Altered Drug-stimulated ATPase Activity in Mutants of the Human Multidrug Resistance Protein*

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The characteristics of P-glycoprotein (MDR1), an ATP-dependent drug extrusion pump responsible for the multidrug resistance of human cancer, were investigated in an in vitro expression system. The wild-type and several mutants of the human MDR1 cDNA were engineered into recombinant baculoviruses and the mutant proteins were expressed in Sf9 insect cells. In isolated cell membrane preparations of the virus-infected cells the MDR1-dependent drug-stimulated ATPase activity, and 8-azido-ATP binding to the MDR1 protein were studied. We found that when lysines 433 and/or 1076 were replaced by methionines in the ATP-binding domains, all these mutations abolished drug-stimulated ATPase activity independent of the MgATP concentrations applied. Photoaffinity labeling with 8-azido-ATP showed that the double lysine mutant had a decreased ATP-binding affinity. In the MDR1 mutant containing a Gly\(^{189}\) to Val replacement we found no significant alteration in the maximum activity of the MDR1-ATPase or in its activation by verapamil and vinblastine, and this mutation did not modify the MgATP affinity or the 8-azido-ATP binding of the transporter either. However, the Gly\(^{189}\) to Val mutation significantly increased the stimulation of the MDR1-ATPase by colchicine and etoposide, while slightly decreasing its stimulation by vinblastine. These shifts closely correspond to the effects of this mutation on the drug-resistance profile, as observed in tumor cells. These data indicate that the S9f-baculovirus expression system for MDR1 provides an efficient tool for examining structure-function relationships and molecular characteristics of this clinically important enzyme.

The overexpression of a 170-kDa membrane protein, termed P-glycoprotein (Pgp)\(^1\) or multidrug resistance transporter (MDR1), is one of the major causes of multidrug resistance of cancer cells to chemotherapy. The human MDR1 protein actively extrudes or interacts with a wide variety of chemically dissimilar and apparently unrelated compounds, e.g. cytotoxic drugs (Vinca alkaloids, colchicine, epipodophyllotoxins, and anthracyclines), calcium channel blockers (verapamil and nifedipine), calmodulin antagonists ( trifluoperazine), or cyclosporines (Gottesman and Pastan, 1993). It has been recently reported that Pgp is also capable of interacting with a variety of small peptides and peptide derivatives (Sharma et al., 1992; Sarkadi et al., 1994; Zhang et al., 1994) as well as hydrophobic fluorescent dyes (Neyfakh, 1988; Homolya et al., 1993; Hollió et al., 1994).

In connection with its transport activity, Pgp has been shown to possess a drug-stimulated ATPase activity in isolated plasma membrane vesicles (Sarkadi et al., 1992; Doige et al., 1992; Shimabuku et al., 1992; Al-Shawi and Senior, 1993), or when the partially purified protein was reconstituted into liposomes (Ambudkar et al., 1992; Doige et al., 1993; Shapiro and Ling, 1994; Urbatsch et al., 1994). Drug transport in vesicles containing partially purified Pgp was shown to be coupled to ATP hydrolysis (Sharon et al., 1993). These studies, in addition to earlier data indicating an energy-requirement for MDR1-dependent drug extrusion clearly demonstrate a direct coupling between drug transport and ATP hydrolysis in the functioning of this protein.

