Phosphorylation Site Mutations in the Human Multidrug Transporter Modulate Its Drug-stimulated ATPase Activity*

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In the human multidrug transporter (MDR1), three serine residues located in the “linker” region of the protein are targets of in vivo phosphorylation. These three serines, or all eight serines and threonines in the linker, were substituted by alanines (mutants 3A and 8A) or with glutamic acids (mutants 3E and 8E). The wild-type and mutant proteins were expressed in baculovirus-infected Spodoptera frugiperda (Sf9) ovarian insect cells, and the vanadate-sensitive, drug-stimulated ATPase activity was measured in isolated membrane preparations. The maximum drug-stimulated MDR1-ATPase activity was similar for the wild-type and the mutant proteins. However, wild-type MDR1, which is known to be phosphorylated in Sf9 membranes, and the 3E and 8E mutants, which mimic the charge of phosphorylation, achieved half-maximum activation of MDR1-ATPase activity at lower verapamil, vinblastine, or rhodamine 123 concentrations than the nonphosphorylatable 3A and 8A variants. For some other drugs (e.g. valinomycin or calcein acetoxymethylester) activation of the MDR1-ATPase for any of the mutants was indistinguishable from that of the wild-type protein. Kinetic analysis of the data obtained for the 3A and 8A MDR1 variants indicated the presence of more than one drug interaction site, exhibiting an apparent negative cooperativity. This phenomenon was not observed for the wild-type or the 3E and 8E MDR1 proteins. The dependence of the MDR1-ATPase activity on ATP concentration was identical in the wild-type and the mutant proteins, and Hill plots indicated the presence of more than one functional ATP-binding site. These results suggest that phosphorylation of the linker region modulates the interaction of certain drugs with MDR1, especially at low concentrations, although phosphorylation does not alter the maximum level of MDR1-ATPase activity or its dependence on ATP concentration.

Overexpression of the human multidrug transporter (MDR1 or P-glycoprotein)† is responsible for the phenomenon of multiple drug resistance in various cancer cell types. MDR1 is an integral plasma membrane protein that acts as an ATP-dependent efflux pump to reduce the intracellular concentration of diverse hydrophobic compounds (1–3). MDR1 belongs to the superfamily of the ATP-binding cassette transporters, containing a tandem repeat of transmembrane domains and conserved nucleotide-binding motifs, connected by a central “linker” region (1, 4, 5).

MDR1 was described as a phosphorylated glycoprotein by several early reports (6, 7), but no clear role for phosphorylation in the transporter activity of MDR1 has yet been established. Numerous studies have suggested a correlation between changes in cellular protein kinase A and protein kinase C activities and the multidrug resistance phenotype. In addition, significant modifying effects of protein kinase activators or inhibitors on drug resistance, drug accumulation, drug binding, and the level of MDR1 phosphorylation have been reported (8, 9). However, the interpretation of these results is difficult, as protein kinases and their modulators may not only affect the phosphorylation of MDR1 but can also influence MDR1 gene expression (10–13). Moreover, most of the protein kinase or phosphatase modulators studied are hydrophobic agents and may interact directly with MDR1 as substrates of the transporter (9, 14–16).

The identification of the major phosphorylation sites within the MDR1 protein provided an opportunity to analyze critically the role of this covalent modification (9, 17). The linker region represents the only documented target for phosphorylation both in vitro and in vivo (18–22). This region contains several consensus sequences for phosphorylation by protein kinase C and protein kinase A, although only four serine residues (Ser661, Ser667, Ser671, and Ser683) are phosphorylated by these kinases in vitro (19, 20, 23). Two of these residues (Ser661 and Ser667) are also phosphorylated by the MDR1-specific V1 kinase isolated from the multidrug resistance KB-V1 cell line (23). Only three of these serine residues, Ser661, Ser667, and Ser671, are phosphorylated in vivo (24).

