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Heterologous Expression Systems for P-glycoprotein: *E. coli*, Yeast, and Baculovirus

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Chemotherapy, though it remains one of the front-line weapons used to treat human cancer, is often ineffective due to drug resistance mechanisms manifest in tumor cells. One common pattern of drug resistance, characterized by simultaneous resistance to multiple amphipathic, but otherwise structurally dissimilar anticancer drugs, is termed multidrug resistance. Multidrug resistance in various model systems, covering the phylogenetic range from bacteria to man, can be conferred by mammalian P-glycoproteins (PGPs), often termed multidrug transporters. PGPs are 170-kD polytopic membrane proteins, predicted to consist of two homologous halves, each with six membrane spanning regions and one ATP binding site. They are members of the ATP-binding cassette (ABC) superfamily of transporters, and are known to function biochemically as energy-dependent drug efflux pumps. However, much remains to be learned about PGP structure–function relationships, membrane topology, posttranslational regulation, and bioenergetics of drug transport. Much of the recent progress in the study of the human and mouse PGPs has come from heterologous expression systems which offer the benefits of ease of genetic selection and manipulation, and/or short generation times of the organism in which PGPs are expressed, and/or high-level expression of recombinant PGP. Here we review recent studies of PGP in *E. coli*, baculovirus, and yeast systems and evaluate their utility for the study of PGPs, as well as other higher eukaryotic membrane proteins.

KEY WORDS: P-glycoprotein; multidrug resistance; MDR; ATPase; drug transport; ATP-binding cassette (ABC) transporters.

INTRODUCTION

Multidrug resistance (MDR) is a common barrier to successful treatment of cancer in man. The hallmark of clinical MDR is resistance to a broad spectrum of amphipathic but otherwise structurally dissimilar antitumor drugs, often of natural origin. These include the *Vinca* alkaloids, anthracyclines, and epipodophyllotoxins, but not antimetabolites, or alkylating and platinating drugs, which are generally of lower structural complexity and higher water solubility. Work carried out over the last 25 years has now firmly established that MDR can be reproduced in tissue culture and animal models, and is frequently mediated by a complex polytopic membrane protein known alternately as P-glycoprotein (PGP; "P" for

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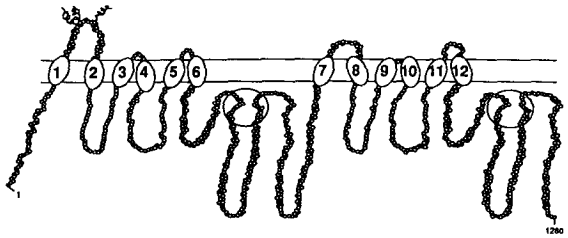

Fig. 1. Topological model of human PGP (*MDR1*) based on amino acid sequence analysis. A twelve-transmembrane domain model is predicted by computer-assisted hydropathy profile and amino acid sequence comparison with bacterial transport proteins (Chen *et al.*, 1986). Putative transmembrane regions are shown as numbered ellipses. Two ATP-binding sites, identified based on homology with other ABC transporters, are circled, and potential sites for N-linked glycosylation are indicated by wiggly lines.

permeability; Juliano and Ling, 1976), or the multidrug transporter (reviewed by Gottesman and Pastan, 1993). The mechanism of MDR mediated by PGP is a reduction in intracellular cytotoxic drug concentration via active transport, against concentration gradients, at the plasma membrane. This results in a reduction of the number of effective drug interactions with biological targets, e.g., microtubules, topoisomerase II, or DNA, and hence a reduction in toxicity (i.e., resistance) in PGP-expressing cells. MDR in cell culture systems can be effectively inhibited by noncytotoxic "reversing agents", such as verapamil, which inhibit transport. Interestingly, work in these model systems has also revealed the interaction of PGP with other types of complex biomolecules, e.g., steroids, peptides, and small proteins (reviewed by Gottesman and Pastan, 1993).

The mammalian PGP (*mdr*) gene family is now comprised of two members from human sources, and three each from the mouse, hamster, and rat (reviewed by Germann *et al.*, 1993). PGPs, along with the cystic fibrosis transmembrane conductance regulator (CFTR), are members of the ATP-binding cassette (ABC) superfamily of transporters (Higgins, 1992), characterized in molecular architecture by a varied number of the same modular unit: a hydrophobic transmembrane domain (usually with six predicted membrane-spanning regions), paired with a hydrophilic nucleotide-binding fold. The initial simultaneous publication of the human *MDR1* (Chen *et al.*, 1986) and mouse *mdr*1a (Gros *et al.*, 1986) cDNA sequences led to the current model of PGP (Gottesman and Pastan, 1988). Hydropathy analysis of the protein encoded by the human cDNA indicates the presence of 12 transmembrane regions in two homologous halves, each with six transmembrane regions and a large intracytoplasmic loop encoding an ATP binding site, and the N-terminal of which has N-linked glycosylation sites (Fig. 1). Although this model has been generally well supported by antibody localization data (Yoshimura *et al.*, 1989), several alternative models have recently appeared, one based on studies in an *E. coli* system (see *E. coli* below).

