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Drug-Stimulated ATPase Activity of the Human P-glycoprotein

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The human multidrug resistance protein, or P-glycoprotein (Pgp), exhibits a high-capacity drug-dependent ATP hydrolytic activity that is a direct reflection of its drug transport capability. This activity is readily measured in membranes isolated from cultured insect cells infected with a baculovirus carrying the human *mdr1* cDNA. The drug-stimulated ATPase activity is a useful alternative to conventional screening systems for identifying high-affinity drug substrates of the Pgp with potential clinical value as chemosensitizers for tumor cells that have become drug resistant. Using this assay system, a variety of drugs have been directly shown to interact with the Pgp. Many of the drugs stimulate the Pgp ATPase activity, but certain drugs bind tightly to the drug-binding site of the Pgp without eliciting ATP hydrolysis. Either class of drugs may be useful as chemosensitizing agents. The baculovirus/insect cell Pgp ATPase assay system may also facilitate future studies of the molecular structure and mechanism of the Pgp.

KEY WORDS: Multidrug resistance; chemotherapy; chemosensitizers; P-glycoprotein; cell-free assay; ATP hydrolysis.

INTRODUCTION

Tumor cells undergoing chemotherapy by specific antineoplastic agents often develop a simultaneous resistance to many other structurally unrelated cytotoxic compounds. The development of such multidrug resistance is often associated with overexpression of the *mdr1* gene, which codes for a 170-kDa integral membrane glycoprotein known as the P-glycoprotein, or Pgp. The Pgp consists of 1280 amino acids and has been proposed on the basis of sequence analyses to contain twelve membrane-spanning hydrophobic helices and two nucleotide-binding sites in a large, cytoplasmic domain. A wide variety of different experimental approaches have established that the Pgp is an ATP-dependent drug pump capable of transporting numerous cytotoxic agents out of cells against a concentration gradient. It is likely that the development of multidrug resistance via the Pgp is responsible for many clinical failures of cancer chemotherapy (Bradley *et al.*, 1988; Gottesman and Pastan, 1988; Juranka *et al.*, 1989; Endicott and Ling, 1989; Pastan *et al.*, 1991).

Whereas the levels of expression of the Pgp in multidrug-resistant tumor cells appear to be more than adequate to spare the cells from the cytotoxic effects of antitumor drugs, the amounts produced in most cases are too low to permit biochemical characterization of the enzymatic properties of the Pgp in isolated membrane preparations. It was expected early on that if the Pgp is indeed a drug extrusion pump driven by ATP hydrolysis, it should exhibit a drug-dependent ATPase activity in broken cell preparations analogous to the ion-dependent ATPase activities of the more well-known transport ATPases such as the Ca^{++}-ATPase of sarcoplasmic reticulum membranes and the Na^+/K^+-ATPase of animal cell plasma membranes (Pedersen and Carafoli, 1987). But for many years, the highest reported specific ATPase activity of the Pgp was no more than a few nmol of ATP hydrolyzed per mg of Pgp protein per minute (Hamada and Tsuruo, 1988a), roughly a thousand times lower than the activities of the

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ion-translocating ATPases, and this activity was only mildly stimulated by drugs thought to be transported by the Pgp (Hamada and Tsuruo, 1988b). Recently, however, a much higher drug-dependent ATPase activity of the Pgp has been measured in several different laboratories (Sarkadi *et al.*, 1992b; Doige *et al.*, 1992; Ambudkar *et al.*, 1992; Al-Shawi and Senior, 1993; Shapiro and Ling, 1994). In this article, the experiments carried out in this laboratory demonstrating a high-capacity drug-stimulated ATPase activity of the Pgp in Pgp baculovirus-infected cultured insect cells are briefly described, along with the results of some more recent experiments on the drug specificity of the multidrug resistance pump. Potential advantages of this system for current and future studies of the Pgp and its various homologues are also discussed.

