ABSTRACT

Structural features of the multidrug resistance protein encoded by the mouse mdr1 gene were studied in multidrug-resistant cell clones stably transfected with a biologically active cDNA clone. Independently derived transfected cell clones, initially selected in Adriamycin, were shown to be cross-resistant to several drugs, including actinomycin D, amacrine, mitoxantrone, VP-16, and vincristine but remained sensitive to cisplatinum, 5-fluorouracil, ara-bnicotinamide, and bleomycin. In drug-resistant transfectants the mdr1 gene product was greatly overexpressed as a polypeptide of apparent molecular weight 160,000–170,000. This protein was present in membrane enriched fractions and could be metabolically labeled with 3Hglucosamine, confirming that the transfected mdr1 gene encodes a membrane glycoprotein. The protein was found phosphorylated on serine residues and was shown to be photolabeled by both the calcium antagonist azidopine and the ATP analogue 8-azido ATP. Tryptic mapping of the ATP-pho-toaffinity labeled protein indicated that ATP cross-linking was site-specific and limited to two discrete peptide fragments of the protein, suggesting that the overexpressed mdr protein is capable of direct and specific ATP binding.

INTRODUCTION

The emergence of cell populations resistant to several structurally and functionally unrelated cytotoxic compounds has been termed multidrug resistance. This phenomenon has been traditionally studied in highly multidrug-resistant cell lines obtained in vitro by stepwise selection with a single cytotoxic drug. In these cells, multidrug resistance is associated with a decreased intracellular drug accumulation, an increased ATP-dependent drug efflux, and concomitant overexpression of a high molecular weight membrane glycoprotein(s), originally termed P-glycoprotein (reviewed in Refs. 1, 2). In drug-resistant cells this protein(s) was shown to be capable of combining photoactivatable analogues of Vinca alkaloids (3, 4). This binding was specifically inhibited by certain calcium channel blockers (4) known to partially revert the multidrug resistance phenotype observed in these cells (5, 6). The group of overexpressed proteins appears to be encoded by a small family of at least three related genes, termed mdr or P-glycoprotein genes (7–10), that become amplified during drug selection, in multidrug-resistant cell lines of mouse (11), hamster (12), and human origins (13).

We have previously reported the cloning of a full length cDNA for a transcriptionally active member of the mouse mdr gene family (7), which will be referred to here as mdr1. Analysis of the nucleotide and predicted amino acid sequences of this clone indicated that the encoded polypeptide was most likely a membrane glycoprotein, highly similar or identical to P-glycoprotein, which contained 12 putative transmembrane domains and a cluster of N-linked glycosylation sites near the amino terminus. It is formed by the internal duplication of a structural unit which encodes three transmembrane loops and a putative nucleotide binding fold (7). The duplicated segment is highly homologous to the energy coupling subunit of bacterial transport proteins particularly HlyB, a protein that participates in the extracellular transport of haemolysin in Escherichia coli (14). These predicted features strongly suggest that the mdr protein(s) participate in energy dependent membrane-associated processes, possibly drug efflux. The cloned mdr1 cDNA was capable of conferring a complete multidrug resistance phenotype when introduced and overexpressed in otherwise drug-sensitive LR73 hamster cells (15).

The independent contribution of different members of the mdr gene family to the multidrug resistance phenotype remains at present unknown. Cell clones stably transfected with full length cDNAs for individual members of the mdr family provide an ideal system to study the structural and functional features of the corresponding mdr protein on an otherwise drug-sensitive cellular background. We have used multidrug-resistant mdr1 transfectants to verify structural and functional features of the overexpressed mdr protein, particularly with respect to the ATP binding property, which was originally predicted by computer-assisted analysis of the full-length cDNA. Our results indicate that the mdr1 gene product is expressed in transfecants as a membrane phosphoglycoprotein of molecular weight 160,000–170,000 exclusively phosphorylated on serine residues. We show, in ligand binding studies, that the overexpressed protein is capable of binding a calcium channel blocker photoactivatable analogue, azidopine, and the photoactivatable analogue of ATP, 8-azido ATP. Our results indicate that binding of 8-azido ATP to the mdr protein is restricted to two discrete peptide fragments.

