ATPase Activity of Purified Multidrug Resistance-associated Protein*

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The multidrug resistance-associated protein (MRP)1 was discovered in multidrug-resistant tumor cells that did not express any of the structurally related P-glycoproteins (1). Several such cell lines have been described (2–7), although drug-resistant protein assayed, and typical Michaelis-Menten behavior was exhibited, yielding estimations of $V_{\text{m}}$ of $-3.0$ nm and $V_{\text{max}}$ of $0.46 \mu$mol mg$^{-1}$ min$^{-1}$. This activity was moderately stimulated by the drugs that others have shown to be transported by MRP-containing membrane vesicles. This stimulation was enhanced by reduced glutathione as is its drug transport, and oxidized glutathione, itself a substrate for transport, caused a strong stimulation. These data describe the first purification of MRP and provide the first direct evidence that the molecule possesses drug-stimulated ATPase activity.

Human multidrug resistance protein (MRP) was expressed at high levels in stably transfected baby hamster kidney (BHK-21) cells. These cells exhibited a pattern of cross-resistance to several different drugs typical of an MRP-mediated phenotype despite the addition of 10 histidine residues at the C terminus to facilitate purification. Consistent with this functional evidence of the presence of MRP at the surface of these transfectants, strong signals were detected by immunoblotting and immunofluorescence using a specific monoclonal antibody to MRP. There was intense uniform staining of the cell surface as well as weaker staining of intracellular membranes. MRP-containing membranes were solubilized in 1% N-dodecyl-$\beta$-d-maltoside in the presence of 0.4% sheep brain phospholipids. Two sequential affinity purification steps on Ni-NTA agarose and wheat germ agglutinin agarose provided substantial enrichment, and contaminating bands were not detected. ATPase activity of the purified protein was assayed in the presence of the phospholipids, which had been maintained throughout all purification steps. ATP was hydrolyzed in proportion to the amount of purified protein assayed, and typical Michaelis-Menten behavior was exhibited, yielding estimations of $K_m$ of $-3.0$ nm and $V_{\text{max}}$ of $0.46 \mu$mol mg$^{-1}$ min$^{-1}$. This activity was moderately stimulated by the drugs that others have shown to be transported by MRP-containing membrane vesicles. This stimulation was enhanced by reduced glutathione as is its drug transport, and oxidized glutathione, itself a substrate for transport, caused a strong stimulation. These data describe the first purification of MRP and provide the first direct evidence that the molecule possesses drug-stimulated ATPase activity.

Experimental Procedures

Materials—AMV reverse transcriptase system was purchased from Life Technologies, Inc. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. Rat monoclonal antibody MRP1 was purchased from Signet. Goat anti-rat antibody-fluorochrome conjugates, colchicine, daunomycin, doxorubicin, vincristine, vincristine, N-acetylglucosamine, imidazole, triethylamine, ATP, ammonium molybdate, sheep brain lipid, L-cysteine, reduced glutathione, oxidized glutathione, and MTT were purchased from Sigma. Sheep anti-rat Ig antibody conjugated with horseradish peroxidase, ECL kit, Hyperfilm ECL film, and $[\gamma\text{-32P}]$ATP were purchased from Amersham. Wheat germ lectin Sepharose 6MB was purchased from Pharmacia. His.Bind Resin was purchased from Novagen. Perchloric acid and bromine solution were purchased from Fisher. N-Dodecyl-$\beta$-d-maltoside was from Calbiochem. Oligonucleotides were synthesized by the Molecular Biology Core of Mayo Clinic Arizona.

Generation of Expressible Human MRP cDNA—2 $\mu$g of total RNA isolated from the J2C Hela cell line selected for doxorubicin resistance by R. Baker (SUNY, Buffalo) was used as template in reverse transcription. As shown in Fig. 1, the following primers were used to amplify individual MRP cDNA fragments according to the original sequence of Cole et al. (1): MRP1S (A GAG ACC ATG GCC CTC CGG GCC TTC TGC AGC G), MRP2A (GCT GGG CAG GAT CCT TGG AGG AGT A), MRP2S (TAC TCC TCC AAG GAT CCT GCC CAG C), MRP4A (CTG ATC ACC ATG GGA AGA ATG TTC), MRP5S (GAA CAT GAA TCC CAT GGT CAT CAG), MRP6A (GCA GTG GAA GAC ACC TAC TCC CCT AGC C), MRP7S (AGG GCA GGT CAA GTC TCT CGT GTA), MRP8A (GCA GTC TGT CCT GGA GCA TAC TCC), and MRP10A (GCA GTT CTA CGC CAA