In addition to being structurally complex, structure–function and drug binding studies indicate that PGP is also functionally complex. Relatively small deletions (e.g., 20–23 amino acids), engineered in various regions of the polypeptide chain, usually result in some loss of function (e.g., Currier *et al.*, 1989; Schinkel *et al.*, 1993). Furthermore, the drug specificity profile of PGP can be altered by point mutations in various parts of its primary structure. In the last six years, using either classical genetic selection, or molecular genetic approaches, five laboratories have reported on 14 mutations of this type in human, mouse, and hamster PGPs (Choi *et al.*, 1988; Gros *et al.*, 1991; Devine *et al.*, 1992; Loo and Clarke, 1993a, b, 1994a). Interestingly, these mutations are located throughout the primary structure of PGP, seven in predicted transmembrane regions (numbers 4, 6, 9, 11, and 12), and the balance in predicted cytoplasmic regions, ranging in N- to C-terminal polarity from amino acid 141 to 830. Mapping of photoaffinity drug labelling sites of PGP with azidopine (Bruggemann *et al.*, 1989, 1992), iodoarylazidoprazosin (Greenberger, 1993), and a forskolin derivative (Morris *et al.*, 1994) indicates the presence of a labelled site in each half of PGP, near or within transmembrane regions 5 and 6, or 11 and 12. Together, these data suggest that the structure of the PGP functional unit is indeed complex and may be cooperative, with the many primary structural determinants of drug transporter activity, which may or may not be interdependent, widely scattered along the polypeptide chain.

In addition to being a multifunctional transporter, PGP is more generally a multifunctional enzyme. Human PGP exhibits both basal and drug-stimulated ATPase activities with drug transporter activity (Sarkadi *et al.*, 1992; Ambudkar *et al.*, 1992; Al-Shawi and Senior, 1993; see minireview by Ambudkar in this issue), which may be regulated by phosphorylation (see minireview of Germann *et al.*, this issue). It has been, in addition, reported to stimulate a volume-, voltage-, and ATP-dependent chloride channel activity (Valverde *et al.*, 1992; Gill *et al.*, 1992).

Many questions related to the complex activities of PGP remain to be answered. Two such questions have driven our research over the last few years. First, which regions of PGP participate and/or cooperate in drug transport? Second, how is ATPase activity coupled to drug transport? To investigate these questions we have long been interested in developing heterologous microbial and viral PGP expression systems which, in contrast to homologous systems, allow the rapid expression of mutant proteins for both genetic and biochemical analysis. This review briefly summarizes recent results from heterologous expression studies of mammalian PGPs in *E. coli*, yeast, and baculovirus, which may have implications for other ABC transporters as well.

E. coli

The appeal of using a prokaryote such as *E. coli* to study mammalian PGPs is twofold. First, if overexpression can be achieved, the purification of large amounts of PGP for biochemical reconstitution and structural studies is possible, and could be achieved more rapidly than if carried out in animal cells. Earlier studies suggested that the glycosylation is dispensable for PGP function (Richert *et al.*, 1988; reviewed in Gottesmann and Pastan, 1993), so that unglycosylated PGP expressed in bacteria should be functional. However, the possible functional consequences of altered phosphorylation of PGP in bacteria are less well understood. Second, if even low-level expression of functional PGP can be achieved in intact cells, the short generation time, ease of drug selection, and ease of plasmid manipulation associated with *E. coli* offers tremendous advantages. Thus if there are no serious obstacles due to the bacterial outer membrane barrier, or to the absence of cholesterol in the bacterial inner membrane, this might allow the rapid identification by selection or by molecular genetics of mutant PGPs with altered drug specificity or transport properties. Unfortunately, in reality, both goals—overexpression of PGP and functional expression of PGP—have been extremely difficult to achieve owing to toxic effects of polytopic transmembrane protein expression in *E. coli*. Since expression at some level must precede functional analysis, initially we consider the prospects of PGP expression at any level in bacteria, based on recent attempts to overexpress other higher eukaryotic membrane proteins, as this is critical for both goals.

To date, there are only two examples of successful high-level overexpression of higher eukaryotic membrane proteins in *E. coli*. The first was the 55-kD bovine 17α-hydroxylase cytochrome P-450 from microsomes, containing a single predicted transmembrane helix, expressed up to 16 mg/liter of *E. coli* culture (Barnes *et al.*, 1991). The second was the 31-kD bovine heart mitochondria oxoglutarate carrier, with six predicted transmembrane helices, expressed at 10–15 mg/liter (Fiermonte *et al.*, 1993). In this case, expressed protein accumulated exclusively in inclusion bodies, with none in the bacterial inner membrane. More typical than these results are the levels of 30–400 molecules per bacterial cell, as reported for the adrenergic receptor (Strosberg, 1992). In one case, overexpression of the 35-kD rat liver mitochondrial proton/phosphate symporter, bearing five predicted transmembrane segments, could only be achieved when truncated forms lacking 64 or more C-terminal amino acids (containing one additional predicted transmembrane region) were fused to the soluble α- or β-subunits of ATP synthase, together with the alkaline phosphatase signal sequence (Ferreira and Pedersen, 1992). Expression of any eukaryotic membrane protein in bacteria is clearly not trivial, and it may be especially difficult with complex polytopic proteins like PGP. The inability to express complex eukaryotic membrane proteins in *E. coli* has previously been ascribed to: (a) toxicity of the foreign gene product, preventing the target plasmid from being established and maintained; (b) instability of the expressed protein, due to rapid degradation by host proteases; (c) major differences in codon usage between the homologous and heterologous systems; and (d) the inability of prokaryotic ribosomes to translate the eukaryotic gene (Ferreira and Pedersen, 1992).