RESULTS AND DISCUSSION

As a laboratory interested primarily in the molecular mechanism of transport ATPases (Scarborough, 1992), we have noted with interest the multidrug resistance phenomenon and the likelihood that the Pgp is an ATP-driven drug extrusion pump. But our experimental involvement with the Pgp developed by a somewhat circuitous route. We were studying the recently cloned human cystic fibrosis protein, the CFTR, expressed in a CFTR baculovirus/Sf9 insect cell expression system (Sarkadi *et al.*, 1992a), and because the CFTR has two regions of substantial homology with proteins known to bind nucleotides (Riordan *et al.*, 1989), an extensive series of experiments was carried out with the expressed CFTR in the insect cell membranes to determine if it catalyzes ATP hydrolysis. The results indicated that the CFTR may catalyze ATP hydrolysis, but the activity detected was quite low. As part of these experiments, we were also able to detect significant ATP hydrolysis above control levels in insect cells infected with a baculovirus containing the cDNA for the human Pgp (Germann *et al.*, 1990), obtained from Dr. M. M. Gottesman. Following up on these observations, it was then discovered that this ATPase activity is markedly stimulated by drugs known to interact with the Pgp, including verapamil, vinblastine, vincristine, daunomycin, quinine, trifluoperazine, nifedipine, and colchicine. Under the optimized reaction conditions developed in these studies, membranes from uninfected Sf9 insect cells exhibit a vanadate-sensitive ATPase activity of about 10 nmol/mg membrane protein/min. In control, β-galactosidase baculovirus-infected cells, the vanadate-sensitive ATPase activity is reduced to progressively lower levels as the time after infection increases. In contrast, in the Pgp baculovirus-infected cells, the vanadate-sensitive membrane ATPase activity increases significantly, to about 30 nmol/mg membrane protein/min, while the vanadate-insensitive fraction remains essentially unchanged. Importantly, when verapamil, a known substrate of the Pgp, is added, the vanadate-sensitive ATPase activity in the Pgp baculovirus-infected cell membranes is stimulated about fivefold, to about 150 nmol/mg membrane protein/min. There is no such vanadate-sensitive, drug-stimulated ATPase activity in membranes from uninfected or β-galactosidase baculovirus-infected cells. The appearance of the drug-stimulated ATPase activity correlates well with the appearance of the *mdr1* gene product detected with a monoclonal anti-Pgp antibody and by labeling with 8-azido-[^{32}P]-ATP and reaches a healthy level of about 3% of the total membrane protein on the third day of infection. Importantly, the maximum specific activity of the drug-stimulated ATPase is about 3–5 μmol/mg Pgp/min, more than three orders of magnitude higher than a minor ATPase activity previously measured for the Pgp (Hamada and Tsuruo, 1988a,b). These results clearly established the existence of a high-capacity, drug-stimulated ATPase activity of the human multidrug resistance protein (Sarkadi *et al.*, 1992b). Subsequent studies have shown that the expressed Pgp has essentially no ATPase activity in the absence of transport substrates such as verapamil. Thus, the drug-dependent Pgp ATPase activity is almost certainly a direct reflection of the transport function of the Pgp. Strong support for this contention has recently been provided by Homolya *et al.* (1993). These investigators have shown convincingly that the ability of a variety of fluorescent compounds to stimulate the Pgp ATPase activity is directly correlated with their ability to be transported by the Pgp.

Drugs capable of interfering with cytotoxic drug transport catalyzed by the Pgp are potentially valuable clinical agents for reversing multidrug resistance (Bradley *et al.*, 1988; Gottesman and Pastan, 1988; Juranka *et al.*, 1989; Endicott and Ling, 1989; Pastan *et al.*, 1991; Akiyama *et al.*, 1988; Jaffrezou *et al.*, 1991; Horio *et al.*, 1991; Miller *et al.*, 1991). Traditionally, such drugs have been identified by screening cultured cells adapted for growth in the presence of

cytotoxic drugs for agents able to resensitize the cells to the killing action of the drugs. Such drugs, termed chemosensitizing agents, are thought to exert their effect by competition with the cytotoxic drugs at the drug binding site of the Pgp, thus raising the intracellular concentrations of the cytotoxic drugs and enhancing their killing action. Unfortunately, there are several drawbacks to this conventional approach to identifying chemosensitizing agents with a high affinity for the Pgp. First, measurement of cell death is a relatively crude assay for the affinity of drugs for the Pgp drug binding site. Second, some agents may enhance the effects of the cytotoxic agents without interacting with the Pgp. Such agents may be useful, but more than likely will enhance the toxicity of the cytotoxic drug to nonmalignant cells as well. Third, because the overall chemosensitization process involves the passage of the chemosensitizing drugs through the plasma membrane into the cells as well as competition for the Pgp drug binding site, the effects of rational functional group changes in known chemosensitizing drugs are not easy to judge, because of the strong likelihood of associated changes in drug permeation rates. Importantly, for related reasons, it is likely that the list of chemosensitizing agents thus far identified is limited to compounds that are able to penetrate the cell plasma membrane. Thus, there may be compounds with very high affinities for the Pgp that fail as chemosensitizers because they are unable to enter cells. If such agents could be identified, subsequent modifications might be made to enhance their permeability properties without affecting their affinity for the Pgp.

