Comparison of the Functional Characteristics of the Nucleotide Binding Domains of Multidrug Resistance Protein 1*

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Multidrug Resistance Protein 1 (MRP1) transports diverse organic anionic conjugates and confers resistance to cytotoxic xenobiotics. The protein contains two nucleotide binding domains (NBDs) with features characteristic of members of the ATP-binding cassette superfamily and exhibits basal ATPase activity that can be stimulated by certain substrates. It is not known whether the two NBDs of MRP1 are functionally equivalent. To investigate this question, we have used a baculovirus dual expression vector encoding both halves of MRP1 to reconstitute an active transporter and have compared the ability of each NBD to be photoaffinity-labeled with 8-azido-[32P]ATP and to trap 8-azido-[32P]ADP in the presence of orthovanadate. We found that NBD1 was preferentially labeled with 8-azido-[32P]ATP, while trapping of 8-azido-[32P]ADP occurred predominantly at NBD2. Although trapping at NBD2 was dependent on co-expression of both halves of MRP1, binding of 8-azido-ATP by NBD1 remained detectable when the N- or proximal half of MRP1 was expressed alone and when NBD1 was expressed as a soluble polypeptide. Mutation of the conserved Walker A lysine 684 or creation of an insertion mutation between Walker A and B motifs eliminated binding by NBD1 and all detectable trapping of 8-azido-ADP at NBD2. Both mutations decreased leukotriene C₄ (LTC₄) transport by approximately 70%. Mutation of the NBD2 Walker A lysine 1333 eliminated trapping of 8-azido-ADP by NBD2 but, in contrast to the mutations in NBD1, essentially eliminated LTC₄ transport activity without affecting labeling of NBD1 with 8-azido-[32P]ATP.

Multidrug Resistance Protein 1 (MRP1) is a member of the ATP-binding cassette (ABC) superfamily of transmembrane transporters that has been shown to confer resistance to a variety of natural product type drugs (1–6). The drug resistance phenotype conferred by MRP1 is similar to that resulting from overexpression of P-glycoprotein (P-gp) (reviewed in Refs. 7–9) and is typically associated with an ATP-dependent decrease in drug accumulation and an increase in drug efflux (4, 6). Although both ABC proteins can function as energy-dependent efflux pumps for a range of natural product type drugs, there is very limited primary structure similarity between them, and phylogenetic analyses suggest that they evolved from different ancestral proteins. There is also considerable evidence that the mechanisms by which MRP1 and P-gp transport drugs are different (reviewed in Ref. 8).

In addition to its ability to confer multidrug resistance, MRP1, unlike P-gp, has been shown by in vitro studies using inside-out membrane vesicles to transport a structurally diverse array of organic, anionic conjugates (reviewed in Ref. 9). These include GSH-, glucuronate-, and sulfate-conjugated aliphatic, prostanoïd, and heterocyclic compounds. The two highest affinity substrates identified to date are the proinflammatoty cysteinyi leukotriene C₄ (LTC₄) (10–12) and the GSH-conjugated epoxide of the potent mutagen, aflatoxin B₁ (13). In addition, MRP1 has been shown to be capable of direct active transport of conjugated bile salts (14) and nonpeptide hormones as well as in vitro synthesized drug conjugates such as doxorubicin-SG (15) and VP-16-glucuronide (14). MRP1 can also transport oxidized glutathione with low affinity but relatively high capacity (16, 17). GSH alone is not actively transported (12, 14, 18, 19). However, it is required for the ATP-dependent transport of unmodified chemotherapeutic agents, such as vincristine and doxorubicin, and xenobiotics, such as aflatoxin B₁ (10, 12, 13, 19). Vincristine reciprocally stimulates ATP-dependent transport of GSH by MRP1, and preliminary estimates of stoichiometry are consistent with the possibility that the two compounds may be co-transported (19). The stimulation of drug transport requires the complete GSH tripeptide but is not dependent on a free thiol, since GS-methyl and, to a lesser extent, GS-ethyl will also stimulate the transport of vincristine (19). Thus, in contrast to P-gp, in vitro transport studies with the compounds tested to date indicate that MRP1 is not capable of ATP-dependent transport of unmodified forms of the drugs to which it confers resistance unless drug transport is stimulated by the presence of GSH. Whether other anions can stimulate drug efflux from intact cells is presently not known.