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1 The abbreviations used are: MRP, multidrug resistance-associated protein; CFTR, cystic fibrosis transmembrane conductance regulator; PAGE, polyacrylamide gel electrophoresis; BHK, baby hamster kidney; DDM, n-dodecyl-$\beta$-d-maltoside; GSSG, oxidized glutathione; LTC4, leukotriene C4; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Ni-NTA, nickel-nitrito-triacetic acid resin; PCR, polymerase chain reaction; DTT, dithiothreitol.
The pellet was resuspended in buffer B (0.6 ml/15-cm-diameter dish) and 125 mM CaCl₂ (26). The cells were shocked 5 h later with 25% packed from BHK cells expressing MRP/His by homogenizing them in 10 mM Tris-HCl (pH 7.4). Nuclei were removed by centrifugation at 10,000 × g for 15 min. The supernatant was applied onto a His.Bind Resin column, which had been pre-equilibrated with buffer B. The column was then washed with 6 column volumes of buffer B containing 0.1% DDMP and 25 mM imidazole. The column was washed once more with 6 column volumes of buffer B containing 0.1% DDMP and 40 mM imidazole. The protein was eluted with 2 column volumes of buffer B containing 0.1% DDMP and 300 mM imidazole. The eluate was diluted with an equal volume of buffer C containing 50 mM Tris-HCl (pH 7.4), 2 mM MgCl₂, 20% glycerol, 0.4% sheep brain lipid, and 0.2% β-mercaptoethanol. The diluted sample was mixed with buffer C pre-equilibrated wheat germ lectin Sepharose 6MB and shaken for 2 h. The wheat germ lectin Sepharose 6MB beads were packed into a small column and washed with 10 column volumes of buffer C. The bound proteins were eluted with 2 column volumes of buffer containing 50 mM Tris-HCl (pH 7.4), 20% glycerol, 0.4% sheep brain lipid, and 0.3 M N-acetylglucosamine.

Assay of ATPase Activity—ATPase activity was assayed employing the methods of Gibson et al. (29) with some modifications. Briefly, the reaction buffer (40 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 10 mM MgCl₂), [γ-³²P]ATP (1 µCi), and ATP (2 µm or as otherwise indicated in the figure legends) was placed in an ice bath and mixed with transported substrates where indicated. The purified MRP protein was added last, mixed well, transferred to a 37 °C water bath, and incubated for 1 h. The reaction was stopped by adding 20 reaction volumes of ice-cold stop buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM KH₂PO₄, 9 mM ATP, 5.6 mM MgSO₄, and 8.2% HClO₄. The released P was precipitated with 21 reaction volumes of ice-cold precipitation solution containing 3% (w/v) ammonium molybdate, 67 mM HCl, 1% (v/v) triethyl-ylamine, and 1% (v/v) bromide water. After incubation on ice for 20 min, the precipitate was separated from supernatant by centrifugation in a microtube for 5 min. The supernatant was transferred to a scintillation vial, and the pellet was washed once more with 2 ml of Tris-HCl. The pellet was dissolved in 0.6 ml of 1 N NaOH and transferred to another scintillation vial. The amounts of ³²P radioactivity in supernatant and pellet were counted separately, and specific ATPase activity was calculated.

RESULTS

Sequence of Expressed MRP cDNA—Sequencing on both strands of the full-length cDNA generated from HeLa cell mRNA as described under “Experimental Procedures” and Fig. 1 indicated identity with the human MRP sequence originally determined by Cole et al. (1) except for single nucleotide substitutions at nine positions. These differences and the resulting amino acid changes are as follows: Thr³⁴¹ to Gly (L80V), Cys⁴⁴⁶ to Thr (T117M), Cys¹³⁰⁴ to Gly (L370V), Thr¹³⁰⁵ to Cys (L582L), Thr¹³⁰⁶ to Cys (L685S), Ala¹³⁰⁷ to Gly (D696G), Cys⁴⁶⁰⁰ to Gly and Gly⁴⁶⁰¹ to Cya (R1282A), and Gly⁴⁷³⁴ to Ala (S1512S). The changes underlined were also found by Zaman et al. (10) in a cDNA synthesized with RNA from a human tumor cell line as template for reverse transcription. L685S and R1282A changes were also found by Stride et al. (31). The locations of the amino acid changes with respect to the putative domain structure and membrane topology are as follows. The first two, L80V and T117M fall within the 220-residue N-
ATPase Activity of Purified MRP