These considerations point to a need for a cell-free assay system for identifying drugs with a high affinity for the Pgp. Until recently, the only such assays available involved binding or uptake studies of radioactive drugs known to interact with the Pgp by isolated membrane vesicles, and the competition with such binding or transport by the test drug (Bradley *et al.*, 1988; Gottesman and Pastan, 1988; Juranka *et al.*, 1989; Endicott and Ling, 1989; Pastan *et al.*, 1991; Akiyama *et al.*, 1988; Jaffrezou *et al.*, 1991; Horio *et al.*, 1988, 1991; Cornwell *et al.*, 1986, 1987; Naito *et al.*, 1988; Kamimoto *et al.*, 1989). White quite useful, these assays can be difficult to interpret due to nonspecific binding of the usually hydrophobic drugs to the membranes, and the relatively low levels of the Pgp in the membrane systems available. The cell-free, Pgp ATPase system described here for measuring drug-Pgp interactions is without such drawbacks, is simple to perform, and is not affected in any obvious way by the permeability characteristics of the drugs that stimulate it. It is thus an ideal system for investigating certain aspects of the interaction of the Pgp with its drug substrates.

Several studies have been carried out utilizing this new system for measuring drug interactions with the Pgp. In the first of these, the interactions between the Pgp and two different types of immunosuppressant drugs known to modulate multidrug resistance in tumour cells were investigated (Rao and Scarborough, 1994). The immunosuppresant macrolides FK506 and FK520 stimulate the Pgp ATPase activity with affinities in the 100 nanomolar range, nearly ten times higher than that of verapamil, the prototype Pgp substrate. On the other hand, the immunosuppressant cyclic peptides, cyclosporin A and dihydrocyclosporin C, do not stimulate the Pgp ATPase activity at all. They do, however, act as potent competitive inhibitors of verapamil-stimulated Pgp ATPase activity, with affinity constants in the 20 nanomolar range. Thus, although these two classes of immunosuppressant drugs affect the Pgp in different ways, they both interact with high affinity at the transported drug binding site of the Pgp. These results provide an important *caveat* with regard to the use of the Pgp ATPase activity as an indicator of drug–Pgp interactions. That is, they show that compounds exist that can interact with high affinity at the drug binding site of the Pgp without eliciting a full catalytic cycle leading to ATP hydrolysis. These effects are not confined to the cyclosporins, since we have noted similar behaviour with other drugs, such as 7-aminoactinomycin D and Hoechst 33342. Therefore, in any search for high-affinity chemosensitizers using this assay or any other drug-stimulated Pgp ATPase assay system, it is advisable to test not only for the ATPase-stimulatory potency of potential agents but also for the inhibition of verapamil stimulation by the same agents. Interestingly, either Pgp ATPase stimulators like the macrolides or verapamil, or dead-end inhibitors like the cyclosporins, can be expected to act as chemosensitizers, because each will curtail cytotoxic drug extrusion by the Pgp. It is interesting to speculate, however, that Pgp ATPase stimulators may be more effective chemosensitizers because they can potentially elicit ATP depletion in drug-resistant cells in addition to their direct effects on cytotoxic drug extrusion by the Pgp.

In another series of experiments, the interactions of several antiestrogens and steroid hormones with

the Pgp were investigated using the Pgp ATPase assay system (Rao *et al.*, 1994). Antiestrogens including tamoxifen, metabolites of tamoxifen, droloxifen, and toremifene stimulated the Pgp ATPase activity, in certain cases with potencies near that of verapamil. Clomifene, nafoxidine, and diethylstilbestrol also acted as Pgp ATPase substrates. Steroid hormones including progesterone, β-estradiol, hydrocortisone, and corticosterone also stimulated the Pgp ATPase activity, with progesterone the most potent. At a concentration of 50 μM, this steroid was as effective as verapamil. Although a variety of explanations have been advanced for the chemosensitizing activity of certain antiestrogens and steroid hormones, the Pgp ATPase assay results suggest that these agents may chemosensitize resistant tumor cells by direct interaction with the Pgp, thereby inferfering with its cytotoxic drug extrusion activity.

