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Using Purified P-glycoprotein to Understand Multidrug Resistance

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Since P-glycoprotein was discovered almost 20 years ago, its causative role in multidrug resistance has been established, but central problems of its biochemistry have not been definitively resolved. Recently, major advances have been made in P-glycoprotein biochemistry with the use of purified and reconstituted P-glycoprotein, as well as membranes from non-mammalian cells containing heterologously expressed P-glycoprotein. In this review we describe recent findings using these systems which are elucidating the molecular mechanism of P-glycoprotein-mediated drug transport.

KEY WORDS: P-glycoprotein; multidrug resistance.

INTRODUCTION: DIRECT VERSUS INDIRECT DRUG TRANSPORT BY P-GLYCOPROTEIN

P-glycoprotein (P-gp, also known as MDR) is a 170-kDa plasma membrane glycoprotein. Expression of class 1 or 2 P-glycoprotein renders cells resistant to a wide spectrum of lipophilic and amphiphilic cytotoxic compounds due to reduced intracellular accumulation. This P-glycoprotein-mediated multidrug resistance (MDR) is thought to be an important cause of failure of cancer chemotherapy, with resulting morbidity and mortality (Gottesman and Pastan, 1993; Childs and Ling, 1994).

The most unusual feature of P-glycoprotein is the enormous variety of chemicals to which it confers resistance. Some examples are given in Gottesman and Pastan (1993). Many of the most commonly used chemotherapy drugs, such as doxorubicin, daunorubicin, vincristine, vinblastine, etoposide, and taxol, are among them. As a result, the mechanism of action of P-glycoprotein is of profound biochemical, as well as clinical, interest.

One of the major outstanding issues concerning P-glycoprotein is whether it functions as a direct drug transporter of broad specificity or whether it affects drug accumulation by an indirect mechanism. Such information will be of primary importance when considering methods of overcoming P-glycoprotein-mediated multidrug resistance in the clinic.

P-glycoprotein belongs to the ATP binding cassette (ABC) superfamily, the members of which are listed in Childs and Ling (1994). Among them are several ATP-dependent, substrate-specific transporters. This relationship engendered the idea that P-glycoprotein is a direct multidrug transporter. Hypothetical direct transport mechanisms include the classical transporter mechanism according to which substrates are encountered in the cytoplasm and pumped directly out of the cell, the "hydrophobic vacuum cleaner" model (Gottesman and Pastan, 1993) according to which P-glycoprotein encounters its lipid-soluble substrates in the plasma membrane and pumps them out of the cell, the "flippase" model (Higgins and Gottesman, 1992; Higgins, 1994) according to which P-glycoprotein transports substrates from the inner leaflet to the outer leaflet of the plasma membrane, and a conjugation model

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in which P-glycoprotein recognizes a common chemical modification such as conjugation to glutathione (West, 1990). The extraordinarily broad substrate specificity of P-glycoprotein, on the other hand, has led to the proposal of alternative, indirect mechanisms by which P-glycoprotein reduces intracellular drug accumulation. One notable hypothesis is that P-glycoprotein regulates the plasma membrane pH gradient (ΔpH) and/or electrical potential ($\Delta\Psi$) (Roepe, 1992; Luz *et al.*, 1994; Roepe *et al.*, 1993, 1994). Many of the known P-glycoprotein substrates are weak bases having positive charge at neutral pH. An increased intracellular pH or reduced negative-inside $\Delta\Psi$ would therefore result in reduced intracellular accumulation of these compounds by passive diffusion across the plasma membrane.