Regarding its molecular architecture, P-glycoprotein is a member of the ABC (ATP-binding cassette) family of membrane transporters, containing 1280 amino acids, which form two symmetrical homologous halves, each with six putative transmembrane domains and an intracellular nucleotide binding domain (Endicott and Ling, 1989; Higgins, 1992). Of major importance would be to learn how ATP binding and hydrolysis is coupled to drug transport within this molecule, that is how the nucleotide binding domains interact with the transmembrane regions when performing active drug extrusion. Both predicted NBDs contain the “homology A” and “homology B” consensus sequences, originally described by Walker et al. (1982). These highly conserved regions are characteristic for several ATP-binding proteins including all the known ABC transporters (Higgins, 1992), and it has been demonstrated that the intact structure of these regions of the NBDs is essential for the drug transporting activity of Pgp. In the mouse MDR1 gene, the conversion of Gly to Ala at positions 431 or
Concerning the site of interaction of Pgp with its transported substrates, e.g. cytotoxic drugs or drug resistance reversing agents, photoaffinity labeling has determined that both halves of Pgp contribute to a single substrate binding site (Brugge mann et al., 1992). Photoaffinity drug labeling experiments combined with trypsin digestion, mapped two major photola beled fragments within, or immediately COOH-terminal to the last transmembrane domains of each half of the molecule, suggesting that some regions of the drug binding sites are in close proximity to the ATP binding regions (Greenberger, 1993).

A key region in substrate handling seems to be the cytoplasmic loop between the second and third transmembrane α-helices. A spontaneous mutation in this region from the wild-type Gly to Val occurred at position 185 in human KB cells, when these cells were selected in high concentrations of colchicine. This substitution greatly increases the relative resistance to colchicine and etoposide (Choi et al., 1988; Kio ka et al., 1989), and influences the photoaffinity labeling of Pgp with different drugs (Safa et al., 1990; Bruggemann et al., 1992). Recent experiments of Loo and Clarke (1994a) indicated that a Gly-Val exchange at five different positions in the cytoplasmic loops of Pgp resulted in a similarly increased resistance to colchicine and adriamycin without altering resistance to vinblastine.

In order to investigate the functional significance of the changes in the nucleotide binding and substrate recognition regions, we expressed the wild-type and some mutant MDR1 proteins in a baculovirus-Sf9 insect cell expression system, and characterized the MDR1-ATPase activity and ATP binding in isolated membrane preparations. As demonstrated earlier, this baculovirus-insect cell expression system can be used for producing large amounts of membrane-inserted human MDR1, which, although underglycosylated, is antigenically and functionally similar to its mammalian counterpart (Germann et al., 1990; Sarkadi et al., 1992; Zhang et al., 1994). In the present experiments we used site-directed mutagenesis to alter single amino acids of the human Pgp in the homology A consensus sequences in the NBDs of the NH2-terminal (Lys433 to Met) and/or COOH-terminal (Lys1076 to Met) halves, and applied the cDNA of the spontaneous Gly to Val to substitute mutation. The MDR1 cDNAs were engineered into baculovirus vectors, recombinant baculoviruses were generated, and after the infection of Sf9 insect cells with the MDR1 viruses the cell membranes were isolated. The expression levels and the functional characteristics of the MDR1 mutants were studied by immunoblotting, by measuring vanadate-sensitive drug-stimulated ATPase activity, and by examining the binding of 8-azido-ATP in the isolated cell membranes.