While we and other groups have used *E. coli* to overexpress predicted soluble fragments of PGP (Bruggemann *et al.*, 1991; Baubichon-Cortay *et al.*, 1994), or fragments with two predicted transmembrane regions (Tanaka *et al.*, 1990), expression of detectable, intact PGP has been difficult. Only recently has moderate-level expression of full-length, functional (see below), mouse PGP (*mdr1*) been reported by the Kaback group, using a *lac*-promoter based plasmid vector and strain UT5600, which is deficient in the ompT outer membrane protease (Bibi *et al.*, 1993). In contrast, our early attempts to express the ($Gly^{185} \rightarrow Val$) form of human PGP (*MDR1*) using the T7 plasmid system of Studier or

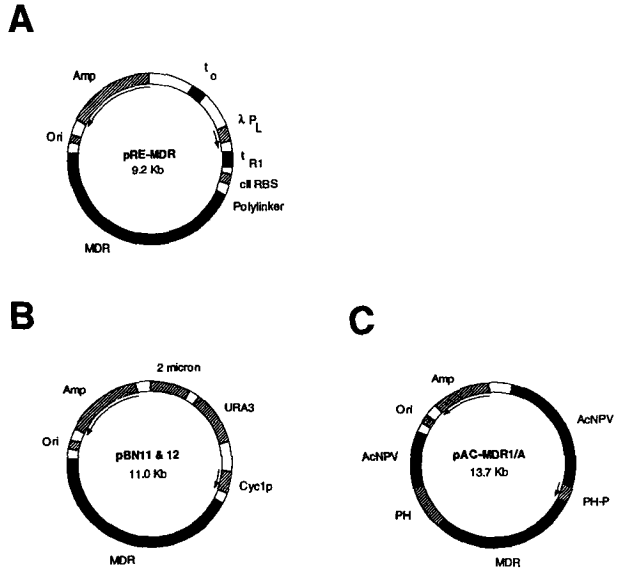

Fig. 2. Examples of expression vectors used for heterologous expression of human PGP (*MDR1*) in *E. coli*, yeast, and baculovirus. (A) pRE-MDR, based on the pRE family of vectors (Reddy, *et al.*, 1989), was constructed for expression in *E. coli* (G. Evans, M. M. Gottesman, and I. Pastan, unpublished data). The inducible bacteriophage λ P_L promoter is flanked by transcription termination sites, t_0 and t_{R1}, which reduce nonspecific and leaky transcription respectively. The human *MDR1* cDNA, encoding a ($Gly^{185} \rightarrow$ Val) mutant PGP and devoid of endogenous 5′- and 3′-untranslated DNA sequences, is fused to the lambda *cII* ribosome binding site (RBS) via a polylinker. The locations of the bacterial replication origin (Ori) and ampicillin selectable marker (Amp) are indicated. (B) pBN11 and pBN12 were constructed for expression in *Saccharomyces cerevisiae* (B. Ni, I. Pastan, and M. M. Gottesman, unpublished data). pBN11 encodes the ($Gly^{185} \rightarrow$ Val) mutant form of human PGP (*MDR1*), and pBN12 encodes the Gly^{185} wild-type human PGP (*MDR1*). Expression is driven by the constitutive yeast cytochrome C promoter (Cyc1p), which is fused transcriptionally to the human *MDR1* cDNA, which is devoid of endogenous 5′- and 3′-untranslated DNA sequences. This vector also contains the URA3 selectable marker, the 2-micron yeast (high copy) replication origin, and bacterial Ori and Amp sequences. (C) pAC-MDR1/A was constructed for expression in the baculovirus system, and is described in detail elsewhere (Germann *et al.*, 1990). Briefly, the human *MDR1* cDNA, with 10 base pairs (bp) of 5′-untranslated DNA and 110 bp of 3′-untranslated DNA, encoding a ($Gly^{185} \rightarrow$ Val) mutant PGP, is fused transcriptionally to the baculovirus polyhedrin promoter (PH-P). This transcription unit is flanked by the polyhedrin (PH) structural gene and by additional *Autographa californica* nuclear polyhedrosis virus (AcNPV) genomic DNA sequences, the 3′ of which bears a poly A addition signal, and both of which allow the formation of recombinant virus (e.g., BV-MDR1) by homologous recombination. This expression vector also contains the bacterial Amp selectable marker, as well as bacterial and M13 origins of DNA replication.