Recent reports show that phosphorylation of MDR1 does not play an essential role in its drug-transporting activity. Mutants in which three to eight serine or threonine residues in the linker region were substituted by nonphosphorylatable alanine, or negatively charged aspartic/glutamic acid residues (mimicking the charge of phosphorylation), all encoded a functional protein; 3A, mutant MDR1 with mutations S661A, S667A, and S671A; 3E, mutant MDR1 with mutations S661E, S667E, and S671E; 8A, mutant MDR1 with mutations S660A, S661A, S667A, S671A, S675A, S683A, and T684A; 8E, mutant MDR1 with mutations S660E, S661E, S667E, T668E, S671E, S675E, S683E, and T684E; AM, acetoxymethylster; Sf9, Spodoptera frugiperda ovarian cells.

* This work was supported in part by research grants from COST, PECO, ACCORD, OMFB, OTKA (F13178, T17602, T8348), and ETT (Hungary), and by the Cancer Research Campaign and Imperial Cancer Research Fund (UK). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: MDR1, human multidrug resistance protein; 3A, mutant MDR1 with mutations S661A, S667A, and S671A; 3E, mutant MDR1 with mutations S661E, S667E, and S671E; 8A, mutant MDR1 with mutations S660A, S661A, S667A, S671A, S675A, S683A, and T684A; 8E, mutant MDR1 with mutations S660E, S661E, S667E, T668E, S671E, S675E, S683E, and T684E; AM, acetoxymethylster; Sf9, Spodoptera frugiperda ovarian cells.

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MATERIALS AND METHODS

Sf9 Cells—Sf9 cells were cultured and infected with baculovirus vectors as described by Germann et al. (31). Baculovirus transfer vectors were constructed by using the human MDR1 cDNA encoding a protein with the following mutants: S661A, S667A, and S671A (3A); S661E, S667E, and S671E (3E); S660A, S661A, S667A, T668A, S671A, S675A, S683A, and T684A (8A); and S660E, S661E, S667E, T668E, S671E, S675E, S683E, and T684E (8E), as described earlier (22, 32). An EcoRI-PstI fragment (nucleotides 1177-3372) was deleted from the wild-type human MDR1 baculovirus expression vector DpACUW21-MDR1 (33), and replaced with the corresponding fragments from the above mutant MDR1 genes. Recombinant baculoviruses carrying the variants of the human MDR1 cDNA were generated using the BaculGold transfection kit (PharMingen), according to the manufacturer’s instructions. In each case the DNA fragments introduced were sequenced to ensure that no additional mutations occurred.

Membrane Preparations—The virus-infected Sf9 cells were suspended in a low ionic strength medium (containing 50 mM Tris-HCl pH 7.0, 50 mM mannitol, 2 mM EGTA, 10

Membrane Preparations—The virus-infected Sf9 cells were suspended in a low ionic strength medium (containing 50 mM Tris-HCl pH 7.0, 50 mM mannitol, 2 mM EGTA, 10 µg/ml leupeptin, 8 µg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol) and disrupted using a glass-Teflon homogenizer. Membrane fractions were isolated by repeated centrifugations and homogenizations, and the membrane protein concentrations were determined as described by Sarkadi et al. (25).

Electrophoresis and Immunoblotting—Membranes were suspended in a disaggregation buffer (25). Samples (20 µl) were run on 6% Laemmli-type gels and electro-blotted onto polyvinylidene difluoride membranes. Quantitative estimation of the expression of human MDR1 was performed using the polyclonal anti-MDR1 antibody 4077 (34) and a secondary antibody (anti-rabbit peroxidase conjugated IgG, 20,000 × dilution, Jackson ImmunoResearch), as described by Müller et al. (35). Horseradish peroxidase-dependent luminescence (ECL, Amersham Corp.) was determined by luminography and quantitated by the BioRad PhosphorImager system.

Immunofluorescence—106 cells were resuspended in phosphate-buffered saline, fixed with 2% formaldehyde, and then incubated with 0.5% saponin for 5 min. Cells were incubated for 45 min in phosphate-buffered saline containing 1% bovine serum albumin, and labeled in the same medium with the monoclonal antibodies MRK 16 and UIC2 (1:100 dilution), as described by Welker et al. (33). Cellular fluorescence was measured with a Becton Dickinson FACScan/fluor flow cytometer, and data analyzed by the Winlist software (Verity Software House, Inc.). Isotype matching nonspecific antibodies were used as controls.