The direct transport hypothesis is supported by several lines of evidence. Photoaffinity analogs of several compounds belonging to the P-glycoprotein-mediated multidrug resistance spectrum, including Rhodamine 123 (Nare *et al.*, 1994; Raviv *et al.*, 1990), verapamil (Safa, 1988), *Vinca* alkaloids (Safa *et al.*, 1986; Cornwell *et al.*, 1986), cyclosporin A (Foxwell *et al.*, 1989), anthracyclines (Beck and Qian, 1992; Busche *et al.*, 1989), forskolin (Morris *et al.*, 1991), azidopine (Safa *et al.*, 1987), prazosin (Greenberger *et al.*, 1990), progesterone (Qian and Beck, 1990), and phenothiazines (Safa *et al.*, 1994), bind with high specificity to P-glycoprotein in the plasma membrane. Single amino acid mutations in P-glycoprotein alter the relative resistance profile of cells to various drugs (Loo and Clarke, 1993, 1994a; Devine *et al.*, 1992; Safa *et al.*, 1990; Gros *et al.*, 1991; Hoof *et al.*, 1994; Devine and Melera, 1994). P-glycoprotein is able to substitute for STE6 as a transporter of yeast a mating factor peptide (Raymond *et al.*, 1992), suggesting direct and broad substrate recognition. P-glycoprotein-expressing 2780^{AD} cells can extrude daunorubicin against a concentration gradient (Lankelma *et al.*, 1990), demonstrating active transport. Yeast secretory vesicles containing mammalian P-glycoprotein are capable of ATP hydrolysis-dependent, concentrative drug uptake under conditions that preclude formation of a favourable ΔpH or $\Delta\Psi$ (Ruetz and Gros, 1994).

In support of the ΔpH/$\Delta\Psi$ hypothesis of indirect drug transport is the observation that multidrug resistant cells often have higher cytoplasmic pH (Keizer and Joenje, 1989; Boscoboinik *et al.*, 1990; Thiebaut *et al.*, 1990, Roepe, 1992; Wei and Roepe, 1994) and reduced plasma membrane $\Delta\Psi$ (Roepe *et al.*, 1993; Hasmann *et al.*, 1989) as compared to their drug-sensitive parents. Experimentally increasing or decreasing the intracellular pH can reduce or increase, respectively, doxorubicin and daunorubicin accumulation (Simon *et al.*, 1994). Observations of a chloride channel activity of P-glycoprotein (Valverbe *et al.*, 1992) and alterations in Na^+-independent Cl^-/HCO_3^- exchange (Luz *et al.*, 1994) and Cl^--dependent intracellular pH regulation in P-glycoprotein-transfected cells (Roepe *et al.*, 1994) suggest a pathway by which P-glycoprotein could influence ΔpH and/or $\Delta\Psi$. Thus the ΔpH/$\Delta\Psi$ hypothesis can partially account for the diversity of cationic compounds in the multidrug resistance spectrum. *Ad hoc* explanations, such as pH-dependent target binding (Roepe, 1992), are required, however, to explain reduced accumulation of uncharged compounds. The hypothesis also fails to account for the direct and specific binding of compounds in the multidrug resistance spectrum to P-glycoprotein (see above) and the drug specificity changes resulting from single amino acid substitutions (see above). Furthermore, Altenberg *et al.* (1993, 1994) found no coupling between Rhodamine 123 transport and intracellular pH or fluxes of Cl^-, Na^+, and HCO_3^- in P-glycoprotein-expressing cells. Similarly, Boscoboinik *et al.* (1990) found no correspondence between reversal of drug resistance by chemosensitizers and alteration of intracellular pH, nor between cytoplasmic acidification by amiloride and reversal of drug resistance. Also, the initial observations of a chloride channel activity of P-glycoprotein have not been supported by later work (Rasola *et al.*, 1994; Dong *et al.*, 1994). Several mobile ionophores inhibited drug efflux from P-glycoprotein-expressing multidrug-resistant K562 cells under conditions in which $\Delta\Psi$ and ΔpH were unchanged (Borrel *et al.*, 1994), indicating a specific effect on P-glycoprotein. Finally, as mentioned above, the P-glycoprotein-containing yeast secretory vesicle system experimentally eliminated the necessity of a ΔpH or $\Delta\Psi$ (Ruetz and Gros, 1994) for drug transport. An indirect mechanism may contribute to drug resistance in some cases, but it does not appear to be an essential feature of P-glycoprotein-mediated multidrug resistance.