using a recombinant *lac*-PGP λ lysogen had limited success. Bacterial clones bearing full-length PGP cDNAs were unstable, a recombinant λ phage with the PGP insert in the sense orientation could not be isolated, and the expression of full-length PGP, or the two halves of PGP independently, was very poor (U. Germann, G. Evans, I. Pastan, and M. M. Gottesman, unpublished data). An attempt to express full-length PGP in *E. coli* as a fusion with β-galactosidase was similarly unsuccessful (K. Ueda, personal communication). More recently we have switched to a heat-inducible, λ P_L promoter-based plasmid system designed for the expression of toxic gene products in *E. coli* (Reddy *et al.*, 1989; Fig. 2A), and in a protease-competent host have demonstrated that low-level expression of a membrane-localized, truncated form of PGP is associated with a 500–1000-fold drop in colony forming ability (G. Evans, M. M. Gottesman, and I. Pastan, unpublished data). The reason for the apparent difference in toxicity of the mouse and human PGPs in bacteria, and the mechanism of the human PGP-associated toxicity, has not been identified. Finally, despite further optimization of the λ P_L system, the recombinant PGP is unstable, and the maximum level attained is less than 0.1% of membrane vesicle protein (G. Evans, M. M. Gottesman, and I. Pastan, unpublished data), compared to 1.0% for human KB-V1 cells (Ambudkar *et al.*, 1992), which bear an amplified human PGP (*MDR1*) gene. In conclusion, PGP overexpression at a level useful for structural studies has not yet been achieved in bacteria, but expression at lower levels is possible.

There are few examples of functional expression of polytopic eukaryotic membrane proteins in intact *E. coli*. One is that of the human erythrocyte glucose transporter reported by the Kaback group, which, when expressed at a low level, allowed glucose uptake in a glucose transport deficient strain (Sarkar *et al.*, 1988). The same laboratory, as mentioned above, has been the first to report functional expression of mammalian (mouse) PGP in bacteria (Bibi *et al.*, 1993). These investigators were able to demonstrate photoaffinity labeling of unglycosylated 140-kD PGP by iodoarylazidoprazosin, resistance (6-fold) of host cells to tetraphenylphosphonium (TPP), a known PGP substrate (Gros *et al.*, 1992), and decreased accumulation of tritiated tetraphenylarsonium (TPA), against $\Delta\psi$, in PGP-expressing cells. It would be interesting to know if the observed TPP resistance is reversible by known PGP "chemosensitizers" (e.g., verapamil). Surprisingly, the mdr88 mutant mouse PGP,

which bears amino acid substitutions within the two predicted ATP-binding folds, but binds ATP analogs normally, and does not confer drug resistance in transfected animal cells, was as active as wild-type PGP in the TPA assay. Thus these authors could not demonstrate genetically that the observed resistance to lipophilic cations was mediated by an ATP-dependent transport activity of mouse PGP. Clearly work with additional mutants is needed to clarify this point. Furthermore, PGP-associated ATPase activity was not reported, so it is not yet known whether this system has potential for study of this activity. To date there has also been no convincing demonstration of PGP-mediated resistance to its known chemotherapeutic substrates in *E. coli*.

The bacterial system for functional expression of mouse PGP described above has also been exploited to advance a new model for the membrane topology of the N-terminal half of PGP. By measuring the activity of alkaline phosphatase, a periplasmic reporter enzyme, associated with a large series of translational fusions of the bacterial alkaline phosphatase to PGP fragments, or in one case full-length PGP, Bibi's group has obtained evidence that the predicted transmembrane region 4 (TM4) of mouse PGP (*mdr1*) is actually periplasmic in the *E. coli* system, and that a region in the predicted second cytoplasmic loop actually forms TM4 (Bibi and Béjá, 1994). This study did confirm the original location of TM1, TM2, TM5, and TM6 predicted by hydropathy profile and generally supported by studies in animal cells (see Introduction). Translocation of a predicted TM (TM3) and a role for the predicted second cytoplasmic loop in membrane orientation have also been reported based on protease protection and glycosylation of hamster PGP (*pgp1*) deletion mutants translated and translocated in cell-free canine pancreas microsomes (Zhang *et al.*, 1993). In contrast, Skach recently reported, based on protease protection of a small set of truncated human PGP (*MDR1*) chimeras expressed in *Xenopus* oocytes, that TM4 is located in the membrane, as predicted by hydropathy profile of the primary amino acid sequence (Skach and Lingappa, 1994). The resolution of this very interesting question of PGP topology will require the direct comparison of the various truncated forms of PGP in the same expression system, and the additional consideration of variants of full-length PGP bearing inserted and substituted topological reporters, to allow for distal topogenic effects of C-terminal sequences, and/or for cooperative topogenic effects in the intact protein.