ATPase Activity—ATPase activity sensitive to vanadate was measured in isolated membranes as described by Sarkadi et al. (25). MgATP concentrations were calculated by considering the presence of EGTA in the medium.

RESULTS

Expression of Mutant Proteins Using the Baculovirus-Sf9 Insect Cells System—Mutant MDR1 proteins altered at the phosphorylation sites were expressed in the baculovirus-Sf9 insect cells system, and drug-stimulated ATPase activity measured in isolated membrane preparations. The serines and/or threonines in the linker region of the protein were replaced by alanines (to prevent phosphorylation) or glutamic acid residues (to mimic the charge of permanent phosphorylation). Two sets of mutants were constructed. In one set the three serine residues phosphorylated in vivo (Ser661, Ser667, and Ser671) were replaced to generate 3A and 3E mutants. In the other set all the eight serines and threonines providing possible additional phosphorylation sites in the linker region (Ser660, Ser617, Ser667, Ser671, Ser675, Ser683, and Thr668, Thr684) were replaced (8A and 8E mutants), to exclude the possibility that when the three primary phosphorylation sites were mutated the other serine/threonine residues may become phosphorylated. The mutant protein containing eight alanine substitutions, when expressed in mammalian cells, has been shown not to be phosphorylated by protein kinase C (22).

As documented in Fig. 1B, immunoreactivity of the 3A and 3E proteins expressed in Sf9 cells was indistinguishable from that of the wild-type MDR1, when examined by using specific monoclonal antibodies. As both MRK 16 and UIC2 recognize complex epitopes of MDR1 on the external membrane surface (36, 37), this finding suggests that the phosphorylation site mutations did not alter the membrane localization of MDR1. In similar experiments we also found the 8A and 8E mutants were normally inserted in the membrane (data not shown).

Maximum Drug-stimulated ATPase Activity of Wild-type and Mutant MDR1—Fig. 2 depicts the mean vanadate-sensitive ATPase activities measured in the Sf9 cell membrane preparations, with or without the addition of 50 μM verapamil. It has previously been shown that this concentration of verapamil achieves maximum stimulation of the ATPase activity of wild-type MDR1 (see Müller et al. (35)). All MDR1 variants exhibited a high level of verapamil-stimulated ATPase activity, and no significant differences in the unstimulated, or maximum verapamil-stimulated ATPase activities could be detected between the wild-type and mutant proteins. The control, β-galactosidase-expressing cell membrane preparations did not exhibit vanadate-sensitive, drug-stimulated ATPase activity. For these, and all subsequent experiments, the 3E and 3A mutants behaved identically to 8E and 8A mutants, respectively. This shows that if the three in vivo phosphorylation-sites are mutated, the other potential sites do not serve as functional substitutors. In the figures below, therefore, the data for only the 3E and 3A mutants are shown.

Apparent K<sub>m</sub> and Hill n Values for Drug Stimulation of MDR1-ATPase Activity—The drug concentration-dependence of the ATPase activity of the wild-type and mutant MDR1 proteins was measured. Fig. 3A shows the data obtained by varying the concentration of verapamil for the 3A and 3E mutants. There was no significant difference (a maximum of 10–15%) between the MDR1-ATPase activities of the two mutants at maximally stimulating (40–60 μM) verapamil concentrations. However, at lower verapamil concentrations, a significant (p < 0.05) difference between the activities of the different mutants was observed: between 1 and 10 μM verapamil the ATPase activity of the 3E MDR1 variant was about 50% higher than that of the 3A variant.

Fig. 3B shows data from Fig. 3A analyzed using a simple Michaelis-Menten kinetic approach, and they are presented in a linearized (Lineweaver-Burk) plot. The double-reciprocal analysis (1/MDR1-ATPase activity versus 1/drug concentra-
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their 3A or 3E counterparts; the apparent $K_m$ of 8E and 8A mutants were 2.2 and 1 $\mu$M, respectively, the estimated $V_{max}$ of 3.1 and 2.8 $\mu$mol of Pi/mg of MDR1 protein/min, respectively.