MATERIALS AND METHODS

Cell Lines and Tissue Culture Conditions. LR73 Chinese hamster cells were transfected with the mammalian expression plasmid pDRX4, containing the biologically active cDNA insert of phage λ DR11, as previously described (15). Drug-resistant colonies were initially selected and subsequently maintained in medium containing Adriamycin at 0.1 μg/ml. Cells were cultured in a minimum essential medium supplemented with 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). For drug-survival experiments, 500 cells were plated in 60-mm dishes containing increasing concentrations of individual drugs and further incubated for 7 days at 37°C without medium change. Colonies were fixed with formaldehyde (4% final), stained with methylene blue, and colonies containing more than 50 cells were scored. The cellular drug resistance was expressed as the D10 value which is the drug dose necessary to kill 90% of the cells. Actinomycin D was obtained from Merck, Sharp and Dohme; Adriamycin from Adria Laboratories; vinblastine from Sigma; mitoxantrone from Lederle; cisplatinum from Bristol; amsacrine was a gift from Dr. J. Hancock (Hotel Dieu Hospital, Quebec); and 5-fluorouracil, arabinos-
cytosine, and bleomycin was obtained from Dr. C. Shustik (Royal Victoria Hospital, Montreal).

Membrane Preparation. Cells grown to confluency in roller flasks were harvested by gentle treatment with Dulbecco’s PBS containing sodium citrate (20 mm) and lacking magnesium and calcium. Cells were washed twice, lysed in hypotonic medium (10 mm TRIS, pH 8.0; 1 mm MgCl2) using a Dounce type of homogenizer and nuclei were eliminated by centrifugation through a cushion of 45% sucrose. In some experiments membranes were further purified by ultracentrifugation of the crude membrane extract on a step gradient containing sucrose at concentrations of 60, 45, 35, and 30%. Membranes floating on the 45 and 35% interfaces were collected and stored frozen at -80°C in PBS containing 10% glycerol. Membrane-enriched fractions prepared by this procedure showed no detectable lactate dehydrogenase activity, a cytotoxic enzyme, and a 25-fold increase of 5’-nucleotidase activity, a marker enzyme of plasma membranes (as measured by assay systems purchased from Sigma diagnostics).

Metabolic and Photoaffinity Labeling. For metabolic labeling with [35S]methionine, cell cultures at 50 to 60% confluence were incubated in methionine free α-MEM medium containing 10% dialyzed fetal calf serum, 5 mm L-glutamine and [35S]methionine at 100 μCi/ml for a minimum of 4 h. For glucosamine labeling, the cells were incubated for 12 h in low glucose (2 mm) RPMI medium, containing 10% dialyzed fetal calf serum and [3H]glucosamine at 25 μCi/ml. For phosphorylation experiments, cells were labeled in phosphate-free medium containing 600 μCi/ml of [32P]orthophosphate for 12 h. In all experiments labeled cells were washed twice with PBS before immunoprecipitation. For azidopine photoaffinity labeling, membranes (2 mg/ml) were incubated in the dark at 20°C for 1 h in a buffer containing 0.04 m potassium phosphate (pH 7.4), calcium chloride (1 x 10^-4 m), dimethyl sulfoxide (0.1%) and [3H]azidopine (0.7 x 10^-4 m; specific activity, 3 Ci/mmol).