**MATERIALS AND METHODS**

Construction of Recombinant Transfer Vectors—In this work the following oligonucleotide primers were used: 5′-GGAAATTTGCT-GGGGTGCTCGT, mdr01 (nt 358-379); 5′-GTCCAAGACAGCAG-CTGATG, mdr02R (nt 659-679); 5′-CCATCTCGAAGAGGATTAAG, abc01 (nt 1204-1224); 5′-CTATTCAGACGACCTGAC, abc22R (nt 3663-3683), 5′-CGCCGCCGACCCACCCGCATTCCA, abc02 (nt 3133-3153); 5′-ATGAGGGTGGCTCTCTT, abc04R (nt 3663-3683). The transfer vector pAC373-MDR1Val185 was prepared as described by Germann et al. (1990); for the preparation of transfer vector pVL941-MDR1Val185 a similar strategy was utilized. The transfer vector pVL1393-MDR1Val185 was constructed by isolating the wild-type MDR1 cDNA from plasmid pHMDRGA (Kio ka et al., 1988). The insert was ligated as a blunt ended 5′-terminus fragment (containing a 10-base pair 5′-untranslated region and a 110-base pair 3′-untranslated region with no polyadenylation site) into the SmaI site of baculovirus transfer vector pVL1393 (Pharmingen). To obtain pVL941-MDR1/G ly185, both pVL941-MDR1Val185 and pVL1393-MDR1Val185 were subjected to BglII digestion. This enzyme cuts MDR1 cDNA at nucleotide positions 258 and 1223 but leaves both vectors unharmed. The approximate 1-kilobase pair BglII insert, originated from pVL941-MDR1Val185, was gel-purified and ligated into the gel-purified fragment of pVL1393-MDR1Val185, which lacked the MDR1 cDNA 5′-translated region. The orientation of the fragment was checked by restriction mapping, the presence of the Gly185 codon was confirmed by sequencing the polymerase chain reaction products generated with primers mdr01-mdr02R; mdr01 was used as sequencing primer. Transfer vectors pVL941-MDR1Val185Met433-Lys185, pVL941-MDR1Val185Lys185Met433, and pVL941-MDR1Val185Met433 were produced by replacing the 1177-3372 EcoRI-PstI region of MDR1 in pVL941-MDR1Val185 with those of pUCFXMDR1/neo MK, KM, and MM, respectively.

Generation of Recombinant Baculoviruses—Recombinant baculovi ruses, carrying the different mutants of the human MDR1 cDNA, were generated by using the Baculovirus Transfer Kit (Pharmingen), according to the manufacturer’s suggestions. Sf9 (Spodoptera frugiperda) cells were infected and cultured according to the procedures described previously (Germann et al., 1990).

Confirmation of Mutations in the Recombinant Baculovirus DNA— For each MDR1 construct the mutation was confirmed by sequencing the respective cDNA from the recombinant baculovirus. Virus supernatants (5 mL), containing wild-type (100×) or mutant virus (100×), were irradiated for 10 min with an UV lamp (max about 250 nm) at a distance of 10 cm. Half of the undiluted virus was plated onto Sf9 cells for plaque assay and the other half was used for generating virus stocks.

**Membrane Preparation and ATPase Measurements**—The virus-infected Sf9 cells were harvested and their membranes were isolated and stored, the membrane protein concentrations were determined as described by Sarkadi et al. (1992). ATPase activity of the isolated Sf9 cell membranes was estimated by measuring inorganic phosphate liberation as described in the same article. The data points in the figures show the means of triplicate determinations in representative experiments. The differences between the ATPase activities measured in the absence and presence of vanadate (100 μM) were calculated.

**Quantitation of the MDR1 Protein by Immunoblotting**—Electrophoresis and immunoblotting with the 4077 polyclonal antibody which recognizes human MDR1 (Tanaka et al., 1990) were carried out as described in Sarkadi et al. (1992). The second antibody was an anti-rabbit peroxidase-conjugated goat IgG (Jackson Immunoresearch), used in 20,000 × dilutions. Horseradish peroxidase-dependent luminescence on the polystyrene dishlow concentration membrane immunoblot (ECL, Amersham) was determined by excising the respective bands from the polystyrene dishlow membrane and measuring their luminescence in a liquid scintillation counter (Beckman LS 6000, SPM mode). The amounts of the expressed MDR1 were calculated from the luminescence readings based on a calibration by a dilution series of standard Sf9-MDR1 membrane preparations.