Fig. 3C presents a Hill plot analysis of the same data points. This representation indicates that the verapamil concentration dependence of the ATPase activity of the 3E mutant can be described by a single linear relationship, providing an apparent Hill $n$ value near to 1. In contrast, the data points for the 3A mutant can be best fitted with a line providing an apparent $n$ value significantly ($p = 0.0002$) smaller than 1 (about 0.7).

Figs. 4 and 5 show a detailed kinetic analysis of the vinblastine and rhodamine 123 concentration dependence of the ATPase activity for the 3A and 3E MDR1 variants. Both drugs are known to stimulate the MDR1-ATPase activity in membrane preparations and of purified, reconstituted MDR1 (25–27). The effects of the phosphorylation site mutations on this activity proved to be similar to those described above for verapamil; there was no significant effect on the ATPase activity at maximally stimulating vinblastine and rhodamine 123 concentrations, but differences between the 3E/8E and 3A/8A mutants were observed at lower concentrations. At maximally stimulating vinblastine concentration, the specific ATPase activities measured in the absence and presence of vanadate were phosphorylation site mutants of MDR1 as described under "Materials and Methods." The amounts of the expressed MDR1 protein are indicated at the bottom. 3A, 8A, 3E, and 8E are phosphorylation site mutants of MDR1 as described under "Materials and Methods." WT, wild-type MDR1; $\beta$-gal, $\beta$-galactosidase-infected control. Molecular mass markers are given in kDa. B, Immunoreactivity of cells expressing MDR1 variants examined by flow cytometry. Phosphorylation mutants (3E and 3A), wild-type MDR1 (WT) and control $\beta$-galactosidase ($\beta$-gal) infected cells were subjected to flow cytometry analysis after staining with monoclonal antibodies MRK16, UIC2 or corresponding isotype control nonspecific antibodies. Isotype controls for each cell type showed similar fluorescence patterns to those seen for the $\beta$-galactosidase-infected cells.

Expression and membrane insertion of the phosphorylation site mutants of human MDR1 expressed in baculovirus-infected Sf9 cells. A, Immunoblot detection of human MDR1 expressed in baculovirus-infected Sf9 cells. Isolated membranes (2 $\mu$g of membrane protein/lane) were subjected to electrophoresis and analyzed by Western blotting using the MDR1-specific polyclonal antibody 4077 as described under "Materials and Methods." The amounts of the expressed MDR1 protein are indicated at the bottom. 3A, 8A, 3E, and 8E are phosphorylation site mutants of MDR1 as described under "Materials and Methods." WT, wild-type MDR1; $\beta$-gal, $\beta$-galactosidase-infected control. Molecular mass markers are given in kDa. B, Immunoreactivity of cells expressing MDR1 variants examined by flow cytometry. Phosphorylation mutants (3E and 3A), wild-type MDR1 (WT) and control $\beta$-galactosidase ($\beta$-gal) infected cells were subjected to flow cytometry analysis after staining with monoclonal antibodies MRK16, UIC2 or corresponding isotype control nonspecific antibodies. Isotype controls for each cell type showed similar fluorescence patterns to those seen for the $\beta$-galactosidase-infected cells.
although the concentration of this drug required to induce half-maximum activation of the ATPase was significantly higher (Fig. 5). In double-reciprocal representations (Fig. 5A), the ATPase activity of the 3E mutant yielded a single linear plot with an apparent $K_m$ value of 56 $\mu$M and an estimated $V_{max}$ of about 3 $\mu$mol of P$_i$/mg of MDR1 protein/min, while the 3A mutant gave two different linear regions with apparent $K_m$ values of 17 and 80 $\mu$M, respectively, and an estimated $V_{max}$ of 2.8 $\mu$mol of P$_i$/mg of MDR1 protein/min. In Hill plots (Fig. 5B), these data can be described by a linear relationship for the 3E mutant ($Hill n_{app} = 1$), while for the 3A mutant the resulting apparent $n$ values for the two different linear regions were 0.5 and 1.