**Table I**

| Drug         | IC<sub>50</sub> of BHK | IC<sub>50</sub> of BHK/MPR | Resistance factor |
|--------------|------------------------|---------------------------|-------------------|
| Daunorubicin | 66                     | 565                       | 9                 |
| Colchicine   | 1100                   | 5900                      | 5                 |
| Vincristine  | 6                      | 135                       | 23                |

**Drug resistance of MRP-expressing BHK cells**

Resistance was determined by MTT assay. Inhibitory concentration (IC<sub>50</sub>) of a drug was defined as the dose that reduced the absorbance at 570 nm to 50% of control value. Resistance factor was calculated as IC<sub>50</sub> of BHK/MPR/IC<sub>50</sub> of BHK.

**Purification of MRP—** A crude microsomal membrane fraction containing a large proportion of the human MRP expressed in BHK cells was employed as starting material. To solubilize the protein from these membranes, the non-ionic detergent, n-dodecyl-β-D-maltoside, was effective in either the presence or absence of added phospholipids. Because of the requirement of P-glycoprotein ATPase activity for phospholipids (37, 38), we included 0.4% sheep brain lipids during DDM solubilization and maintained its presence throughout each step of the purification. Fig. 3 illustrates the fractionation of total membranes and MRP during the two-step purification. Under the conditions of loading and washing of Ni-NTA agarose beads, which were arrived at empirically (see “Experimental Procedures”), the MRP/His was quantitatively bound and, upon elution with elevated imidazole (lanes 5), was already very highly enriched and readily detectable by Coomassie Blue staining. However, when the gel was loaded to allow detection at this level, the presence of multiple additional bands was observed. The N-linked oligosaccharide chains demonstrated to be present on MRP (24) made the use of the lectin-affinity chromatography an obvious choice for further purification. When the MRP-containing material eluted from the Ni-NTA agarose beads, which were applied to wheat germ lectin Sepharose, not all MRP was bound. Some was detectable in both the flow-through (lane 6) and wash (lane 7). However, a larger proportion did bind specifically via N-acetylglucosamine-containing determinants and was eluted by a high concentration of this monosaccharide (lane 8). Importantly, most of the contaminating bands in the nickel bead eluate could be detected in the flow-through and wash from wheat germ lectin beads and not in the N-acetylglucosamine eluate (lanes 6–8). Hence, these two successive affinity purification steps yield a very highly enriched preparation of the MRP glycoprotein in a lipid matrix, which might be expected to provide an approximation of its native environment. We have not quantitated purification factors or yields at each step, but the entire procedure produces, from one 150-cm<sup>2</sup> plate of confluent cells, approximately 2 μg of purified MRP. Aside from the major MRP monomer band of approximately 190 kDa, the only additional staining band present in the final preparation has an apparent molecular weight approaching that of a homodimer, although its size has not been more...
accurately determined. This band clearly contains MRP as indicated by its detection with the specific monoclonal antibody as well as by staining (Fig. 3). Although it is more evident once the protein has been purified to at least the Ni-NTA column stage, a small amount was detected even in the membranes before solubilization. A similar large immunoreactive band is observable in immunoblots of other drug-resistant cells expressing high levels of MRP (11, 33).

**ATPase Activity of Purified MRP**—To determine if purified MRP eluted from wheat germ lectin beads in the absence of detergent but in the presence of 0.4% sheep brain lipid-pos-
sessed intrinsic ATPase activity, the release of \(^{32}\)P from \([\gamma^{32}\text{P}]\text{ATP}\) was assayed. Fig. 4A indicates that indeed ATP was hydrolyzed in direct proportion to the amount of purified MRP present, at least over a range of 50–250 ng, indicating that it is active as an ATPase.