Thus, the current evidence from complex systems support the ability of P-glycoprotein to function as a direct multidrug transporter. Definitive understanding, and differentiation between alternative models of direct transport, however, will most readily come from studies of drug transport by purified, reconstituted P-glycoprotein, where complete control can be

exerted over experimental conditions. In addition, the use of purified P-glycoprotein will allow assessment of hypotheses of alternative P-glycoprotein functions, including chloride channel (Valverde *et al.*, 1992; Gill *et al.*, 1992) and ATP channel (Abraham *et al.*, 1993), which are based on *in vivo* experiments.

PURIFICATION AND ATPase ACTIVITY OF P-GLYCOPROTEIN

P-glycoprotein was identified as a plasma membrane protein of multidrug-resistant cell lines (Juliano and Ling, 1976). It was first purified from cultured Chinese hamster ovary cells by Riordan and Ling (1979). At that time, the activity of P-glycoprotein was not known, so no functional assays could be performed. Although the drug accumulation defect of multidrug-resistant cells was known to be energy dependent (See *et al.*, 1974), sequence information was not yet available to suggest that P-glycoprotein was an ATPase.

The next reported purification of P-glycoprotein used immunoaffinity chromatography with the monoclonal antibody MRK-16 (Hamada and Tsuruo, 1988a). The protein was extracted from multidrug-resistant cultured human leukemia cells with CHAPS, a gentle zwitterionic detergent. By that time, P-glycoprotein had been shown to be responsible for multidrug resistance. Drug-sensitive cells acquired multidrug resistance when transfected with the gene for P-glycoprotein (Gros *et al.*, 1986a; Ueda *et al.*, 1987). The deduced amino acid sequence of P-glycoprotein showed it to be related to several transport proteins and to have two putative nucleotide binding domains (Chen *et al.*, 1986; Gros *et al.*, 1986b). It was speculated, therefore, that P-glycoprotein functioned as an ATP-dependent drug transporter. Accordingly, Hamada and Tsuruo studied the ATPase activity of their purified protein, which was very low at 1.2 nmol/min-mg (Hamada and Tsuruo, 1988a), and of MRK-16-immunoprecipitated P-glycoprotein, which was higher at 50 nmol/min-mg (Hamada and Tsuruo, 1988b). The ATPase activity of the immunoprecipitated P-glycoprotein was stimulated 50% by micromolar concentrations of verapamil and trifluoperazine.

A β-galactosidase-P-glycoprotein fusion protein was purified by immunoprecipitation (Shimabuka *et al.*, 1992). This preparation had a specific ATPase activity of 180 nmol/min-mg. The N-terminal half of the protein, prepared by the same method, had an ATPase activity of 148 nmol/min-mg. The ATPase activity of the whole or N-terminal half of P-glycoprotein fused to β-galactosidase was stimulated by phospholipids.

Partially purified preparations of P-glycoprotein extracted with detergent from cultured mammalian cell membranes were reported (Ambudkar *et al.*, 1992; Doige *et al.*, 1992), with further characterization of their ATPase activities. Doige *et al.* (1992) used a CHAPS extraction procedure with multidrug-resistant Chinese hamster ovary CH^RC5 cells, resulting in partially purified P-glycoprotein with an extrapolated specific activity of about 500 nmol/min-mg. Ambudkar *et al.* (1992) used an octylglucoside extraction technique in the presence of phospholipids and glycerol combined with lectin affinity chromatography to obtain partially purified P-glycoprotein with an extrapolated specific ATPase activity of about 1 μmol/min-mg. Ambudkar *et al.* (1992) also reconstituted their P-glycoprotein into lipid vesicles with retention of ATPase activity. Whereas Doige *et al.* (1992) reproduced the 50% stimulation of the ATPase activity of P-glycoprotein in detergent solution seen by Hamada and Tsuruo (1988b), Ambudkar *et al.* (1992) found higher degrees of stimulation by vinblastine and some chemosensitizers when the P-glycoprotein was reconstituted.