As documented in Table I, the data obtained for the stimulation of the wild-type MDR1-ATPase by verapamil, vinblastine, or rhodamine 123, were qualitatively similar to those found for the 3E mutant, although the variability was somewhat greater. A plausible explanation for the more variable apparent $K_m$ values for the wild-type protein is that, in most Sf9 membrane preparations, the MDR1 protein is fully phosphorylated (31) such that it behaves similarly to the 3E mutant. However, the absolute level of phosphorylation of the wild-type enzyme may depend on the actual membrane preparation (e.g. the length of virus infection before membrane...
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Effects of phosphorylation site mutations on the estimated apparent $K_m$, $V_{\text{max}}$, and Hill $n$ values of the MDR1-ATPase activity

| Activating agent | Wild-type | 3 A mutant | 3 E mutant |
|------------------|-----------|------------|------------|
| Verapamil        | $K_m$ ($\mu M$) | 0.8–3 | 1 | 2.2 |
|                  | $V_{\text{max}}$ | 2.6–3.2 | 2.7 | 3.1 |
|                  | Hill plot, $n_{\text{app}}$ | 0.9–1.0 | 0.7 | 0.9 |
| Vinblastine      | $K_m$ ($\mu M$) | 0.5–0.8 | 0.5 | 0.8 |
|                  | $V_{\text{max}}$ | 1.5 | 1.3 | 1.7 |
|                  | Hill plot, $n_{\text{app}}$ | 0.8–1.0 | 1.0/0.5/1.0 | 1.0 |
| Rhodamine 123    | $K_m$ ($\mu M$) | 30–50 | 17 | 56 |
|                  | $V_{\text{max}}$ | 2.6–3.2 | 2.8 | 3.0 |
|                  | Hill plot, $n_{\text{app}}$ | 0.8–1.0 | 0.5/1.0 | 1.0 |
| Valinomycin      | $K_m$ ($\mu M$) | 0.5 | 0.5 | 0.5 |
|                  | $V_{\text{max}}$ | 2.8 | 2.8 | 2.8 |
|                  | Hill plot, $n_{\text{app}}$ | 1.0 | 1.0 | 1.0 |
| Calcein AM       | $K_m$ ($\mu M$) | 0.8–1 | 0.8 | 0.8 |
|                  | $V_{\text{max}}$ | 3.8–4.1 | 4.1 | 4.1 |
|                  | Hill plot, $n_{\text{app}}$ | 1.0 | 1.0 | 1.0 |

*a Estimated $V_{\text{max}}$ = $\mu$mol of P/mg of MDR1 protein/min.

Table I: Effects of phosphorylation site mutations on the estimated apparent $K_m$, $V_{\text{max}}$ and Hill $n$ values of the MDR1-ATPase activity.

FIG. 5. Effects of rhodamine 123 concentration on the MDR1-ATPase activity of the 3A and 3E mutant proteins. A, double-reciprocal plot of the rhodamine 123 concentration dependence of MDR1-ATPase activity. MgATP concentration was 3 mM. B, Hill plot of the same data as in A.

preparation, which varied between 70 and 76 h in our laboratory. It was not possible to address this question by treating the Sf9 cell membranes with alkaline phosphatase before the ATPase measurements as the process of the removing the phosphatases strongly reduced any measurable MDR1-ATPase activity.

The data summarized in Table I show another important conclusion. Some drugs did not show any difference in MDR1-ATPase stimulation or apparent $K_m$ values between the wild-type and the mutant proteins. These included a high affinity, transported peptide substrate of MDR1, valinomycin (17, 38) (Table I, row 4), and the hydrophobic molecule calcein AM (Table I, row 5).