**Photoaffinity Labeling with 8-Azido-ATP**—For the photoaffinity labeling of the wild-type or mutant MDR1 protein, isolated Sf9 cell membranes (150 μg of protein) were incubated in a reaction buffer of Tris (50 mM), mannitol (50 mM), EDTA (2 mM), dithirotetra (1 mM), MgCl2 (5 mM), pH 7.0, in a final volume of 50 μL. In the presence of 70 μM final concentration of 8-azido-ATP (Sigma), containing 0.1 MBq (32P)–8-azido-ATP (ICN Biomedicals, 196 GBq/mmol), and in some samples various concentrations (0.1–1 mM) of ATP or AMP. The samples in a drop on a Parafilm-covered glass plate, cooled to 4°C, and kept on ice, were irradiated for 30 min with an UV lamp (max about 250 nm) at a distance of 10 cm.
Expression and Photoaffinity Labeling—In the first set of experiments we compared the expression levels of the mutant MDR1 proteins in the baculovirus-infected SF9 cells by quantitative immunoblotting. As shown in Fig. 1A, the expression of the various MDR1 constructs in the isolated SF9 cell membranes yielded approximately the same amounts of immunoreactive proteins, while the β-galactosidase-infected cells had no measurable amount of MDR1. The MDR1 expression levels varied from cell batch to batch within about 20%, and the mean values (in micrograms/mg of membrane protein) for each mutant used in the further experiments are given in Fig. 1. These values were obtained from the quantitation of the ECL luminescence of the peroxidase-labeled MDR1-specific bands on the immunoblots, and as a reference we used the previously estimated MDR1 expression level of 30 μg/mg membrane protein for the MDR1-Val185 construct (Sarkadi et al., 1992).

Fig. 1. A, Immunoblot detection of human MDR1 expressed in SF9 cells by baculovirus. Isolated membranes of baculovirus-infected SF9 cells were subjected to electrophoresis and to immunoblotting with the anti-MDR1 polyclonal antibody 4077, as described under “Materials and Methods.” Peroxidase-dependent luminescence on the immunoblots was quantitated with liquid scintillation counting. The amounts of the expressed MDR1 protein are indicated on the bottom of the figure (V, Val185; G, Gly185; KM, Met433; MM, Met433 and Met1076 in MDR1). B, [α-32P]8-azido-ATP labeling of wild-type and mutant P-glycoproteins. Isolated SF9 cell membranes were incubated in the presence of 35 μM final concentration of 8-azido-ATP, containing [α-32P]8-azido-ATP, and irradiated with an UV lamp. The membranes were then precipitated with trichloroacetic acid, washed, and dissolved in electrophoresis buffer, and run on 4-12% gradient or 6% gels. The proteins were electroblotted, the blots dried and subjected to autoradiography (see Materials and Methods”). C, [α-32P]8-azido-ATP, and immunoblotting of the wild-type and the MM mutant P-glycoproteins. Samples were prepared as described for B. 8-Azido-ATP labeling was carried out either in the presence of 1 mM AMP (lanes 2 and 5), or 1 mM ATP (lanes 3 and 6). The identity of the [α-32P]8-azido-ATP-labeled MDR1 band was assured by immunostaining with MDR1-specific antibody (4077) on the same blot (lanes 1 and 4).

As demonstrated in Fig. 1B, in contrast to the β-galactosidase expressing cell membranes, an additional 8-azido-ATP-labeled band, corresponding to the antigenically identified MDR1 protein, was observed in the membranes expressing either the wild-type or any of the mutant MDR1 constructs. We found that 8-azido-ATP binding and photoaffinity labeling required the presence of Mg2+ (1 mM EDTA eliminated labeling). At the 8-azido-ATP concentration applied in these experiments (35 μM), the photoaffinity labeling of the different MDR1 proteins were not significantly different (Fig. 1B). As shown in Fig. 1C, the addition of excess cold ATP (1 mM) abolished radioactive 8-azido-ATP labeling of the expressed MDR1 proteins, while 1 mM AMP was ineffective in this respect.