The dependence of the rate of hydrolysis on ATP concentra-
tion shown in Fig. 4B exhibited typical Michaelis-Menten be-
behavior and, when expressed as a Lineweaver-Burk plot in Fig. 4C, showed a linear relationship, yielding a \(K_{m}\) for ATP of approximately 3 mM and a \(V_{max}\) of 0.46 \(\mu\text{mol} \text{ mg}^{-1} \text{ min}^{-1}\). This maximal specific activity is of the same order as the basal-
pecific ATPase activity of P-glycoprotein in the absence of drugs (37–39).

**Effect of Transported Substrates on MRP ATPase**—MRP-
containing membrane vesicles have been shown to transport glutathione and glucuronide conjugates of a range of hydropho-
obic compounds including some of the cancer drugs to which MRP overexpressing cells are resistant (9, 11, 13, 14, 19–21, 40). Although there is some controversy over whether conjuga-
tion of substrate is essential to transport by MRP (41), it is presumed that the presence of a conjugating agent such as glutathione is necessary (12, 19). We first tested the influence of the cysteinyl leukotriene, LTC\(_4\), one of the best characterized MRP sub-
strates for transport (9, 11, 13, 42), on ATPase activity (Fig. 5). 1 nM LTC\(_4\) caused an increase in specific activity of approxi-
ately 25%, and this enhancement increased further to as much as 60% at 10 \(\mu\text{m}\). Hence, while MRP is clearly not de-
pendent on such substrates for its ATPase activity, there is significant stimulation.

We next tested the influence of the cancer drugs to which the MRP-transfected cells exhibited cross-resistance. Fig. 6A shows that the anthracyclines, daunomycin and doxorubicin, also both cause moderate augmentation of MRP ATPase, with doxorubicin having a greater influence at higher concentra-
tions. The vinca alkaloids, vinblastine and vincristine, had similar stimulatory effects, with the latter eliciting its activa-
tion at lower concentrations. Increasing concentrations of col-
chicine caused a smooth rise in ATPase-specific activity. Hence, while the response of MRP ATPase to these drugs is moderate, it is quite similar in magnitude to that of P-glycoprotein ATPase (37–39).

Because glutathione-conjugated compounds seem to be the preferred substrates for transport by MRP (9, 11, 13, 42), we also examined the influence of reduced and oxidized glutathione in the presence and absence of doxorubicin (Table II). Reduced glutathione alone had a minor stimulatory effect whereas the oxidized form, which has itself been reported to be transported by MRP (15), caused a much stronger stimulation. Interestingly, the addition of GSH together with doxorubicin seemed to increase activity more than when GSSG was com-
bined with the anthracycline. Interpretation of this observation in the presence of only purified MRP and no conjugating or other enzymes is not obvious at this stage. Nevertheless, it does seem that the presence of glutathione increases the drug acti-
vation and that oxidized glutathione alone is capable of sub-
stantial activation. These observations support the conclusion that several substrates for transport by MRP enhance but are not required for ATPase activity of the protein.

**DISCUSSION**

We have developed stable mammalian cell transfectants ca-
pable of high level expression of functional MRP designed to facilitate its purification. This enabled its separation from other membrane proteins by two successive affinity chromatog-
raphy steps in the continuous presence of membrane lipids. The purified glycoprotein hydrolyzed ATP with a specific activity of approximately 0.5 \(\mu\text{mol} \text{ mg}^{-1} \text{ min}^{-1}\) and a \(K_{m}\) for ATP of about 3 mM. Activity could be stimulated by a maximum of 2-fold by compounds that have been previously shown to be transported by MRP-containing membrane vesicles.

The stable BHK cell lines, in which integrated MRP se-
quences were highly amplified because of strong selection of the dihydrofolate reductase-containing pNUT (25) plasmid with methotrexate, seemed to produce the mature glycoprotein at higher levels than some other heterologous expression sys-
tems, surprisingly, even including the baculovirus-insect cell system (18). This could reflect the fact that this amplification at the DNA level may to some extent mimic the situation in MRP overexpressing cells selected for resistance to drugs which it transports. Transcription from the metallothionein gene pro-
moter in the pNUT plasmid also occurs at a high level. Pulse-
chase experiments also revealed that the core-glycosylated form present in the endoplasmic reticulum matured with the addition of complex oligosaccharide chains in a relatively effi-
cient fashion (data not shown). The 190-kDa band representing this mature form of the protein is formed on passage through the Golgi complex, but our immunocytochemical observations (Fig. 2) indicated that a large amount of the protein is present at the cell surface in the steady state, indicating that most of it had proceeded beyond the Golgi. Much of the protein has been reported to be in intracellular membranes in some cells and tissues, and one report (43) described observations consistent with the possibility that the development of MRP-based drug
resistance correlated with the conversion of a lower molecular mass band to the mature 190-kDa band (43). It was the principal form of the molecule in the transfected BHK cells, although a larger band, either a dimer or a monomer in stable association (resistant to SDS and DTT) with another molecule, is also present.