The ATPase activity of P-glycoprotein has also been studied in plasma membranes purified from hamster cells (Al-Shawi and Senior, 1993; Garrigos *et al.*, 1993) or insect cells expressing human P-glycoprotein (Sarkadi *et al.*, 1992; Loo and Clarke, 1994b; Rao and Scarborough, 1994). In each case, at least twofold stimulation of the ATPase activity was observed in the presence of some chemosensitizers. Loo and Clarke (1994b) found that both halves of P-glycoprotein were necessary for drug stimulation of the ATPase activity, but not for the basal ATPase activity.

The maximum unstimulated specific ATPase activity reported for P-glycoprotein is about 3 μmol/min-mg, comparable to many membrane ATPases involved in transport. The foregoing work also established that the ATPase activity of P-glycoprotein is inhibited by orthovanadate and sulfhydryl modifying reagents. Al-Shawi and Senior (1993) showed that inhibition of the ATPase activity of P-glycoprotein by *N*-ethylmaleimide could be prevented by coincubation with MgATP, suggesting that the relevant cysteine residues are those in or near the nucleotide binding domains.

Although much progress was made in the biochemical characterization of the ATPase activity of P-glycoprotein, uncertainty remained regarding the identification of the ATPase activity entirely with P-glycoprotein because of the use of only partially purified preparations or whole plasma membranes. We characterized the ATPase activity of a highly purified preparation of P-glycoprotein in detergent solution and after reconstitution into artificial lipid vesicles (Shapiro and Ling, 1994). The P-glycoprotein was purified from plasma membranes of multidrug-resistant Chinese hamster ovary CH^RB30 cells using extraction with Zwittergent 3-12, anion exchange chromatography, and immunoaffinity chromatography with the monoclonal antibody C494. Because of the high purity of our preparation and characterization of inhibitor sensitivities, contaminating ATPases could be excluded. Subsequently, Urbatsch *et al.* (1994) reported on the ATPases activity of virtually pure, reconstituted P-glycoprotein. Their protein was purified by extraction of highly purified multidrug-resistant hamster cell plasma membranes with octylglucoside followed by dye affinity chromatography. The specific ATPase activity of their preparation was high, 2.1 μmol/min-mg. Our purified P-glycoprotein had a lower specific ATPase activity of 320 nmol/min-mg. Importantly, however, the degree by which the ATPase activity of our purified, reconstituted P-glycoprotein was stimulated by chemosensitizers was considerably greater, up to 10-fold by verapamil, than that of any other ATPase-active P-glycoprotein preparation.

CHEMOSENSITIZERS AND THE ATPase ACTIVITY OF P-GLYCOPROTEIN

It is generally found with ATP-dependent transport systems that ATP hydrolysis and transport are tightly coupled such that ATP is not hydrolyzed unless substrate is transported. It is not yet known whether ATP hydrolysis by P-glycoprotein is tightly coupled to transport *in vivo*. If so, the high basal rates of ATP hydrolysis by most P-glycoprotein preparations may indicate damage to the protein. On the other hand, ATP hydrolysis by P-glycoprotein may be only loosely coupled to transport. In that case, constitutively high basal ATPase activity of P-glycoprotein in multidrug-resistant cells would result in a high rate of cytoplasmic ATP consumption. Indeed, this phenomenon has been observed *in vivo* (Broxterman and Pinedo, 1991). The physiological substrate of P-glycoprotein, if there is one, has not yet been identified, however, so it is possible that ATP consumption by P-glycoprotein *in vivo* is due to active transport.