In a similar series of experiments, Zhang *et al.* (1994) demonstrated interactions of several prenylcysteine compounds with the Pgp using the Pgp baculovirus/insect cell system. On the basis of these results, it was suggested that these compounds are possible physiological substrates of the Pgp. Similar suggestions have arisen in our studies in each case where the Pgp ATPase substrate is present endogenously in humans. But judging from the rather amazing diversity among the compounds we and others have found to be Pgp substrates using the unambiguous cell-free Pgp ATPase assay, if the physiological function of the Pgp is to transport all of these various compounds, it is a very promiscuous transporter indeed.

Although the baculovirus/insect cell Pgp expression system coupled with the cell-free drug-stimulated Pgp ATPase assay we have developed is among the most attractive systems available for studying the Pgp, two *caveats* regarding this system are worthy of mention. First, the Pgp that is expressed in this system as we have used it has a valine at residue 185 in place of a glycine, stemming from the fact that the gene used for production of the baculovirus was obtained from a drug-resistant cell line (Choi *et al.*, 1988). As such, information obtained regarding this Pgp may not be entirely relevant to the normal form of the human Pgp. On the other hand, such information may actually be more relevant to the Pgp that is overexpressed in drug-resistant tumor cells. Second, in our hands and apparently others, the production of baculoviruses containing Pgp cDNAs is much more difficult than it is with numerous other cDNAs that we have experience with. Moreover, our first attempts to use this system for site-directed mutagenesis produced results (unpublished) that were not the same as those of another laboratory. This, together with the known existence of Pgp homologues in insects, raises the remote but troublesome possibility that the baculoviruses emerging from the selection procedure may contain Pgp genes different from those supplied in the original transfer plasmids. Problems similar to this arising from recombination events are legendary in yeast. Thus, it may be necessary to sequence the isolated baculovirus Pgp DNA regions in order to be sure of the sequences of the Pgps expressed in this system.

In addition to its utility in screening for high-affinity transport substrates of the Pgp, the baculovirus/insect cell Pgp ATPase system offers additional promose for studies of the human Pgp and Pgps other than the human *mdr1* gene product. First, the amounts of the Pgp produced by this system are among the highest currently available. This, together with a simple ATPase assay for Pgp function, should facilitate efforts to solubilize and purify this transporter in the quantities needed for biochemical studies of its structure and molecular mechanism. Production of amounts of purified Pgp sufficient for crystallization trials is feasible, with the additional advantage of the presence of little or no carbohydrate that could interfere with crystallization. Second, of the various Pgp expression systems available, this system may be the most facile for site-directed mutagenesis studies of numerous aspects of Pgp function, if the potential problem of unwanted mutation can be dismissed or circumvented. Third, using recombinant baculoviruses prepared in the laboratory of Dr. J. Croop, we have been able to directly measure the ATPase activities of two drosophila homologues of the Pgp (unpublished results). This augurs that it should be possible to develop Pgp baculovirus/insect cell expression and ATPase assay systems for other Pgp homologues of medical and general interest. New human Pgp-like multidrug resistance proteins such as that recently discovered by Cole *et al.* (1992) would be of great interest in this regard, as would the Pgp homologues involved in antigen presentation (Monaco *et al.*, 1990; Deverson *et al.*, 1990; Trowsdale *et al.*, 1990; Spies *et al.*, 1990). The Pgps of problematic protozoans such as *Plasmodium falciparum* and *Leishmania*, spp. (Croop, 1993) may also be amenable to study by this approach. Of probable greatest interest in this regard would be the Pgp homologues of

P. falciparum, one or more of which appears to be involved in multidrug resistance in malaria (Foote *et al.*, 1990; Cowman *et al.*, 1991; Barnes *et al.*, 1992; Krogstad *et al.*, 1992). The emergence of a large number of drug-resistant strains of *P. falciparum* in the last several decades could actually provide a rich source of select mutants for studies of the drug transport characteristics of the *P. falciparum* Pgps. Thus it can be expected that high-capacity Pgp ATPase assay systems such as that described here for the human *mdr1* gene product will emerge for a variety of other Pgps in the foreseeable future.

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