Expression of the Human Multidrug Resistance cDNA in Insect Cells Generates a High Activity Drug-stimulated Membrane ATPase

(Received for publication, December 9, 1991)

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Drug-resistant tumor cells actively extrude a variety of chemotherapeutic agents by the action of the multidrug resistance (MDR1) gene product, the plasma membrane P-glycoprotein. In this report we show that the expression of the human MDR1 gene in cultured SF9 insect cells via a baculovirus vector generates a high activity vanadate-sensitive membrane ATPase. This ATPase is markedly stimulated by drugs known to interact with the P-glycoprotein, such as vinblastine and verapamil, and the ability of the various drugs to stimulate the ATPase corresponds to their previously observed affinity for this transporter. The drug-stimulated ATPase is not present in uninfected or mock-infected SF9 cells, and its appearance correlates with the appearance of the MDR1 gene product detected with a monoclonal anti-MDR protein antibody and by labeling with 8-azido-ATP. The drug-induced ATPase requires magnesium ions, does not utilize ADP or AMP as substrates, exhibits a half-maximal activation at about 0.5 mM MgATP, and its maximal activity (about 3–5 nmol/mg MDR protein/min) approaches that of the well characterized ion transport ATPases. These results provide the first direct demonstration of a high capacity drug-stimulated ATPase activity of the human multidrug resistance protein and offer a new and simple assay for the investigation of functional interactions of various drugs with this clinically important enzyme.

A major obstacle in the efficient chemotherapy of human malignancies is the development of a simultaneous resistance to a variety of structurally unrelated cytotoxic drugs. The emergence of this multidrug resistance is frequently associated with the expression of a membrane protein which most probably actively extrudes these drugs from the resistant tumor cells (1–5). This 130–170-kDa membrane transport protein (its actual molecular weight depends on the state of glycosylation) has been termed the multidrug resistance (MDR) protein or P-glycoprotein. This transporter is encoded by the MDR1 gene, and the expression of the MDR1 cDNA in drug-sensitive cells has been demonstrated to confer a multidrug resistance phenotype (8–12). The MDR protein shares significant sequence homology with several bacterial transporters (13) and with mammalian proteins such as the cystic fibrosis transmembrane conductance regulator (for reviews see Refs. 4–7). The MDR protein consists of 1280 amino acids and has been proposed to contain 12 membrane-spanning hydrophobic domains, two nucleotide-binding domains, and specific N-linked glycosylation sites (4–12). Certain aspects of this proposed transmembrane topography and a tissue-specific, variable glycosylation which probably does not affect the multidrug transporter function have been confirmed by biochemical approaches (14–16).

It has been demonstrated that drug efflux associated with elevated P-glycoprotein is an energy-dependent process (1–3, 16, 18–20, 24). The activity of the multidrug transporter in drug-resistant cells is associated with rapid cellular ATP depletion when ATP resynthesis is inhibited (25). Membrane vesicles containing the protein exhibit an ATP-dependent drug uptake (22, 23). The protein binds ATP, and mutagenesis of the proposed nucleotide-binding domains has been shown to result in a failure of the expressed protein to confer drug resistance (4, 7, 26). All these data collectively suggest that the MDR gene product is a membrane ATPase which functions as an active drug transporter. However, no definitive data are as yet available indicating that the MDR protein exhibits a high capacity, drug-specific ATPase activity. In one report relevant in this regard, a purified P-glycoprotein preparation was reported to exhibit an ATPase activity (27), but the extremely low specific activity (a maximal activity of 1–3 nmol/mg MDR protein/min) could not account for the high levels of ATP consumption and drug transport catalyzed by this protein in tumor cell membranes. In this report we demonstrate that the expression of the MDR1 gene in SF9 (Spodoptera frugiperda ovarian) insect cells results in the appearance of a drug-stimulated ATPase activity with a high specific activity approaching that of the ion-transporting ATPases.