Effects of Phosphorylation Site Mutations on the MgATP Concentration Dependence of MDR1-ATPase Activity—To examine the effects of the phosphorylation site mutations on the MgATP concentration dependence of MDR1-ATPase activity, ATPase measurements were carried out at maximally stimulated drug concentrations, varying the MgATP concentrations between 0.1 and 10 mM in the incubation medium. Fig. 6A shows the results of such an experiment using 50 $\mu M$ verapamil, for both the 3A and 3E mutants, presented at a double-reciprocal plot (1/MDR1-ATPase activity versus 1/MgATP concentration). This plot gave a linear relationship for both MDR1 variants, and neither the $V_{\text{max}}$ (2.6 versus 3.0) nor the $K_m$ MgATP values (0.17 mM versus 0.20 mM) were significantly different between the two mutants ($p = 0.06–0.8$ for data measured at 0.07–5 mM MgATP). A similar linear plot, yielding estimated $V_{\text{max}}$ values around 3 $\mu$mol of P/mg of MDR1 protein/min and a $K_m$ for MgATP about 0.19–0.26 mM, was obtained for the wild-type, as well as for the 8A ($K_m$, 0.19 mM, $V_{\text{max}}$ 2.7 $\mu$mol of P/mg of MDR1 protein/min) and 8E ($K_m$, 0.20 mM, $V_{\text{max}}$ 3.0 $\mu$mol of P/mg of MDR1 protein/min) mutants. It is important to note that the Hill plots (Fig. 6B) yielded apparent $n$ values of about 1.4 for all the mutants and the wild-type MDR1 protein ($n_{\text{app}}$ for 3A, 8A, 3E, 8E, and wild-type MDR1 were 1.44, 1.38, 1.42, 1.37, and 1.41, respectively), indicating the presence of more than one ATP-binding sites with an apparent positive cooperativity. Similar results were obtained for the MgATP-dependence of the ATPase activity in the mutant MDR1 proteins in the presence of maximally activating calcein AM or rhodamine 123 concentrations (data not shown).

Discussion

Human MDR1 is phosphorylated by protein kinase C and protein kinase A at several serine and threonine residues in the linker region which separates the two halves of the molecule. Mutation of these phosphorylation sites does not have any major effect on drug transport by MDR1 or on its ability to confer drug resistance on cells (21, 22). In this study we have investigated the role of linker phosphorylation in modulating the drug-stimulated ATPase activity of MDR1. Wild-type MDR1, and mutant proteins altered in the linker phosphorylation sites, were expressed to similar levels in baculovirus membranes and shown to be membrane localized. Assay of MDR1-ATPase activity in such membranes has previously been shown to provide a direct and sensitive assay for subtle changes in MDR1 activity in response to the energy-donor ATP, and also for transported substrates (25, 35, 39).

The linker region of MDR1 has eight potentially phosphorylatable serine/threonine residues although only three of these are phosphorylated in vivo (24). Proteins in which these three sites, or all eight potential sites, were mutated behaved indistinguishably. Thus, when the primary sites were mutated it did not appear that secondary sites could substitute. It should be noted that MDR1 does not appear to be phosphorylated at any site outside the linker and, thus, all potential phosphorylation sites are altered in these mutants (18–22).

Maximum stimulation of MDR1-ATPase activity by all the
drugs tested was similar for the wild-type and all mutant MDR1 proteins. This is consistent with the finding that phosphorylation of MDR1 has no detectable effect on drug resistance, drug accumulation, drug binding or drug transport capacity (21, 22).  

The finding that the maximal ATPase activity was not altered in the mutant proteins provides strong evidence that the mutations do not have a nonspecific effect on the folding or activity of MDR1.

When the drug concentrations required to achieve half-maximum activation of MDR1-ATPase activity were studied, the wild-type and 3E/8E mutants behaved indistinguishably. This is consistent with the fact that in insect cell membranes MDR1 is phosphorylated and the glutamate for serine/threonine phosphorylation of MDR1 has no detectable effect on drug resistance, drug accumulation, drug binding or drug transport capacity (21, 22).

The drug-stimulated ATPase activity of the nonphosphorylatable 3A/8A mutant was also indistinguishable from wild-type for a subset of drugs including calcein AM and valinomycin. However, the stimulation of ATPase activity of the 3A/8A MDR1 proteins by a different subset of drugs, including verapamil, rhodamine 123 and vinblastine, showed two significant differences from that of the wild-type protein: (i) plots of drug-concentration against ATPase activity were biphasic, indicating multiple drug interaction sites; (ii) higher drug concentrations were required to achieve half-maximal ATPase stimulation. Ahmad et al. (40) have also presented evidence that exchanging Ser671 to asparagine reduced ATPase activity by about 50% at 10 μM verapamil when expressed in Sf9 cells.