In the following experiments we have analyzed the effects of different 8-azido-ATP concentrations on the level of MDR1
Drug-stimulated ATPase of MDR1 Mutants

Fig. 3. Vanadate-sensitive MDR1-ATPase activity in isolated membranes of Sf9 cells, stimulation by verapamil. ATPase activity of the isolated Sf9 cell membranes was estimated by measuring inorganic phosphate liberation, as described under "Materials and Methods." The data points in the figures show the mean ± S.D. of three to five determinations for each preparation. The differences between the ATPase activities measured in the absence and presence of vanadate (100 μM), respectively, are plotted. Verapamil, when indicated (darker columns), was applied in a concentration of 20 μM; V, Val185; G, Gly185; MK, Met433; KM, Met1076; MM, Met183 and Met1076 in MDR1.

Fig. 4. MgATP concentration dependence of vanadate-sensitive ATPase activity in isolated Sf9 cell membranes. ATPase activity of the isolated Sf9 cell membranes was estimated by measuring inorganic phosphate liberation as described under "Materials and Methods." The data points in the figures show the mean ± S.D. of three determinations for each membrane preparation. The differences between the ATPase activities measured in the absence and presence of vanadate (100 μM), respectively, are plotted. MgATP concentrations were varied as shown on the abscissa. V, Val185; G, Gly185; MM, Met433 and Met1076 in MDR1.

Fig. 4 shows the MgATP dependence of vanadate-sensitive ATPase activity of β-galactosidase-infected and various MDR1-expressing Sf9 cell membranes. Both the wild-type and the Gly185, Val mutant MDR1 containing membranes show a verapamil-stimulated ATPase activity which reaches maximum levels at about 3 mM MgATP, with an apparent K_m for ATP about 0.5–0.8 mM. MgATP concentrations above 10 mM slightly inhibit this drug-stimulated ATPase. In contrast, the β-galactosidase expressing membranes, or the membranes containing either the KM, MK, or MM mutants of MDR1 (in Fig. 4 we present only the MM construct), have no significant vanadate-sensitive verapamil-stimulated ATPase activity in the whole range of MgATP concentrations examined. Thus, although present in comparable amounts in the isolated membranes and showing 8-azido-ATP labeling, these ATP-binding site mutants have no detectable MDR1-related ATPase activity.

As shown above, in the case of the Gly185 to Val mutant we could not see a significant difference in the maximum level of verapamil-stimulated ATPase activity or in its MgATP concentration dependence. However, according to the data in the literature (Choi et al., 1988; Kioka et al., 1989) these mutants have different drug specificities. Fig. 5 presents the effects of increasing concentrations of several MDR1-interacting drugs on the vanadate-sensitive ATPase of the two different (Gly185 and Val185) MDR1-expressing cell membranes. In this figure we present the vanadate-sensitive MDR1-dependent ATPase activity values corrected for the amount of the MDR1 protein in the isolated Sf9 membranes (see Fig. 1). As shown, the verapamil-stimulation curves for the two different MDR1 proteins are similar, although slight differences can be observed. The concentration required for 50% of maximal stimulation (AC50) for verapamil is slightly higher in the case of the Gly185 (1.8 μM) than for the Val185 (1.2 μM) MDR1 construct, and the inhibition of this latter enzyme is more pronounced at higher verapamil concentrations. In fact, this latter difference may explain the somewhat higher apparent AC50 values calculated in the case of the Gly185 form. The vinblastine activation of the two different MDR1-ATPases are also similar: in both cases the maximum activity is obtained at about 2 μM vinblastine, with an apparent AC50 of about 0.5 μM.
Drug-stimulated ATPase of MDR1 Mutants

In contrast to verapamil and vinblastine, the activation of the two MDR1 forms is distinctly different by colchicine and VP-16 (etoposide). These compounds activate the Val185 MDR1 at lower concentrations and to much higher maximum levels, than the wild-type MDR1. The AC50 value for colchicine in the Gly185 MDR1 membrane was estimated to be about 60 μM, and the maximum stimulation level is less than 20% of that by verapamil. In the case of etoposide, in the Gly185 MDR1 membranes the AC50 value is estimated to be about 0.1 μM. The maximum stimulation is again 65% of that by verapamil, while in the case of the Gly185 MDR1 the AC50 value is about 1.5 μM for the Gly and about 4 μM for the Val enzyme), although in this case the levels of maximum activations are about the same.