**EXPERIMENTAL PROCEDURES

Recombinant baculovirus carrying the human MDR1 gene was generated, and SF9 (S. frugiperda) cells (Invitrogen) were infected and cultured according to the procedures described previously (28). The MDR cDNA contains a mutation from Gly to Val at amino acid position 185 (33). Recombinant virus containing the β-galactosidase gene was provided by Invitrogen. The virus-infected SF9 cells were cultured in T150 culture flasks with about 2 × 10⁶ cells/30 ml of medium for the times indicated. The cells were harvested by scraping them into Tris-mannitol buffer (50 mM Tris, pH 7.0, with HCl containing 300 mM mannitol and 0.5 mM phenylmethylsulfonyl flu-
oride). For membrane preparation the cells were lysed and homoge-
nized using a glass-Teflon tissue homogenizer in TMEP (50 mM Tris, pH 7.0, with HCl, containing 50 mM mannitol, 2 mM EGTA, 10 µg/ml leupeptin, 8 µg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluo-
ride, and 2 mM β-mercaptoethanol), and the undisrupted cells and much of the vesicles were removed by centrifugation at 500 x g for 10 min. The supernatant fluid was then centrifuged for 60 min at 100,000 x g and the pellet containing the membranes resuspended in TMEP at 4 °C, and the membranes were stored at -70 °C.

For immunoblotting, membrane suspensions were precipitated with trichloroacetic acid (4% (w/v) final concentration), centrifuged for 5 min at 5,000 x g, and then suspended in disaggregation buffer (50 mM Tris, pH 6.8, with H₃PO₄, containing 2% SDS (w/v), 15% glycerol (w/v), 2% β-mercaptoethanol (v/v), 1 mM EDTA, and 0.02% (w/v) bromphenol blue). Samples were disaggregated at room temperature for 20 min at a protein-concentration of about 1 mg/ml. Electrophoresis and immunoblotting of the samples was carried out using Bio-Rad Mini-Protean ready gels (4–15% acrylamide gradient) in a Mini-Protein electrophoresis cell and a Mini-Trans-Blot cell, as described previously (34). Gels were run for 60 min at 110 V in a buffer containing 25 mM Tris-glycine, pH 8.3, and 0.2% (w/v) SDS. Electrophoretic of the proteins onto polyvinylidine difluoride mem-
branes (Bio-Rad, 0.2-µm pore size) was carried out in a Tris buffer containing a high concentration of glycine and no methanol (0.7 M glycine, 25 mM Tris, pH 7.7) at 60 V for 1.5 h with a cooling unit. For immunodetection of the proteins, polyvinylidine difluoride mem-
branes with the blotted proteins were incubated in TBS-Tween solutions (0.05% Tween 20 in 50 mM Tris, pH 7.4 with HCl containing 5% (w/v) Carnation nonfat dry milk for 60 min at room temperature. Monoclonal anti-MDR protein antibody (C219, P-glycocheck, Centocor Diagnostics) was diluted 500-fold in the TBS-Tween-milk buffer and incubated with the blots for 60 min. The blots were then washed twice for 15 min each in TBS-Tween solution. Peroxidase-labeled blots were developed by the enhanced chemiluminescence method, using the Amersham Corp. kit. Quantitative densitometry of the x-ray films from the luminograms or the Coomassie Blue stained bands was performed with a Hoefer model GS-300 scanning densitometer interfaced with an Apple II computer.

For 8-azido-ATP labeling, isolated Sf9 membranes (50 µg of membrane protein in 0.25 ml final volume) were incubated on ice in a buffer containing 50 mM Tris-mannitol (pH 6.8), 2 mM EGTA, 2 mM dithiothreitol, 50 mM KCl, and 2 mM MgCl₂, and radiolabeling was started by the addition of 10 µM final concentration (5–10 µCi) of α-
32P-labeled 8-azido-ATP (ICN, 314.5 GBq/mmol). After 10 min of incubation on ice the samples were illuminated for 10 min at a distance of about 3 cm with a short wavelength (254 nm) UV lamp. The reactions were precipitated with trichloroacetic acid contain-
ing 10 mM inorganic phosphate and 2 mM ATP and centrifuged for 5 min at 5,000 x g. The pellet was washed 3 times with the same trichloroacetic acid solution and then resuspended in 50 µl of disaggreg-
ation buffer neutralized with 5 µl of 1 M Tris. The proteins were then electrophoresed and blotted as described above and the dried blots subjected to autoradiography.