For competition experiments, 0.7 μM [3H]azidopine was incubated for 1 h at 20°C with membrane fractions (2 mg/ml) in the presence of 50 μM nifedipine, 50 μM verapamil, or 50 μM vinblastine. The mixtures were incubated for 10 min at 20°C on ice. After removal of residual perfusion acid, the oxidized protein was digested with trypsin (5 mg/ml) at 37°C for 16 h. Digestion products were spotted onto cellulose thin-layer plates (Polygram cel 400; Machery-Nagel) and electrophoresed for 30 min at 1000 V, in buffer containing pyridine, acetic acid, and water at a ratio of 10:100:1800 (pH 3.5). The second-dimensional thin-layer chromatography was carried out in the presence of n-butanol, pyridine, water, and acetic acid in a 4:3:3:1 ratio. For phosphoamino acid determination, the immunoprecipitated 32P-labeled protein was recovered from the gel and hydrolyzed at 100°C for 2 h with double-distilled 6 N HCl. Phosphoamino acids were separated by two-dimensional electrophoresis on cellulose thin-layer plates (cel 300) in the presence of buffers adjusted to pH 1.9 (88% formic acid, acetic acid, water, at a 50:156:1794 ratio) and pH 3.5 (as above), respectively.

RESULTS

Drug-sensitive LR73 cells were transfected with the biologically active cDNA clone of λ phage DR11, constructed in the expression vector pDREX4 (15), and drug-resistant colonies were selected in Adriamycin. Three independent colonies (clones 1A, 5, 8) were expanded in culture and their degree of cross-resistance to several drugs was quantitated. Results of this analysis are shown in Table 1 for clone 1A. Similar results were obtained for the two other independent transfectants (data not shown). Adriamycin-resistant cell lines stably transfected with the mouse mdrl cDNA expressed the classical pleiotropic drug-resistance phenotype: they were resistant to anthracyclines, Vinca alkaloids, actinomycin D, VP-16, amsacrine, and mitoxantrone but remained susceptible to drugs that are not traditionally part of the multidrug resistance spectrum such as bleomycin, cis-platinum, arabinoxytosine, and 5-fluorouracil (Table 1). Although small clone-to-clone variations were observed, the overall level of drug resistance of the transfected cells varied between 7- (amsacrine) and 30-fold (mitoxantrone).

Extensive cDNA sequence homology indicates that the cloned mouse mdrl cDNA and hamster pgp cDNAs (P-glycoprotein genes) are members of the same gene family (8). Therefore, we used the anti-hamster P-glycoprotein monoclonal antibody C219 (18) in an attempt to identify in transfectants the protein encoded by the mdrl cDNA. LR73 hamster control cells and drug-resistant transfectants 1A, 5, and 8 were labeled with [35S]methionine and total cellular proteins were immunoprecipitated with the C219 antibody (Fig. 1A). A specific protein species of apparent molecular weight of 160,000-170,000 was detected in the three transfectant cell clones but was undetected in the control, drug sensitive, LR73 cells. To determine whether mdrl was expressed as a membrane protein in transfectants, metabolic labeling with [35S]methionine was carried out on cells from clone 1A and membrane enriched fractions were prepared by ultracentrifugation on discontinuous sucrose gradients. The labeled membrane fractions were immunoprecipitated and analyzed for the presence of the mdrl protein by SDS-PAGE. Results presented in Fig. 1B (Lanes 1 and 2) indicate that the specific protein was present in membrane-enriched fractions sedimenting on the 35 and 45% sucrose gradient fractions. The labeled membrane fractions were immunoprecipitated and analyzed for the presence of the mdrl protein by SDS-PAGE. Results presented in Fig. 1B (Lanes 1 and 2) indicate that the specific protein was present in membrane-enriched fractions sedimenting on the 35 and 45% sucrose gradient fractions. Moreover, when equal amounts of proteins obtained from whole cell lysates and membrane enriched fractions were analyzed by immunoblotting, an approximately 20-fold enrichment for the mdrl gene product was observed in the membrane-enriched fraction (Fig. 1C). Finally, the radiolabeled mdrl protein could also be immunoprecipitated from membrane-enriched fractions prepared from cells grown in medium containing [3H]glucosamine (Fig. 1B, Lanes 3 and 4). Taken together these results confirm that in the mdrl transfectants, the encoded protein is expressed as a membrane glycoprotein.

