

Bishop, L., Agbayani, R., Ambudkar, S. V., Maloney, P. C., and Ames, G. F-L. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 6953–6957.

Chen, C-J, Chin, C. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson I. B. (1986). *Cell* **47**, 381–389.

Chen, C. J., Clark, D., Ueda, K., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1990). *J. Biol. Chem.* **265**, 506–514.

Childs, S., and Ling, V. (1994). In *Important Advances in Oncology: The MDR Superfamily of Genes and its Biological Implications* (DeVita, V. T., Hellman, S., and Rosenberg, S. A., eds.). Lippincott, Philadelphia, pp. 21–36.

Cole, S. P. C., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M. V., and Deeley, R. G. (1992). *Science*, **258**, 1650–1654.

Dano, K. (1973). *Biochim. Biophys. Acta*, **323**, 466–483.
Davidson, A. L., and Nikaido, H. (1990). *J. Biol. Chem.* **265**, 4254–4260.
Davidson, A. L., Shuman, H. A., and Nikaido, H. (1992). *Proc. Natl. Acad. Sci. USA* **89**, 2360–2364.
Decottignies, A., Kolackowski, M., Balzi, E., and Goffeau, A. (1994). *J. Biol. Chem.* **269**, 12797–12803.
Dhir, R., and Gros P. (1992). *Biochemistry* **31**, 6103–6110.
Doige, C. A., and Ames, G. F. L. (1993). *Annu. Rev. Microbiol.* **47**, 291–319.
Doige, C. A., Yu, X. H., and Sharom, F. J. (1992). *Biochim. Biophys. Acta* **1109**, 149–160.
D'Souza, P., Ambudkar, S. V., August, J. T., and Maloney, P. C. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 6980–6984.
Endicott J. A., and Ling V. (1989). *Annu. Rev. Biochem.* **58**, 137–171.
Eytan, G. D., Borgnia, M. J., Regev, R., and Assaraf, Y. G. (1994). *J. Biol. Chem.* **269**, 26058–26565.
Fojo, A. T., Akiyama, S., Gottesman, M. M., and Pastan, I. (1985). *Cancer Res.* **45**, 3002–3007.
Foote, S. J., Thompson, J. K., Conman, A. F., and Kemp, D. J. (1989). *Cell* **57**, 921–930.
Gartner, J., Moser, H., and Valle, D. (1992). *Nature Genetics* **1**, 16–23.
Germann, U. A., Willingham, M. C., Pastan, I., and Gottesman, M. M. (1990). *Biochemistry* **29**, 2295–2303.
Gill, D. R., Hyde, S. C., Higgins, C. F., Valverde, M. A., Mintenig, G. M., and Sepúlveda, F. V. (1992). *Cell* **71**, 23–32.
Glisson, B., Gupta, R., Smallwood-Kentro, S., and Ross, W. (1986). *Cancer Res.* **46**, 1934–1938.
Gottesman, M. M., and Pastan, I. (1988). *J. Biol. Chem.* **263**, 12163–12166.
Gottesman, M. M., and Pastan, I. (1993). *Annu. Rev. Biochem.* **62**, 385–427.
Gros, P., Ben Neriah, Y., Croop, J., and Housman, D. E. (1986). *Nature* (*London*) **323**, 728–731.
Gros, P., Croop, J., and Housman, D. E. (1986). *Cell* **47**, 371–380.
Higgins, C. F. (1992). *Annu. Rev. Cell Biol.* **8**, 67–113.
Higgins, C. F., and Gottesman, M. M. (1992). *Trends Biochem. Sci* **17**, 18–21.
Horio, M., Gottesman, M. M., and Pastan, I. (1988). *Proc. Natl. Acad. Sci. USA* **85**, 3580–3584.
Hyde, S. C., Emsley, P., Hartshorn, M. J., Mimmack, M. M., Gileadi, U., Pearce, S. R., Gallagher, M. P., Gill, D. R., Hubbard, R. E., and Higgins, C. F. (1990). *Nature* (*London*) **346**, 362–365.
Juliano, R. L., and Ling, V. (1976). *Biochim. Biophys. Acta* **455**, 152–162.
Lockwich, T., Ambudkar, S. V., Chauthaiwale, J., and Ambudkar, I. S. (1995). *Biophys. J.*, **68**, A387.
Luz, J. G., Wei, L-Y., Basu, S., and Roepe, P. D. (1994). *Biochemistry* **33**, 7239–7249.