YEAST

Heterologous expression of PGPs in the yeast *Saccharomyces cerevisiae*, a simple eukaryote, has many of the same advantages as *E. coli*. If the yeast cell wall barrier and the existence of other drug efflux pumps in yeast (Balzi and Goffeau, 1991) is not a serious impediment to drug uptake, then structure–function studies of PGP in intact cells should be possible. In fact, the potential for overexpression is perhaps greater in yeast than in *E. coli* due to the presence of eukaryotic membrane biogenesis and trafficking machinery much more like that of the homologous PGP system. Recently, several laboratories have reported the functional expression in yeast of human and mouse PGPs using a variety of inducible and constitutive yeast promoters. Raymond *et al.* (1992) initially demonstrated that mouse PGP (*mdr3*) expressed under control of the yeast alcohol dehydrogenase (ADH) promoter was underglycosylated but could partially complement a *STE6* deletion mutant by allowing export of **a**-factor mating pheromone, a farnesylated dodecapeptide. Yeast *STE6* encodes the **a**-factor membrane transporter, a member of the ABC transporter superfamily. There was no cytotoxicity associated with PGP expression in this system. Kuchler and Thorner (1992) reported a similar study using the *STE6* promoter to express a variant form of human PGP (*MDR1*) bearing the ($Gly^{185} \rightarrow$ Val) mutation, which alters the substrate specificity of authentic PGP (Choi *et al.*, 1988). In this study, PGP could not complement the *STE6* defect but could confer resistance to valinomycin, a peptide antibiotic. In addition, though underglycosylated, human PGP found in yeast plasma membranes and total membranes could be specifically photoaffinity labelled with 8-azido-ATP. More recent studies (Kuchler *et al.*, 1992; B. Ni, I. Pastan, and M. M. Gottesman, unpublished data) suggest that the wild-type but not the Val^{185} variant human PGP has only at best a very limited ability to complement *STE6* function in yeast. Taken together with data seen below on drug resistance, these complementation data indicate that recombinant mouse and human PGPs, though underglycosylated, are probably targeted to the yeast plasma membrane, though in one case a significant amount was localized in the endoplasmic reticulum based on indirect immunofluorescence studies (Kuchler and Thorner, 1992). These recombinant PGPs also appear to function as transporters. These data confirm in a yeast expression system that (a) the ($Gly^{185} \rightarrow$ Val) mutation of human

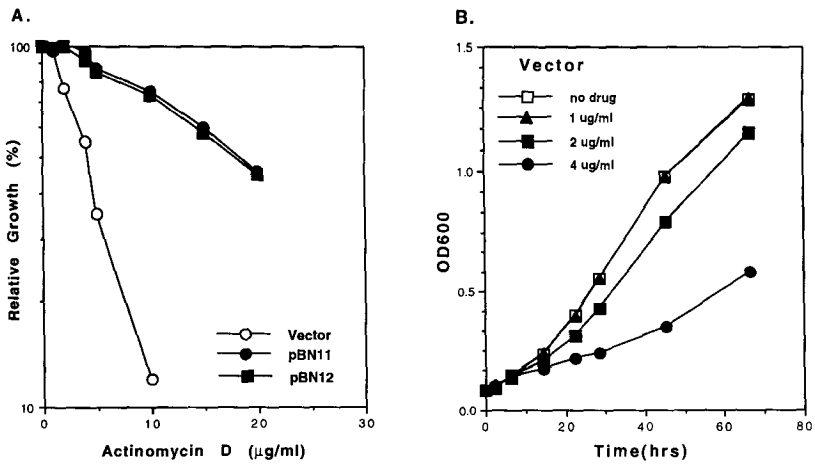

Fig. 3. Reduced ability of actinomycin D to inhibit the growth of yeast transformants expressing human PGPs (*MDR1*). Yeast transformants were grown in modified synthetic liquid medium (B. Ni, I. Pastan, and M. M. Gottesman, unpublished data) to mid-exponential phase, diluted into the same medium to an OD_{600} of 0.1, and grown for 1 h with shaking before addition of the drug. In (A), the growth of yeast transformed with pBN11 (solid circles), pBN12 (solid squares), or vector alone (open circles) and treated with drug was monitored by OD_{600} after a 22-h incubation (A). The structure of the pBN11 ($Gly^{185} \rightarrow$ Val mutant PGP) and pBN12 (Gly^{185} wild-type PGP) expression vectors is shown in Fig. 2B. In (B), the growth rate of yeast transformed by vector alone and treated with no drug (open squares), 1 μg/ml (filled triangles), 2 μg/ml (filled squares), or 4 μg/ml (filled circles) drug was monitored in the same way.

PGP is associated with altered substrate specificity, and (b) complex glycosylation is not required for PGP function.