Phosphorylation has been suggested as a postranslational modification implicated in the regulation of P-glycoproteins (21, 22). Metabolic labeling of LR73 and drug-resistant clone 1A cell cultures with [32P]orthophosphate followed by immunoprecipitation of whole cell lysates with the C219 antibody indicated that the overexpressed mdrl protein encoded by the transfected gene was phosphorylated in this clone (Fig. 2A). Subsequent phosphoamino acid analysis shown in Fig. 2B indicated that the protein was phosphorylated exclusively on serine residues whereas no radioactivity was found associated

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4 The abbreviations used are: PBS: phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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with phosphothreonine or phosphotyrosine (Fig. 2B).

Multidrug resistance can be partially overcome by exposing drug-resistant cells to a group of calcium channel blockers, including verapamil (5, 6). Since these compounds can specifically compete with the binding of photoactivatable drug analogues to P-glycoprotein (4, 27), it is speculated that they share a common or overlapping binding site on this protein. To determine whether the M, 170,000 polypeptide encoded by the transfected mdr1 gene is capable of binding calcium channel blockers, photoactivatable [3H]azidopine (16) was cross-linked with phosphothreonine or phosphotyrosine (Fig. 2B).

Drug doses for ADM, VBL, VP-16, ACTD, AMSA, and MIT are expressed in nanograms per milliliter and in micrograms per milliliter for CISP, 5FU, ARAC, and BLEO.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>ADM</th>
<th>VBL</th>
<th>VP16</th>
<th>ACTD</th>
<th>AMSA</th>
<th>MIT</th>
<th>CISP</th>
<th>5FU</th>
<th>ARAC</th>
<th>BLEO</th>
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<tr>
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<td>4.5</td>
<td>76</td>
<td>3.5</td>
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<tr>
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<td>360</td>
<td>23</td>
<td>640</td>
<td>21</td>
<td>21</td>
<td>1.03</td>
<td>2.1</td>
<td>4.5</td>
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</table>

DISCUSSION

A large body of biochemical data on multidrug resistance has been obtained from the study of highly drug-resistant cell lines produced by stepwise selection procedures (1, 2). These studies have consistently linked the emergence of drug resistance to the amplification and overexpression of one or more members of the mdr/P-glycoprotein gene family. We have shown that a full-length cDNA clone for one of these genes, mdr1, can efficiently confer drug resistance to otherwise drug-sensitive cells in transfection experiments (15). We feel that the study of multidrug resistant cell clones obtained by direct transfection of a biologically active cDNA presents distinct advantages: (a) drug-resistant transfectants are obtained by single-step drug selection, therefore minimizing the risk that additional genetic alterations, resulting from long periods of exposure to high drug concentrations, may alter the phenotypic expression of mdr proteins; (b) multiple members of the mdr family are amplified in drug resistant cell lines (11–13) and it is necessary to introduce and express individual mdr cDNA clones in drug sensitive cells to clarify the independent contribution of each of these genes to the final resistance phenotype; (c) functional analysis of the individual mdr protein(s) can be greatly enhanced by the use of site-directed mutagenesis of the corresponding cDNA and subsequent testing of mutant clones in transfection experiments.