**DISCUSSION**

P-glycoprotein, the product of the human MDR1 gene, is responsible for an ATP-dependent extrusion of numerous cytotoxic drugs from a wide variety of cancer cells, thus a thorough knowledge concerning its molecular mechanism of action would be of utmost importance for a clinical intervention. In vitro expression systems combined with functional assays of the protein may greatly facilitate such studies and may allow a deeper understanding of the structure-function connections within the protein in its natural membrane environment.

A recombinant baculovirus-Sf9 insect cell expression system for producing large amounts of human multidrug transporter has been developed by Germann et al. (1990). The major advantages of this system are that the Sf9 cells perform most of the higher eukaryotic post-translational modifications, including glycosylation and phosphorylation, and seem to insert foreign proteins into the cell membrane in a correct transmembrane orientation (O'Reilly et al., 1992). Antibodies raised against different regions of MDR1 recognized the Sf9-expressed protein, and 8-azido-ATP and specific drug binding was also retained by the molecule (Germann et al., 1990; Sarkadi et al., 1992). The molecular mass of the Sf9-expressed MDR1 is slightly smaller than in most mammalian cells (about 130–140 kDa), representing an underglycosylated form of the protein. However, previous studies indicated that the variable levels of glycosylation in various tissues has no significant effect on the function of MDR1 (Greenberger et al., 1987), and Schinkel et al. (1993) demonstrated that Pgp mutants lacking the N-glycosylation sites produced a drug resistance pattern indistinguishable from that of fully glycosylated wild-type MDR1.

In the experiments presented above we have used the baculovirus-Sf9 cells expression system to produce wild-type and mutant forms of the human MDR1 protein. As shown in Fig. 1, all the expressed mutants showed electrophoretic mobilities comparable to that of the wild-type P-glycoprotein and were recognized by a polyclonal antibody specific for the NH2-terminal half of MDR1 (antibody 4077; see Tanaka et al. (1990)). A similar antibody recognition of all the expressed proteins could be seen by the commercially available C219 monoclonal antibody and by the polyclonal antibody 4007, recognizing the COOH-terminal half of MDR1 (Tanaka et al., 1990). Since our experiments showed a highly selective and quantitative recognition of Pgp by antibody 4077 in several cell types (Homolya et al., 1993), the quantitative assessment of the expression levels was carried out by using this antibody and the ECL measurements in scintillation counter (see “Materials and Methods”).

In the studies reported here we used baculovirus-infected Sf9 membrane preparations with roughly similar MDR1 expression levels and provide for each mutant the measured specific protein values (Fig. 1A). In each case the respective MDR1 form was produced via baculoviruses containing the MDR1 cDNA in a pVL941 virus vector. It should be noted that MDR1 expression levels were variable when using different baculovirus vectors: e.g. MDR1 in pVL1393 had an expression level of less than one-third of that in the pVL941. Still, the molecular masses, the antibody recognition, and all the functional characteristics were similar in the MDR1 preparations prepared by different baculovirus vectors.

For the functional characterization of the mutant MDR1 forms we have studied 8-azido-ATP binding, as well as the drug-stimulated vanadate-sensitive ATPase activity related to the expressed protein. The photoaffinity analog, 8-azido-ATP has been successfully used to specifically label various ATP-binding proteins, including Pgp (Cornwell et al., 1987; Sarkadi...
Drug-stimulated ATPase of MDR1 Mutants