ATPase activity in these studies was measured in insect cell membranes while previous transport studies have been carried out using yeast and mammalian cells. However, it seems unlikely that this significantly affects the conclusions. In mammalian cells no effect of phosphorylation on drug transport could be detected, but higher verapamil concentrations were required to inhibit calcein AM and vinblastine transport by the 8A mutant MDR1 protein, entirely consistent with the present results in insect cells.

Many lines of evidence show that drug transport and ATPase activity by MDR1 are coupled. Thus the MDR1-ATPase activity is stimulated by transported drugs in a dose-dependent fashion (26, 27, 29, 41–45). Drug transport and ATPase activities are both vanadate-inhibitable (26, 41, 45), and compounds which bind tightly to MDR1 act as potent inhibitors of both drug transport and drug-stimulated ATPase activity (26, 38). For reconstituted MDR1, the rate of ATP-driven 86Rb+-valinomycin uptake correlates with ATP hydrolysis (46), and the stimulation of MDR1-ATPase by several compounds directly correlates with their ability to be transported (39). Finally, mutations which affect substrate specificity of MDR1 have corresponding effects on its ATPase activity (35, 47) (for reviews, see Refs. 17 and 30). Thus, changes in ATPase activity in the phosphorylation mutants might be expected to correlate with changes in the drug transport properties of MDR1. Since the effects of the phosphorylation mutants on drug-stimulated ATPase activity were relatively small and were only observed at subsaturating drug concentrations, these may not have been detectable by measuring drug resistance or drug transport in intact cells, and reflect the high sensitivity of the ATPase assay.

What is the significance of the observation that phosphorylation affects the ability of only a subset of drugs to modulate ATPase activity? The finding that phosphorylation has no effect on calcein AM transport (22) or stimulation of ATPase activity (this study) makes calcein AM an ideal tool for use in the functional diagnostics of the multidrug resistance protein (48, 49) in tumor cells which may have variable patterns of phosphorylation. This is in contrast to, for example, rhodamine 123 whose transport may be influenced by variable phosphorylation states of MDR1. It should also be noted that the effects of phosphorylation on the verapamil stimulated MDR1-ATPase activity may explain the somewhat variable effects of phosphorylation on the verapamil stimulated MDR1-ATPase activity in different laboratories (35, 47).

In this study we have found that phosphorylation of MDR1 does not affect dramatically, but modulates gently its ATPase activity, and may therefore regulate the drug transport properties of MDR1. These effects may be significant under in vivo conditions when the level of the transported anticancer drugs is relatively low. Still it is not unreasonable to suppose that the principal role of phosphorylation of the linker region may reflect an alternative function of MDR1, that is regulation of cell swelling-activated chloride channels (50–52). Significantly, phosphorylation of the linker of MDR1 influences its ability to

$^a$ C. F. Higgins, R. Callaghan, and H. R. Goodfellow, unpublished data.

$^b$ H. R. Goodfellow, R. Callaghan, and C. F. Higgins, unpublished results.
act as a channel regulator (32).

The biphasic correlation between drug concentration and ATPase stimulation observed for the nonphosphorylated MDR1 is best interpreted in terms of multiple drug-interacting sites on MDR1. In the non-phosphorylated state, these sites show significantly different drug affinities and an apparent negative cooperativity, while in the fully phosphorylated form of the enzyme the two sites are indistinguishable. Several published studies provide evidence that MDR1 has multiple drug binding/interaction sites (53–58),4 although the precise role of these sites in MDR1 function are still obscure. Photoaffinity labeling and other studies (59, 60) (for reviews, see Refs. 17 and 30).

In summary, the results presented here indicate that phosphorylation of the anti-MDR1 polyclonal antibodies. The technical help by Ilona Zombori and Györgyi Demeter is gratefully acknowledged.

Acknowledgments—We thank Professor M. M. Gottesman for providing the anti-MDR1 polyclonal antibodies. The technical help by Ilona Zombori and Györgyi Demeter is gratefully acknowledged.

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