Additional studies have reported PGP-mediated resistance to cytotoxic drugs in yeast. Raymond *et al.* (1994), extending their complementation study in the same system described above, reported PGP (*mdr3*)-mediated resistance to the antifungal agent FK520, a structural homolog of the immunosuppressant FK506, a known PGP substrate and reversing agent (Saeki *et al.*, 1993). Importantly, a mutant ($Ser^{939} \rightarrow$ Phe) was shown to have a decreased ability to confer resistance to FK520, an effect paralleled in mammalian cell types. Attempts to demonstrate PGP-mediated resistance to chemotherapeutic drugs (e.g., vinblastine, colchicine, and adriamycin) in intact cells were unsuccessful, due to the intrinsic resistance of yeast cells to these drugs. This was perhaps due, at least in part, to the presence of a cell wall in yeast, as it was possible to demonstrate PGP-mediated transport of vinblastine in plasma membrane vesicles derived from these cells (see below). PGP expressed in this system could be photoaffinity labelled with iodoarylazidoprazosin, and could transport the known substrate vinblastine in an ATP-dependent and verapamil-sensitive manner in intact spheroplasts. This system may prove useful in identifying other important regions or residues within PGP that are essential for function.

In contrast to the results of Raymond *et al.*, (1994), we have recently been able to demonstrate human PGP (*MDR1*)-mediated resistance to chemotherapeutic drugs in intact yeast cells, using a mutant strain with altered membrane properties. Saeki *et al.* (1991) had earlier studied the drug-binding activity of human PGP expressed in yeast using the acid phosphatase promoter and discovered that the major sterol component of the yeast plasma membrane, ergosterol, inhibited or altered the drug binding ability of PGP. Utilizing an *erg6* deletion strain deficient in sterol transmethylase activity, which is necessary for ergosterol biosynthesis, PGP has been expressed in both low and high copy number plasmids (for example, see Fig. 2B), importantly with no detrimental effects on the growth rate of the cell (B. Ni, I. Pastan, and M. M. Gottesman, unpublished data). Resistance to adriamycin, daunomycin, actinomycin D, and valinomycin could be demonstrated (approximately 4-fold for each drug; for example, see Fig. 3), and in some cases the pattern of resistance differed between the

wild-type and mutant ($Gly^{185} \rightarrow Val$) gene products. Also, PGP could be photoaffinity labelled with azidopine and iodoarylazidoprazosin, which were competed by known PGP substrates and reversing agents (e.g., verapamil). ATP-dependent drug transport was confirmed using plasma membrane vesicles made from yeast cells overexpressing PGP. Thus this system could also allow for the dissection of PGP structure and function relationships in yeast using clinically utilized PGP substrates.

The biochemical and bioenergetic characteristics of PGP expressed in yeast have been extensively explored by Gros and coworkers in the last four years. Recently, Ruetz *et al.* (1993), again using the ADH promoter, expressed mouse PGP (*mdr3*) in inside-out plasma membrane vesicles and demonstrated ATP-dependent, osmotically sensitive transport of vinblastine and colchicine, which was inhibitable by verapamil. The transport properties of a variant of mouse PGP (*mdr3*) bearing the ($Ser^{939} \rightarrow$ Phe) mutation were quantitatively and qualitatively similar to those seen in animal cells. Unfortunately, the basal or drug-stimulated ATPase activities of PGP could not be detected in this system.

Most recently, a powerful secretory vesicle (SV) system based on the *sec 6-4* mutant strain of *Saccharomyces cerevisiae* has been exploited in the study of PGPs. In this system, used previously to study the yeast plasma membrane ATPase (Nakamoto *et al.*, 1991), the mutant accumulates 95–120 nm post-Golgi SVs containing an abundance of newly synthesized protein en route to the plasma membrane. These vesicles offer several advantages to vesicles prepared from plasma membranes. First, these SVs are tightly sealed since they are protected from proteolytic attack by the plasma membrane itself during removal of the cell wall. Secondly, the membrane proteins expressed are uniformly in an "inside-out" orientation, making them useful for both ATPase and drug uptake studies. Thirdly, owing to the abundance of the SVs and the ease of their purification, this system has a high potential for the overexpression and purification of membrane proteins. Again using the yeast ADH promoter, Ruetz and Gros (1994a,b) have reported the functional overexpression of all three mouse PGPs (*mdr1*, *mdr2*, and *mdr3*) in this system. It was shown that PGP encoded by *mdr2*, which does not transport drugs, acted as a phosphatidylcholine (PC) translocase, transporting PC from the outer to inner membrane leaflet of SVs in an ATP- and magnesium-dependent manner (Ruetz and Gros, 1994b). Vesicles derived from cells expressing PGPs encoded by *mdr1* or *mdr3* were shown to accumulate vinblastine in an ATP-dependent manner, which could be inhibited by verapamil. The observed transport was independent of membrane potential and proton movements, and furthermore colchicine accumulation into the sealed vesicles could be demonstrated against a steep concentration gradient. Taken together, the recent data of Ruetz *et al.* (1993) and of Ruetz and Gros (1994a) suggest that recombinant mouse PGPs (*mdr1* and *mdr3*) made in yeast are indeed functional as ATP-dependent drug efflux pumps. Unfortunately, however, perhaps due to endogenous ATPase activities or inhibitors, direct biochemical measurement of drug-dependent ATPase activity has not yet been possible in these or any other yeast expression systems examined to date (C. Hrycyna, unpublished data).