The ATPase activity of the isolated Sf9 cell membranes was estimated by measuring inorganic phosphate liberation. Membrane suspensions (about 10 µg of membrane protein, as determined by a modified Lowry method (35)) were incubated at 37 °C in 0.1 ml of a medium containing 50 mM Tris-Mes (pH 6.8), 2 mM EGTA, 2 mM dithiothreitol, 50 mM KCl, and 5 mM sodium azide, and the ATPase reaction was started by the addition of 5 mM MgATP. The indicated drugs (obtained from Sigma) were added in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the assay medium was 1%. Control experiments indicated that dimethyl sulfoxide at this concentration had no appreciable effect on the ATPase activity. The reactions were stopped by the addition of 0.1 ml of 5% SDS solution and the amount of inorganic phosphate determined immediately.

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RESULTS AND DISCUSSION

Sf9 insect cells were infected with a recombinant baculo-
virus containing the MDR1 cDNA, constructed and charac-
terized as described (28). As controls, uninfected Sf9 cells and cells infected with a baculovirus construct containing the β-
galactosidase gene were also examined. The cells were dis-
rupted by homogenization in a low ionic strength medium, and after a low speed pelleting of undisrupted cells and nuclear debris, the light membrane fraction was collected by high speed centrifugation. Fig. 1, upper panel, shows the time course of the expression of the MDR protein as detected by immunoblotting with an anti-P-glycoprotein monoclonal antibo-
ty C219 (29). The approximately 130-kDa immunoreac-
tive protein appears in the light membrane fraction of the MDR1 baculovirus-infected Sf9 cells after the 1st day and reaches its maximal intensity on the 3rd day of infection. It is not detectable in the uninfected or β-galactosidase baculo-
virus-infected cell membranes. The 130-kDa immunoreactive protein represents an underglycosylated form of the MDR protein, as previously observed in the Sf9 cells (28). The expression of the MDR1 gene product at day 3 produced a visible protein band on Coomassie Blue-stained gels (not shown). Based on densitometric analyses of the stained gels, the expressed MDR protein amounted to about 3% of the total protein content in the light membrane fraction. Fig. 1, lower panel, shows the results obtained upon photolabeling of the light membrane fractions isolated from uninfected or MDR1 baculovirus-infected Sf9 cells with 32P-labeled 8-azo-
ido-ATP. The proteins were labeled in the isolated membranes,
precipitated with trichloroacetic acid, and subjected to gel electrophoresis, blotting, and autoradiography. In addition to a few endogenous azido-ATP binding proteins, the MDR1 baculovirus-infected cells, in contrast to the uninfected cells, showed substantial labeling in the 130-kDa region. The labeling in this region exactly overlapped the anti-MDR protein monoclonal antibody staining. These data indicate that the expressed MDR1 gene product in Sf9 cell membranes is capable of binding the ATP analogue, 8-azido-ATP, as has also been shown in tumor cells (21).

Fig. 2 shows the ATPase activity, measured as inorganic phosphate (P_i) liberation from 5 mM MgATP, in the light membrane fractions of β-galactosidase baculovirus-infected (lower panel) or MDR1 baculovirus-infected (upper panel) Sf9 cells, as a function of the time after infection. Membrane ATPase activity was determined in the presence of 5 mM sodium azide, an inhibitor of F1-Fo ATPases, 1 mM ouabain, an inhibitor of the Na^+,K^+-ATPase, and 2 mM EGTA, an inhibitor of calcium-dependent ATPase activity. These reagents have previously been shown not to interfere with P-glycoprotein-dependent drug transport (4, 23). The membranes of the uninfected cells (day 0) had an ATPase activity of about 30 nmol/mg membrane protein/min, 30-40% (about 10 nmol/mg membrane protein/min) of which was inhibited by 100 μM sodium orthovanadate. In the β-galactosidase baculovirus-infected cells this vanadate-sensitive ATPase activity was reduced to even lower levels as the time after infection increased. In contrast, in the MDR1 baculovirus-infected cells, the activity of the vanadate-sensitive membrane ATPase increased significantly to about 30-40% (about 10 nmol/mg membrane protein/min) of which was inhibited by 100 μM sodium orthovanadate. In the β-galactosidase baculovirus-infected cells this vanadate-sensitive ATPase activity was reduced to even lower levels as the time after infection increased.