Maloney, P. C., and Ambudkar, S. V. (1989). *Arch. Biochem. Biophys.* **269**, 1–10.
McGrath, J. P., and Varshavsky, A. (1989). *Nature* (*London*) **340**, 400–404.
Monaco, J. J., Cho, S., and Attaya, M. (1990). *Science* **250**, 1723–1726.
Moscow, J. A., and Cowan, K. H. (1988). *J. Natl. Cancer Inst.* **80**, 14–20.
Mosser, J., Douar, A-M., Sarde, C-O., Kioschis, P., Feil, R., Moser, H., Poustka, A-M., Mandel, J-L., Aubourg, P. (1993). *Nature* (*London*) **361**, 726–730.
Newman, M. J., and Wilson, T. H. (1980). *J. Biol. Chem.* **255**, 10583–10586.
Ng, W. F., Sarangi, F., Zastawny, R. L., Veinot-Drebot, L. and Ling, V. (1989). *Mol. Cell. Biol.* **9**, 1224–1232.
Pedersen, P. L., and Amzel, L. M. (1993). *J. Biol. Chem.* **268**, 9937–9940.
Racker, E., Violand, B., O'Neil, S., Alfonzo, M., and Telford, J. (1979). *Arch. Biochem. Biophys.* **198**, 470–477
Rasola, A., Galietta, L. J. V., Gruenert, D. C., Romeo, G. (1994). *J. Biol. Chem.* **269**, 1432–1436.
Riordan, J. R. (1993). *Annu. Rev. Physiol.* **55**, 609–630.
Riordan, J. R., Rommens, J. M., Kerem, B. S., Alon, N., Rozmahel, R., Grzelczak, A., Aielenski, J., Lok, S., Plavsic, N., Chou, J-I., Drumm, M. L., Iannuzzi, M. C., Collins, F. S., and Tsui, L-C. (1989). *Science* **245**, 1066–1073.
Roepe, P. D., Wei, L. Y., Cruz, J., and Carlson, D. (1993). *Biochemistry* **32**, 11042–11056.
Roninson, I. B. (1991). *Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells*, Plenum Press, New York.
Ruan, Z-S., Vellareddy, A., Crawford, I. T., Ambudkar, S. V., Rhee, S. Y., Allison, M. J., and Maloney, P. C. (1992). *J. Biol. Chem.* **267**, 10537–10543.
Ruetz, S., and Gros, P. (1994). *Cell* **77**, 1071–1081.
Sarkadi, B., Price, E. M., Boucher, R. C., Germann, U. A., and Scarborough, G. A. (1992). *J. Biol. Chem.* **267**, 4854–4858.
Shapiro, A., and Ling, V. (1994). *J. Biol. Chem.* **269**, 3745–3754.
Sharom, F. J., Yu, X., and Doige, C. A. (1993). *J. Biol. Chem.* **268**, 24197–24202.
Shyamala, V., Baichwald, V., Gant, T. W., Beall, E., and Ames, G. F-L. (1991). *J. Biol. Chem.* **266**, 18714–18719.
Smit, J. J. M., Schinkel, A. H., OudeElferink, R. P. J., Groen, A. K., Wagenaar, E., van Deemter, L., Mol, C. A. M., Ottenhoff, R., van der Lugt, N. M. T., van Roon, M. A., van der Valk, M. A., Offerhaus, G. J. A., Berns, A. J. M., and Borst, P. (1993). *Cell* **75**, 451–462.
Trowsdale, J., Hanson, I., Mockridge, I., Beck, S., Townsend, A., and Kelly, A. (1990). *Nature* (*London*) **348**, 741–743.
Ueda, K., Cornwell, M. M., Gottesman, M. M., Pastan, I., Roninson, I. B., Ling, V., and Riordan, J. R. (1986). *Biochem. Biophys. Res. Commun.* **141**, 956–962.
Ueda, K., Cardarelli, C., Gottesman, M. M., and Pastan, I. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 3004–3008.
Urbatsch, I. L., Al-Shawi, M. K., and Senior, A. E. (1994). *Biochemistry* **33**, 7069–7076.
Valverde, M. A., Diáz, M., Sepúlveda, F. V., Gill, D. R., Hyde, S. C., and Higgins, C. F. (1992). *Nature* (*London*) **355**, 830–833.
Wang, X. Y., Wall, D. M., Parkin, J. D., Zalcberg, J. R., and Kemm, R. E. (1994). *Clin. Exp. Pharmacol. Physiol.* **21**, 101–108.
Willingham, M. C., Cornwell, M. M., Cardarelli, C. O., Gottesman, M. M., and Pastan, I. (1986). *Cancer Res.* **46**, 5941–5946.
Zeidel, M. L., Ambudkar, S. V., Smith, B. L., and Argre, P. (1992). *Biochemistry* **31**, 7436–7440.