The protein with the addition of 10 histidine residues at the C terminus was apparently active because the cells exhibited a cross-resistance pattern similar to that reported for several different MRP-expressing cell types, although the resistance to vincristine was higher than in some of the others. Because these cells had never been subjected to any selection pressure by this drug or the anthracyclines, it seems extremely likely that resistance is solely due to their expression of MRP.

Since the objective was to purify a fully functional molecule, we solubilized MRP-containing membranes in a non-ionic detergent in the presence of excess lipid. Lipid was present throughout the purification, and in the final step, elution from the lectin beads, detergent was omitted. DDM had been used previously by Loo and Clarke (44) for the purification of P-glycoprotein by Ni-NTA affinity chromatography. They stated that phospholipids interfered with that step. While perhaps not optimal, the data in Fig. 3 suggest that the binding to and elution of MRP from Ni-NTA agarose was quite efficient. It is possible that the presence of phospholipid micelles with which other hydrophobic proteins might associate contributed to the significant contamination that remained following this step. However, if that were the only explanation, one might have expected the contaminants to also carry through the wheat germ lectin affinity step, and they do not. Although not optimized for yield, the purification provides abundant amounts for enzymatic assays and is sufficient for many but not all other purposes; 100 µg was obtained in a preparation using routine cell culture methods and scale up by at least one order of magnitude is immediately feasible.

Human MRP purified in this way has ATPase activity that is at least superficially similar to that of P-glycoprotein in that the V_max is in the µmol mg⁻¹ min⁻¹ range and the K_m is in the mM or physiological range. It is important to note that this K_m for ATP is much higher than that determined in very rigorous transport assays with several different substrates in MRP-containing membrane vesicles (13, 14). We have no explanation of this apparent discrepancy at this stage, but as mentioned further below there is still no compelling evidence that MRP, P-glycoprotein, or other structurally related members of that class of molecules function mechanistically as classical transport ATPases with obligatory tight coupling between ATP hydrolysis and translocation of solute. However, there are also several possible technical reasons for the discrepancy. The membrane vesicles in which the transport assays have been performed contain many proteins in addition to MRP, which could conceivably increase its affinity for ATP. On the other hand, despite our attempt to maintain a membranous environment, the purified molecule could well have lost a higher ATP...
affinity, which it may process in its truly native state. It will be necessary to characterize MRP ATPase much more extensively in terms of inhibitor sensitivity and other properties. In vitro mutagenesis should readily reveal the contribution of each of the two nucleotide binding domains to activity.

The extent of ATPase activation by drugs to which MRP overexpressing cells are resistant in the presence or absence of GSH or by GSSG is modest and similar to that of P-glycoprotein, this has been considered by some investigators to reflect high basal activity (38), possibly due to activation by lipids. We have not yet attempted to distinguish between the possibilities of artifactualy elevated basal activity and low inherent sensitivity to transported substrates in the case of MRP ATPase.

Nevertheless, from previous work and the experiments described in this paper, it would seem that MRP, like P-glycoprotein, now qualifies as a transport ATPase, at least according to the loose criteria that it contributes to the transport of conjugated hydrophobic xenobiotics and metabolites and has ATPase activity that is influenced by these substances. However, in comparison with prototypic transport ATPases such as sodium and calcium pumps, for example, dependence of ATP hydrolysis on solute that are able to be translocated is much less complete, and it has not yet been shown that MRP alone is sufficient for transport. Demonstration of the latter property has been difficult even for P-glycoprotein, which has been much more extensively studied (45).

However, given the hydrophilicity imparted upon the hydrophobic substrates by conjugation with either a cysteiny peptide or a glucuronide moiety, it may be easier to perform appropriate transport assays with large proteoliposomes containing only purified MRP than in the case of P-glycoprotein.

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