Stimulation of the ATPase activity of P-glycoprotein by some chemosensitizers and MDR drugs is an intriguing phenomenon. Stimulation of ATPase activity by substrates for transport is expected if ATP hydrolysis and transport are coupled, however loosely, yet not all known substrates of P-glycoprotein significantly stimulate ATP hydrolysis (Shapiro and Ling, 1994 and references therein; Rao and Scarborough, 1994). Some of the chemosensitizers that stimulate ATP hydrolysis, such as verapamil, are also transported by P-glycoprotein (Yusa and Tsuruo, 1989; Cano-Gauci and Riordan, 1987), suggesting that they may act as competitors with drugs for transport. Some ATPase-stimulating chemosensitizers, such as progesterone (Rao *et al.*, 1994), are not transported, however (Yang *et al.*, 1990; Ueda *et al.*, 1992). They may function by further uncoupling transport from ATP hydrolysis.

Some compounds that act as chemosensitizers inhibit ATP hydrolysis by P-glycoprotein. We have found that the flavonoid quercetin increases accumulation of the P-glycoprotein substrate Hoechst 33342 by multidrug-resistant CH^RC5 cells (Shapiro, A. B., and Ling, V., in preparation). We have also found that Rhodamine 123 accumulation by CH^RB30 cells is enhanced by *n*-(9-anthroyloxy) stearic acids ($n = 3$, 6, 7, 9, 12) (Pawagi, A., Shapiro, A. B., and Ling, V., submitted). In both cases, the chemosensitizers inhibit the ATPase activity of purified P-glycoprotein. Thus there is a complex relationship between chemosensitizers and the rate of ATP hydrolysis by P-glycoprotein.

DRUG TRANSPORT BY PURIFIED, RECONSTITUTED P-GLYCOPROTEIN

The purported function of P-glycoprotein is to reduce intracellular accumulation of cytotoxic lipophilic agents. Most frequently, P-glycoprotein is assumed to act as a drug pump, although there is uncertainty as to the means by which P-glycoprotein brings about reduced intracellular drug accumulation (see Gottesman and Pastan, 1993 and above). Drug transport has been measured in plasma membrane vesicles from multidrug-resistant cells expressing P-glycoprotein (Doige and Sharom, 1992; Guiral *et al.*,

1994; Horio *et al.*, 1988; Kamimoto *et al.*, 1989) and vesicles from yeast expressing P-glycoprotein (Ruetz *et al.*, 1993; Ruetz and Gros, 1994). These studies have shown that drug transport by vesicles containing P-glycoprotein is dependent upon ATP hydrolysis and inhibited by chemosensitizers. Because these systems contained many components, however, it could not be concluded that P-glycoprotein, acting alone, was sufficient for drug transport. It has been suggested that P-glycoprotein-mediated drug transport requires other cellular activities, such as cytochrome P450 (Gatmaitan and Arias, 1993) or glutathione-*S*-transferase (West, 1990). Also, these systems did not distinguish between transport from the aqueous versus lipid phase, an important mechanistic issue.

Only one study of drug transport by partially purified P-glycoprotein reconstituted into artificial lipid vesicles has been published to date (Sharom *et al.*, 1993). ATP-dependent, concentrative uptake of colchicine was demonstrated. This work also did not address the question of whether transport was from the aqueous or lipid phase. Furthermore, because impurities remained in the partially purified P-glycoprotein, it could not be concluded definitively that P-glycoprotein alone was sufficient for drug transport.

We have recently succeeded in demonstrating ATP-dependent, chemosensitizer inhibited, concentrative transport of two P-glycoprotein substrates, daunorubicin and Hoechst 33342, by highly purified, reconstituted P-glycoprotein (Shapiro and Ling, manuscript submitted), thereby proving that P-glycoprotein by itself is capable of active drug transport. We used fluorescence methods that made it possible to follow transport in real time and, in the case of Hoechst 33342, to show that the transported substrate was removed from the lipid phase. This result supports the "hydrophobic vacuum cleaner" hypothesis of P-glycoprotein function (Gottesman and Pastan, 1993), according to which P-glycoprotein recognizes its lipophilic substrates within the plasma membrane and pumps them out of the cell, in some cases before they enter the cytoplasm (Homolya *et al.*, 1993). Furthermore, by using a protonophore as well as measuring the intraliposomal pH during transport, we were able to eliminate a requirement for a pH gradient for transport.