In contrast, in the MDR1 baculovirus-infected cells, the activity of the vanadate-sensitive membrane ATPase increased significantly to about 30-35 nmol/mg membrane protein/min, while the vanadate-insensitive fraction remained unchanged. Importantly, when verapamil, a known substrate of the MDR pump (4, 5, 17, 18, 23, 24), was added, the vanadate-sensitive ATPase activity in the MDR1 baculovirus-infected cell membranes was stimulated about 5-fold (day 3) to about 150 nmol/mg membrane protein/min. This activity is 10-50-fold greater than the vanadate-sensitive ATPase activity in the uninfected or β-galactosidase baculovirus-infected cell membranes. There was no such drug-stimulated ATPase activity in the uninfected or β-galactosidase baculovirus-infected cell membranes. The appearance of the vanadate-sensitive, verapamil-stimulated ATPase activity in the MDR1 baculovirus-infected cells correlated with the appearance of the MDR protein detected on the immunoblots (Fig. 1).

The vanadate-sensitive, drug-stimulated ATPase activity in the MDR1 baculovirus-infected Sf9 cells was linear with respect to time for up to 20 min at 37 °C and with respect to the membrane concentration between 50 and 200 μg/mL, and was absolutely dependent on the presence of magnesium in the assay medium. Calcium ions (0.1-1 μM, buffered with EGTA) had no appreciable effect. ADP or AMP (1-5 mM) was not hydrolyzed, while 5 mM GTP was hydrolyzed at about 50-60% the rate of 5 mM ATP. The approximate K_i for vanadate inhibition in the presence or absence of verapamil was 10 μM.

Fig. 3 shows the dependence of the ATPase activity in the MDR1 baculovirus-infected Sf9 cell membranes on the MgATP concentration in the assay in the presence or absence of verapamil. As shown, verapamil substantially increases the maximal activity but does not measurably change the apparent K_m for MgATP. Half-maximal activation of the ATPase was obtained at about 0.5 mM ATP in both cases. Although somewhat higher than that reported for vesicle drug transport (23), this value is in the physiological range of cellular ATP concentrations and could thus explain why P-glycoprotein-dependent drug extrusion appears to be so sensitive to cellular ATP depletion (16, 19, 24). The maximal activity of the drug-stimulated MDR ATPase, based on the estimated 3% contribution of the expressed protein to the total amount of membrane protein (see above) is about 5-50 μmol/mg MDR protein/min, approximately 1,000-fold greater than the activity previously associated with the isolated MDR protein (27). The MDR protein-dependent ATP hydrolytic capacity observed here approaches the magnitude of the ATPase activity of the P-type ATPases in a variety of different membranes (30, 31) and thus could account for the ATP consumption observed in the drug-transporting cells (25).

In addition to the ATPase inhibitors already mentioned, we have tested the effects of several other agents on the drug-stimulated ATPase activity. NaN_3 (10 mM) and okadaic acid (200 nM), both inhibitors of several classes of phosphatases, had no inhibitory action on the drug-stimulated ATPase in MDR1 baculovirus-infected Sf9 cell membranes, while oligomycin abolished this ATPase activity at a concentration of 20 μg/mL. Detergents such as Triton X-100, deoxycholate, and CHAPS inhibited the MDR ATPase at extremely low concentrations (0.005-0.05%). CHAPS completely abolished the drug-stimulated ATPase activity at 0.1% (about 1.6 mM), which is below the critical micelle concentration (6-10 mM), for this detergent. Thus a direct interaction of the hydropho-
bic drug-binding site of the enzyme within the membrane, as proposed by Gros et al. (8) and Busche et al. (32), with the detergents may occur. This detergent inhibitory effect is the most plausible explanation for the extremely low ATPase activity (1–3 nmol/mg MDR protein/min) reported for the purified MDR protein (27), since it was isolated and assayed in the presence of CHAPS.

Fig. 4 shows the effects of several drugs reported to interact with the P-glycoprotein (see Refs. 4, 5, 23, and 24) on the vanadate-sensitive ATPase activity in the MDR1 baculovirus-infected SF9 cell membranes. Vinblastine, trifluoperazine, and verapamil, drugs which compete for azidopine binding in the MDR1-expressing SF9 membranes (28), activated the membrane ATPase in the 0.1–10 μM concentration range, consistent with their effect on the reversal of drug resistance and P-glycoprotein function in intact cells in this concentration range (4, 5). In contrast, 5-fluorouracil, which is not a good substrate of the MDR protein (4, 7), produced only a slight activation and only at a much higher concentration.