BACULOVIRUS

The advantage of heterologous expression of PGP in the baculovirus/insect cell system is the very high potential for overexpression and biochemical characterization in this system. This system is useful both for studies in crude membrane vesicles from infected cells, and for purification, reconstitution, and structural studies. Because recombinant baculovirus interferes with normal cell growth, this system is not useful for drug resistance studies in intact cells, but has been used for PGP substrate accumulation studies in intact cells (Rao *et al.*, 1994b). Five years ago we reported the expression of human PGP (*MDR1*) in this system (Germann *et al.*, 1990). Human PGP was among the earliest examples of membrane transports expressed in this system and remains the only mammalian PGP expressed in insect cells to date.

The original recombinant baculovirus, BV-MDR1, contains the human *MDR1* cDNA with a ($Gly^{185} \rightarrow$ Val) mutation downstream from the powerful, late-acting polyhedrin promoter (see Fig. 2C). In Sf9 insect cells infected with this virus, glycosylation of PGP could not be detected, but PGP was phosphorylated. As seen in Table I, subcellular distribution and photoaffinity drug binding profiles were similar to those of authentic PGP expressed in human KB-V1 cells (Germann *et al.*, 1990). In cells infected by the BV-MDR1 virus, PGP is associated with ATPase (Sarkadi *et al.*, 1992) and drug transporter (Rao *et al.*, 1994b) activities quantitatively similar to, or in excess of, those seen in homologous (animal cell) systems.

Table I.[a] Inhibition of [^{3}H]Azidopine Photoaffinity Labeling of the Human Multidrug Transporter in Plasma Membranes of Multidrug-Resistant Human KB-V1 Cells and BV-Mdr1-Infected Sf9 Insect Cells

Drug	KB-V1	Sf9-MDR1
Vinblastine	+++[b]	+[c]
Daunomycin	+++	+++
Colchicine	—[d]	—
Vincristine	++[e]	+
Verapamil	++	++
Quinidine	+	++
Nifedipine	++	++
Diltiazem	++	++
Actinomycin D	+++	+++

[a] Reproduced from *Biochemistry* (Germann *et al.*, 1990) with permission.
[b] Means more than 80% inhibition.
[c] Means 30–50% inhibition.
[d] Refers to no detectable inhibition.
[e] Means 50–80% inhibition.

Based on Western blot and vanadate-inhibitable ATPase activity, it is estimated that PGP accounts for about 3% of total membrane vesicle protein (Sarkadi *et al.*, 1992), which is 3-fold higher than that in KB-V1 cells (1%, see above). Pgp expression is generally maximal 3 days postinfection, with various multiplicities of infection. At present, no systematic comparison of different insect cell lines (e.g., Sf21 and High Five™), or of different media, or of different vectors has been reported. Thus it is possible that the expression of PGP in this system has not yet been optimized.

Functional overexpression of active PGP in insect cells has provided new insights into the ATPase activities of the multidrug transporter. Using plasma membrane vesicles prepared from BV-MDR1 (see Fig. 2C) infected Sf9 cells, Sarkadi *et al.* (1992) demonstrated PGP-associated, substrate (i.e., drug and reversing agent)-stimulated ATPase activity. Importantly, the maximal, drug-stimulated specific activity measured in this study (5 μmol/mg PGP protein/min) approached that of the ion-transporting ATPases, and was high enough to account for the levels of ATP depletion and drug transport previously observed in tumor cells which express PGP. This level of ATPase activity is somewhat lower than that estimated for purified PGP based on reconstitution studies with authentic human PGP (*MDR1*; 15–38 μmol/mg protein/min; Ambudkar *et al.*, 1992), but this may be explained by the relatively lower glycolytic activity and ATP levels of insect cells (see Ambudkar *et al.*, 1992). Also, this study provided the first demonstration of high-level ATPase activity of an ABC transporter (or M-type ATPase) from higher eukaryotes. The simple crude membrane vesicle ATPase assay described therein was soon used by several laboratories to screen potential PGP substrates and inhibitors, based on the relatively stringent substrate-dependence of PGP ATPase activity. Stimulatory effects on PGP ATPase activity, generally 2-5 fold, have now been ascribed to a wide array of compounds, including proposed physiological PGP substrates and inhibitors, such as progesterone, β-estradiol, and tamoxifen (Rao *et al.*, 1994a); immunosuppressant drugs, such as FK506 and FK520 (Rao and Scarborough, 1994); various hydrophobic peptides and their derivatives (Sarkadi *et al.*, 1994); and prenylcysteine compounds, derived from prenylated proteins (Zhang *et al.*, 1994). While many of these compounds are known to be substrates or inhibitors of PGP, direct membrane transport assays have not been carried out for several of these compounds, and thus this screening method has not yet been validated in all cases. Interestingly, we have found that most of the PGP ATPase activity in the crude Sf9 membranes used in the drug screening assays is not associated with plasma membranes. Although some of the recombinant PGP is associated with the plasma membranes, the majority of the protein is retained in a membrane fraction in the cell and can be separated from other membrane fractions by centrifugation of the post-nuclear lysate at 5000 $\times$ ***g***. Both the plasma- and intracellular-membrane fractions exhibit similar verapamil-stimulated PGP ATPase activity. However, the octylglucoside-solubilized protein from these membranes elutes at different salt concentrations during anion exchange chromatography (S. Ambudkar, U. Germann, I. Pastan, and M. M. Gottesman, unpublished data). This heterogeneity may be due to differences in the posttranslational modifications of PGP and/or the lipid composition of these membranes, which could affect drug transport and drug modulation of PGP-ATPase activity.