In this paper we report on the characterization of the mouse mdr1 gene product in cell clones stably transfected with the corresponding cDNA. In these cells the protein is overexpressed as a membrane glycoprotein of approximately M, 170,000, in agreement with the predicted sequence of the protein deduced from the cDNA (7). The protein encoded by the transfected mdr1 gene is recognized by the anti-hamster P-glycoprotein monoclonal antibody C219 (18), confirming that the two proteins are encoded by closely related members of the same gene family, as previously suggested by the strong nucleotide sequence homology of the respective cloned cDNA (8). P-glycoprotein(s) have been previously reported to be phosphorylated in highly multidrug-resistant cell lines: in multidrug-resistant derivatives of the mouse macrophage line J774 (25), phosphorylation analysis reveals that the protein is phosphorylated on both serine and threonine residues, while in human multidrug-resistant K562/ADM leukemic cells, phosphorylation is...
Fig. 1. Expression of the \textit{mdr1} protein in cell clones stably transfected with the corresponding cDNA clone. \textit{A}, \[^{35}S\]methionine-labeled proteins of a whole cell lysate from drug-sensitive LR73 cells (Lane 1) and multidrug-resistant \textit{mdr1}-transfectant cell clones 1A (Lane 2), 5 (Lane 3), and 8 (Lane 4) were immunoprecipitated with the monoclonal antibody C219 and separated by SDS-PAGE on a 7.5% gel. All three multidrug-resistant cell clones express a protein with apparent molecular weight of \(M, 170,000\) which is not detectable in the drug-sensitive LR73 cells.\textit{B}, cells from the \textit{mdr1}-transfectant clone 1A were labeled with \[^{35}S\]methionine (Lanes 1, 2) or \[^{3}H\]glucosamine (Lanes 3, 4) and cell homogenates fractionated on discontinuous sucrose gradients. Radiolabeled \textit{mdr} protein was immunoprecipitated and are shown in Lanes 5 and 6, respectively. \textit{mdr} protein was photoaffinity labeled with the ATP analogue 8-azido ATP and immunoprecipitated with the monoclonal antibody C219 and separated by SDS-PAGE on a 7.5% acrylamide gel and transferred to a nitrocellulose membrane. The blot was incubated for 12 h with antibody C219 at a concentration of 100 \(\mu g/ml\). Immune complexes were revealed by treating the blot with \[^{125}I\]protein A followed by autoradiography for 24 h. Arrows in \textit{B} and \textit{C}, \(M, 170,000\) \textit{mdr1} gene product.

Fig. 2. Phosphorylation of the \textit{mdr1} protein. \textit{A}, drug-sensitive LR73 and multidrug-resistant \textit{mdr1}-transfectant cell clone 1A cell cultures were incubated for 16 h with 600 \(\mu Ci/ml\) \[^{32}P\]orthophosphate. Proteins from crude membrane preparations were immunoprecipitated and separated by SDS-PAGE on a 4 to 10% gradient gel: Lane 1, LR73 cells; Lane 2, drug-resistant \textit{mdr1}-transfectant clone 1A. Arrow, phosphorylated \textit{mdr1} gene product with apparent electrophoretic mobility of \(M, 170,000\). \textit{B}, the \[^{32}P\]-labeled immunoprecipitated protein was electroeluted from the gel, acid digested, and analysis of phosphoamino acids was carried out as described ("Materials and Methods"). The predicted positions of phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) are indicated. Only phosphoserine was detected in the \[^{32}P\]-labeled \textit{mdr} protein.

Restriction to serine residues (26). In multidrug-resistant \textit{mdr1} transfectants the overexpressed \textit{mdr} protein appears to be phosphorylated solely on serine residues. A possible explanation for differences in the phosphoamino acid content is that threonine phosphorylation of the protein is specific to the macrophage line used in those studies. Alternatively, threonine phosphorylation may be limited to an antigenically related protein species encoded by another member of the \textit{mdr} gene family. Indeed, drug-resistant J774 cells selected with different drugs are known to express antigenically cross-reactive but distinct \textit{mdr} proteins (17).