et al., 1992; Al-Shawi et al., 1994). In the past few years several studies have demonstrated a high activity vanadate-sensitive, drug-stimulated ATPase, directly connected to the presence of MDR1 in isolated membranes (Sarkadi et al., 1992; Doige et al., 1992; Shimabuku et al., 1992; Al-Shawi and Senior, 1993; Loo and Clarke, 1994b), or in partially purified and reconstituted Pgp preparations (Ambudkar et al., 1992; Doige et al., 1993; Shapiro and Ling, 1994; Urbatsch et al., 1994; Sharom et al., 1993). The MDR1-ATPase has been reported to have a relatively low affinity for ATP, with $K_m$(MgATP) values ranging between 0.5 and 0.8 mM (Sarkadi et al., 1992; Ambudkar et al., 1992; Urbatsch et al., 1994), in accordance with a strong effect of ATP depletion on drug extrusion in intact cells (Endicott and Ling, 1989; Gottesman and Pastan, 1993). All the available evidence strongly suggests that this MDR1-specific ATPase is closely coupled to the ATP-dependent transport function of this protein. In fact, 8-azido-ATP, until exposed to UV light, was shown to be an ATP-like substrate of MDR1, while covalently bound 8-azido-ATP abolished further ATP splitting by the transporter (Al-Shawi et al., 1994).

The combination of the ATP binding and hydrolysis assays is believed to provide valuable information about the interaction of genetically manipulated Pgp both with its energy-donor substrate and the transported species. In order to address these questions, the first set of mutants examined in this work was prepared to contain point mutation(s) in the predicted nucleotide binding domains. Both of the predicted NBDs of P-glycoprotein contain the highly conserved A and B consensus motifs, which were originally described by Walker et al. (1982), from sequence comparisons of a large number of bacterial and eukaryotic ATP-binding proteins. The glycine-rich A motif (believed to form a loop between a $\beta$ bend and an $\alpha$ helix) plays a crucial role in ATP utilization, and the conserved lysine residue in the Walker A sequence is thought to interact directly with one of the phosphate groups of the ATP molecule. Mutational analysis of this A motif in ATP-binding proteins showing ATPase activity, such as the $\alpha$ and $\beta$ subunits of Escherichia coli F$_1$-ATPase (Parsonage et al., 1988), and the yeast STE6 transporter (Berkower and Michaelis, 1991), indicated that substitution of the conserved lysine residue jeopardizes transport activity. The experiments of Azzaria et al. (1989) demonstrated that in mouse Pgp the replacement of these key lysines by arginines in either one of the NBDs eliminated the drug resistance, suggesting that both NBDs are required for the activity of the transporter, although in this mutant ATP binding was conserved. It is interesting to note that when the wild-type and the same double lysine-to-arginine mutant of the mouse MDR1 were expressed in E. coli, the wild-type MDR1-dependent drug efflux was retained by the mutant (Bibi et al., 1993).

Unpublished experiments of Morse et al. showed that when mouse cells were transfected with human MDR1 in which lysines 433 and/or 1076 were replaced by methionines, these mutant MDR1 proteins did not confer a multidrug-resistant phenotype. In the experiments described here we demonstrate that when the MDR1 mutants carrying the same lysine/methionine substitutions were expressed in Sf9 cells, each of these point mutations abolished the drug-stimulated ATPase activity, independent of the MgATP concentrations applied. At the same time, specific high affinity photoaffinity labeling of MDR1 by 8-azido-ATP (completely inhibitable by excess cold ATP) was only altered in the double lysine to methionine (MM) mutant, and even in this case the labeling of MDR1 was only slightly decreased at 8-azido-ATP concentrations above 25 $\mu$M. It is to be noted that Morse et al. observed a significant change of 8-azido-ATP binding in the mutant MDR1 proteins when examined at low (2.5 $\mu$M) 8-azido-ATP concentrations.

The ATP concentrations producing half-maximal stimulation of the MDR1-ATPase activity (see Sarkadi et al. (1992) and Shapiro and Ling (1994), and Fig. 4 of this paper), or of the MDR1-dependent drug transport (see Gottesman and Pastan (1993)) are in the range of 0.3–0.5 mM. Thus the functional role of the high-affinity ATP binding by MDR1, as seen in the azido-ATP binding experiments, as well as its alteration in the MM mutant, cannot be properly appreciated as yet. Still, our data indicate that drug-stimulated ATPase activity is absent if these highly conservative residues are altered in any of the two NBDs of MDR1, and while single NBD mutations have no major effect on ATP binding at low ATP concentrations, the double lysine to methionine mutation considerably alters this ATP binding.