More recent technical advances have increased the appeal of the baculovirus system. The engineering of improved expression vectors and baculovirus recombination targets has greatly reduced the time required for isolation of recombinant viruses (see Kitts and Possee, 1993), and has thus increased the feasibility of studies with mutant proteins. Loo and

Clarke (1994b) expressed the amino and carboxyl halves of human PGP (*MDR1*) either independently or together, and observed substrate-stimulated ATPase activity only with coexpression. This provides further evidence that the functional unit of PGP is complex and cooperative (see Introduction), and in addition indicates that covalent linkage of these two homologous domains is not necessary for their cooperative function. By cooexpression of protein kinase Ca and either wild-type or ($Ser^{671} \rightarrow Asn$) mutant human PGP (*MDR1*), Ahmad *et al.* (1994) demonstrated increased ATPase activity of the wild type, but not mutant PGP mediated by phosphorylation (see minireview of Germann *et al.* in this issue).

Another technical advance is the use of engineered tags to improve protein purification. For example, placement of six tandem histidine residues at either terminus of a recombinant protein allows its relatively straightforward purification by metal-chelate chromatography (Janknecht *et al.*, 1991). This approach has been used to great advantage for soluble proteins in the recent literature (see, for example, Alnemri *et al.*, 1993), but has not yet been reported for a membrane protein. Recently we have expressed in baculovirus a variant of human PGP (*MDR1*) bearing a six-histidine tag at its C-terminus (D. Chen, M. M. Gottesman, and I. Pastan, unpublished data), in order to achieve a high degree of purification in a single step using a nickel adsorbent. As a final note, CFTR (see Introduction), another well-characterized ABC transporter, has also been studied in the baculovirus system. Both wild-type CFTR and its most common variant in the Caucasian population, the ΔPhe^{508} mutant, have been expressed in insect cells and purified, and their anion conductance properties characterized in reconstituted liposomes (Kartner *et al.*, 1991; Bear *et al.*, 1992; Li *et al.*, 1993).

CONCLUSIONS AND FUTURE DIRECTIONS

We have reviewed recent contributions from *E. coli*, yeast, and baculovirus heterologous expression systems to our understanding of multidrug transport mediated by mammalian PGPs. Work from a large number of groups has shown that these systems can faithfully model many of the structural and functional properties of authentic PGP. Much of the results from PGP membrane topology experiments in bacteria is consistent with that from studies in mammalian cells, and with the hydropathy models; the ability of wild-type mouse and human PGPs to complement the *STE6* defect in yeast and to confer a drug-resistant phenotype on yeast (see Fig. 3), suggests that PGP can function in yeast; and the biochemical characteristics (e.g., the specific drug-stimulated ATPase activity) of recombinant PGP from baculovirus are very similar to those of authentic PGP made in mammalian cells (see the section on baculovirus). However, because any one of these systems clearly does not model all of the properties of PGP observed in mammalian cells, caution and patience are warranted in attempting to extrapolate these results to the native context of PGP. One possible difference between heterologous and homologous systems which requires more attention is the differing lipid content of the plasma membranes of different organisms, which seems likely to play a role in determining drug specificity of PGP (see Gottesman and Pastan, 1993). However, we do not yet understand heterologous or homologous systems well enough to accurately interpret all of the differences seen between the two.

Based on the results discussed in this review, the promise of *E. coli*, yeast, and baculovirus heterologous expression systems for the future study of PGPs, and perhaps of other ABC transporters as well, can be evaluated. For biochemical experiments in crude membranes, baculovirus is clearly the system of current choice, owing to high levels of expression (> 3% of total membrane protein) and easily measurable basal and drug-stimulated ATPase activities. For purification, reconstitution, and structural experiments, the baculovirus or yeast secretory vesicle systems will be useful, but the former may yield greater amounts of recombinant protein. The potential for drug resistance studies in intact cells is greatest in the yeast systems (Raymond *et al.*, 1994; B. Ni, I. Pastan, and M. M. Gottesman, unpublished data), but still suffers from limitations due to the intrinsic resistance of yeast to many important anticancer drugs. The utility of *E. coli* heterologous expression systems in the study of PGPs still remains to be proven.

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