The use of highly purified P-glycoprotein reconstituted into artificial lipid vesicles provides a powerful system for studying in detail the function of P-glycoprotein. We have used our system to examine the direct effects of chemosensitizers of P-glycoprotein. We have recently studied the effects of the flavonoid quercetin. This compound and two other flavonoids have been reported to enhance efflux of doxorubicin and 7,12-dimethylbenz(a)anthracene from multidrug-resistant MCF-7 and HCT-15 cells (Phang *et al.*, 1993; Critchfield *et al.*, 1994). Scambia *et al.* (1994) found, however, that quercetin inhibited efflux of doxorubicin from MCF-7 cells. In our hands, quercetin acted as a chemosensitizer, increasing accumulation and decreasing efflux of the P-glycoprotein substrate Hoechst 33342 by multidrug-resistant CH^RC5 cells. We used our transport assay with purified, reconstituted P-glycoprotein to study the direct effects of quercetin on P-glycoprotein (Shapiro and Ling, in preparation). We found that quercetin potently inhibited Hoechst 33342 transport in this system, and that this inhibition was due to inhibition by quercetin of the ATPase activity of P-glycoprotein, probably combined with formation of a noncovalent, nontransported complex between quercetin and Hoechst 33342. The use of purified, reconstituted P-glycoprotein to study drug transport by P-glycoprotein enabled us to separate the direct effects of quercetin on P-glycoprotein from the numerous other effects of quercetin on living cells.

CONCLUSION

Recent progress in P-glycoprotein biochemistry, particularly the availability of purified and reconstituted, transport-active P-glycoprotein, will pave the way in the near future for important advances in our understanding of this important and enigmatic protein. Of particular interest will be detailed information regarding the mechanisms of transport and chemosensitization, but other aspects of P-glycoprotein biochemistry deserve attention.

P-glycoprotein is phosphorylated *in vivo* (Gottesman and Pastan, 1993) but it is not presently clear how phosphorylation affects function. *In vivo* experiments can be difficult to interpret because of the pleiotropic effects on cells of alterations in phosphorylation. With purified, reconstituted P-glycoprotein, however, the effects of changes in phosphorylation on ATPase or transport activities can be readily examined after treating the P-glycoprotein with kinases or phosphatases. Our preliminary experiments indicate slightly enhanced ATPase and Hoechst 33342 transport activities when the P-glycoprotein is

phosphorylated by protein kinases A and C (Shapiro and Ling, unpublished observations).

P-glycoprotein exists *in vivo* in an oligomeric state (Poruchynsky and Ling, 1994), but the functional consequences of oligomerization are unknown. The role of oligomerization could be studied using purified, reconstituted P-glycoprotein by chemical crosslinking or varying the number of P-glycoprotein molecules per liposome.

There is evidence that P-glycoprotein can occur in more than one topological form (Zhang *et al.*, 1993; Zhang and Ling, 1991, 1993; Skach *et al.*, 1993; Bibi and Béjà, 1994), with some of the predicted transmembrane helices being outside the membrane bilayer. The different topologies may represent different functional states of P-glycoprotein. Purified, reconstituted P-glycoprotein could be used to explore this relationship given the availability of a method to determine the topology of the protein and the ability to prepare liposomes containing P-glycoprotein having different transmembrane topologies.

The basis for the extremely broad substrate specificity of P-glycoprotein is currently not well understood. Some effort has been made to define a pharmacophore for recognition by P-glycoprotein (Klopman *et al.*, 1992; Dellinger *et al.*, 1992; Tang-Wai *et al.*, 1993) and to determine or predict the drug binding sites (Greenberger *et al.*, 1991; Pawagi *et al.*, 1994). The availability of purified P-glycoprotein will facilitate identification of drug binding sites by chemical crosslinking and structural methods.

Finally, purified P-glycoprotein is required for efforts to learn about its three-dimensional structure. Valuable structural details could come from electron microscopy, fluorescence resonance energy transfer, spectroscopy, and other techniques.

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