A clearcut difference in the maximal activation of the ATPase in the MDR1-expressing cells by the different drugs was also observed (Fig. 4). This is probably due to the fact that the drugs which activate the ATPase also have an inhibitory action at higher concentrations. Thus, the different maximal ATPase values may represent conflicting effects of activation and inhibition by the interacting drugs. In support of this interpretation, when present in inhibitory concentrations (500 μM), trifluoperazine abolished the activation of the ATPase by 5 μM verapamil (data not shown).

Table I compiles quantitative estimates of the concentrations of several drugs required for half-maximal activation of the vanadate-sensitive ATPase and the respective increases in the maximal ATPase activity in the MDR1 baculovirus-infected SF9 cell membranes. All of the drugs used in these experiments have been shown to interact with the MDR drug transporter, and all had a strong activating effect on the ATPase activity in concentrations corresponding to their described effects on drug binding or movement in tumor cells (4, 5, 7). There was no apparent correlation between the drug concentrations required for half-maximal activation and the maximal ATPase activities observed. It is important to note that none of the drugs presented in Table I activated ATPase activity in uninfected or β-galactosidase-infected SF9 cell membranes.

The mechanism of ATP hydrolysis by MDR protein is unclear at present. The aspartyl-phosphate phosphorylation site, with amino acids (I/L)CSDKTGTILTX(N, conserved from fungi to humans in the P-type ATPases (30, 31), is not found in the MDR1 amino acid sequence. However, an alternative site for covalent phosphoryl-enzyme complex formation may be present in MDR protein and other members of this family. Although the absence of the formation of a phosphorylated intermediate for the MDR protein was reported earlier (23), the large amount of MDR protein expressed in the SF9 cells provides a useful system for re-investigating this question in efforts to gain insight into the molecular mechanism of the MDR ATPase reaction.

The data in this paper demonstrate the presence of a heretofore undetected large capacity, drug-stimulated, vanadate-sensitive membrane ATPase activity directly related to the presence of the human MDR1 gene product. This finding should facilitate future studies of the structure and molecular mechanism of the various members of this newly recognized family of transport proteins. The present discovery of a potent ATPase activity associated with the MDR protein indeed raises the expectations that functions other than a C1- channel activity may be found for another member of this family, the cystic fibrosis transmembrane conductance regulator. Moreover, the system described allows estimations of the drug affinity and ATPase capacity of various natural and site-directed MDR mutants. By measuring the characteristics of the drug-stimulated ATPase activity of mutant MDR proteins expressed in SF9 cells, the analysis of function-reaction relationships in the MDR protein can be further extended.

The relatively simple assay technique described and the availability of the SF9 insect cell-baculovirus expression system may also provide a valuable tool for examining the functional interactions of newly developed chemotherapeutic agents with the multidrug transporter. Thus, cytotoxic drugs which do not activate the MDR ATPase or drugs which are not toxic but which activate the ATPase and compete with the therapeutic drugs can be selected and evaluated for a possible clinical application to overcome drug resistance in tumor cells.

### Table I

| Drug        | Estimated concentration (μM) | Maximal activation* |
|-------------|-----------------------------|---------------------|
| Vinblastine | 0.5                         | 3.1                 |
| Vinristine  | 0.5                         | 3.0                 |
| Verapamil   | 0.5                         | 5.2                 |
| Daunomycin  | 2                           | 2.1                 |
| Quinine     | 3                            | 2.5                  |
| Trifluoperazine | 5                        | 4.5                  |
| Nifedipine  | 10                          | 5.0                 |
| Colchicine  | 20                          | 2.2                 |

*Increase in activity over that in the absence of drug.

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**Fig. 4.** Effect of several drugs on the vanadate-sensitive ATPase activity in isolated membranes of MDR1 baculovirus-infected SF9 cell after 3 days of infection. ○, no drug added; ○, verapamil; ▲, trifluoperazine; ▲, vincristine; ◆, 5-fluorouracil. The differences between the ATPase activities measured in the absence and presence of vanadate (100 μM) are plotted.
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