In a previous study, aimed at investigating the role of various parts of the MDR1 protein in its function, the separately expressed NH$_2$-terminal half of the human Pgp showed an ATPase activity comparable to that of the full-length protein, but did not confer drug resistance when expressed in mammalian cells (Shimabuku et al., 1992). The authors suggested that the NH$_2$-terminal NBD contains all the residues required to hydrolyze ATP, without necessarily interacting with the COOH-terminal binding site. A key problem in the studies of Shimabuku et al. (1992) was the relatively low level of the ATPase activity in the isolated full-length or truncated MDR1 (about 150 nmol/mg MDR1 protein) and the lack of drug-stimulation in either case. Convincing data for the role of the two halves of MDR1 have recently been provided by Loo and Clarke (1994b). When the two halves of MDR1 were expressed in Sf9 cells separately, both proteins showed a low level of ATPase activity with no substrate stimulation, while their co-expression restored high-activity, drug-stimulated ATPase.

The data presented in this paper, in accordance with the experiments of Azzaria et al. (1989) and Loo and Clarke (1994b), strongly support the idea that the interaction of two functional NBDs is essential both for drug extrusion and drug-stimulated hydrolysis of ATP by MDR1. At the same time it is still unclear whether the concerted hydrolysis of two ATP molecules may be required to transport one molecule of drug.

Another key issue to be addressed in the structure-function studies is the site(s) of interaction of the multidrug resistance protein with its transported substrates. A single point mutation in human Pgp, a spontaneous exchange of Gly to Val at position 185 (Choi et al., 1988), was reported to result in an increased relative resistance to colchicine and etoposide, while unchanged or slightly reduced resistance toward vinblastine and vincristine (Choi et al., 1988; Currier et al., 1992; Cardarelli et al., 1995). In this study we have reproduced the Gly$^{185}$ to Val point mutation in the baculovirus-Sf9 expression system for MDR1. Our experiments showed no significant alteration in the maximum activity of the MDR1-ATPase or in its activation kinetics by verapamil and vinblastine, and this mutation did not modify the MgATP affinity or the 8-azido-ATP binding of the transporter either. However, the Gly$^{185}$ to Val mutation significantly increased the stimulation of the MDR1-ATPase by colchicine and etoposide, while slightly decreasing its stimulation by vincristine. These shifts closely correspond to the effects of this mutation on the drug-resistance profile in intact tumor cells. Moreover, the data indicating that the Gly$^{185}$ to Val exchange, while increasing colchicine extrusion and colchicine stimulation of the MDR1-ATPase activity, reduces the binding of this drug to the MDR1 protein (Safa et al., 1990), may suggest that in the molecular mechanism of drug extrusion, ATP splitting is required for the dissociation of the drug from the transporter protein. Altogether the data pre-
sent in this paper further support the usefulness of the MDR1-ATPase assay in isolated Sf9 membranes for a functional characterization of molecular alterations in the P-glycoprotein.

While the present paper was under revision, a publication by U. S. Rao (1995) reported the expression and partial characterization of the Gly185 to Val MDR1 mutants in Sf9 cells. The related findings of the two reports are mostly in accordance, although Rao (1995) obtained a higher maximum activity and a lower Kₘ for the Val185 MDR1-ATPase than for the Gly185 protein when using verapamil. Since in the experiments of Rao (1995) the maximal MDR1-ATPase activity/mg of membrane protein when using verapamil. Since in the experimentsof Rao (1995) reported the expression and partial characterization of molecular alterations in the P-glycoprotein.

Drugs-stimulated ATPase of MDR1 Mutants

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