Multidrug resistance can be partially overcome if cells are grown in the presence of calcium channel blockers (5, 6). Such
Fig. 3. Cross-linking of azidopine to the mdr1 protein. A, membrane-enriched fractions were prepared from both drug-resistant 1A and drug-sensitive LR73 cells and UV-irradiated in the presence of [3H]azidopine. Lanes 1 and 2, profiles of labeled membrane proteins from LR73 and 1A cells, respectively. Proteins from [3H]azidopine cross-linked membrane fractions were immunoprecipitated with the antibody C219: Lane 3, LR73 cells; Lane 4, drug-resistant 1A cells. Arrow, location of a transfectant-specific [3H]azidopine-labeled protein with apparent molecular weight of 170,000. B, competition of azidopine binding to membrane proteins from drug-resistant LR73 cells and 1A cells. Lanes 4 and 5, protein profiles of membranes from 1A and LR73 cells, respectively, which were cross-linked to azidopine by UV irradiation at 20°C. The binding of azidopine to a M, 170,000 protein in 1A membranes (arrow) could be competed out by verapamil (Lane 1), vinblastine (Lane 2), and nifedipine (Lane 3).

Fig. 4. Tryptic map of 8-azido-ATP coupled mdr1 protein. ATP-photoaffinity labeled mdr1 protein was subjected to complete digestion with trypsin and digestion products were separated by electrophoresis (first dimension) and chromatography (second dimension). For electrophoresis, the origin (O) and positions of the anode and cathode are indicated. Arrow, direction of thin layer chromatography (TLC); arrow heads, two photoaffinity labeled tryptic peptides.

Treatment results in an increased intracellular drug accumulation in these cells, presumably because calcium channel blockers directly compete with drug molecules for the drug efflux pump. The suggestion that calcium channel blockers and chemotherapeutic drugs share a common or overlapping binding site on the efflux pump is supported by the observation that either group of compounds can compete with the binding of photoactivatable analogues of the other to P-glycoprotein (4, 27). In these studies, [3H]azidopine bound specifically to at least two (16) and possibly three (27) antigenically related overexpressed protein species. Moreover, the binding of [3H]azidopine was highly specific and limited to one tryptic fragment of the protein (27). It is tempting to speculate that the proteins identified in those studies are encoded by closely related but distinct members of the mdr gene family. Results from our study clearly indicate that the protein encoded by the transfected mdr1 cDNA is capable of combining [3H]azidopine with very high specificity.

We and others have proposed an energy-dependent efflux function for the mdr protein, with ATP coupling playing a central role in the efflux mechanism (2, 7, 28). We have shown (Fig. 1B) that the mdr protein expressed in drug-resistant transfecants can cross-link the ATP analogue 8-azido ATP. Similar photoaffinity labeling of P-glycoprotein was reported in the case of multidrug-resistant human KB carcinoma cells (29). To investigate the specificity of ATP binding to the protein and eventually characterize its functional role in drug resistance, we generated peptide maps from the photoaffinity labeled protein. These maps showed that the coupling of ATP was highly specific and limited to two discrete peptides. It is not possible yet to determine if these two peptides correspond to fragments overlapping the two predicted nucleotide binding folds in the mdr protein or correspond to photoaffinity labeling of two peptides surrounding a single functional nucleotide binding fold. It is interesting to note that the tryptic map of the photoaffinity labeled mdr protein reported here is similar (showing a weakly and a strongly labeled peptide) to that of 8-azido ATP photolabeled E. coli RecB and RecD proteins (30), two proteins which each contains the consensus sequence for a nucleotide binding fold present in mdr1 (31, 32).

Studies presented in this paper describe the model system we wish to utilize to characterize the functional role and structural features of individual members of the mdr gene family: we have recently obtained the complete nucleotide sequence of a second transcriptionally active member and have identified a third transcriptionally active mdr gene.7 The systematic study of these genes in transfection experiments using full length cDNA clones should clarify their role in the establishment of multidrug resistance and may help clarify their role in normal physiological processes.

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Characterization of the Multidrug Resistance Protein Expressed in Cell Clones Stably Transfected with the Mouse *mdr1* cDNA

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