The predicted topology of MRP1 differs from most other members of the ABC superfamily, including P-gp. Rather than being composed of only two nucleotide binding domains (NBDs) and two polytopic membrane-spanning domains (MSDs), MRP1 has a third MSD that is formed by the first 200 amino
acids of the protein. This domain consists of five putative transmembrane helices with an extracellular NH₂ terminus, the location of which has been verified experimentally by N-glycosylation site mutagenesis (29). Three recently identified human MRP-related proteins (MRP2, MRP3, and MRP6) also contain a third MSD and have been shown, or predicted, to have extracellular NH₂ termini (21–25). However, the amino acid sequences of the MSDs are relatively poorly conserved when compared with the remainder of the proteins. Two other MRP-related proteins have been identified that contain only two MSDs (MRP4 and MRP5) (26, 27), suggesting that the third MSD may have been acquired by fusion between a gene specifying a more typical four-domain ancestral protein and genes encoding other integral or membrane-associated proteins.

In prokaryotic transporters such as the histidine permease of *Escherichia coli*, the four domains of the active transporter are separate polypeptides. Both ATP-binding subunits are identical, and inactivation of one results in a transporter that has 50% of the ATPase and transport activities of a wild-type complex (28). In eukaryotic transporters such as the P-gps, the two NBDS, although not identical, are highly conserved, but in contrast to the histidine permease, inactivation of either NBD completely abolishes ATPase and transport activities of the protein (29–32). Compared with P-gp, the two NBDS of the MRP-related proteins are considerably more divergent. One of the major differences between the two NBDS of the MRP-related proteins is a conserved “deletion” of 13 amino acids between the Walker A and B motifs of NBD1 that is not present in the second NBDS of the MRPs or either of the NBDS of P-gp (1, 7, 9). However, it does occur in NBD1 of the cystic fibrosis transmembrane conductance regulator (CFTR), a member of the ABC superfamily that functions as an ATP-gated chloride channel (33). In addition, NBD1 and NBD2 of the MRP-related proteins are more similar to the corresponding NBD of CFTR than they are to each other, consistent with the evolution of CFTR and the MRP-related proteins from a common four-domain ancestor (34).

To investigate possible functional differences between the NBDS of MRP1, we have directly examined binding of 8-azido-ATP and trapping of 8-azo-[³²P]ADP by each NBD when they are expressed individually or in combination. We have shown previously that it is possible to reconstitute an active transporter by co-infecting *Spodoptera frugiperda* Sf21 cells with viruses encoding the two halves of MRP1 (35). However, the efficiency of reconstitution is limited by the fact that all cells in the infected population do not express equivalent amounts of both halves of the molecule. We have circumvented this problem by using a virus with a dual expression cassette encoding both halves of MRP1, and we show that membrane vesicles prepared from such cells transport LTC₄ with initial uptake rates comparable with those of vesicles containing similar amounts of the intact protein. We have also examined binding of 8-azo-[³²P]ATP and trapping of 8-azo-[³²P]ADP by each of the NBDs expressed as soluble proteins in Sf21 cells. These studies indicate that the two NBDS of MRP1 differ markedly with respect to their ability to bind 8-azo-[³²P]ATP and to trap 8-azo-[³²P]ADP. The lack of functional equivalence between the two NBDS is supported by studies in which we show that inactivation of each of the NBDS in the reconstituted transporter has different effects on LTC₄ transport activity and the ability to label the co-expressed wild-type NBD with 8-azo-ATP.

**Materials and Methods**

**Generation of Constructs—** Recombinant donor plasmids encoding full-length MRP1, as well as the NH₂-and COOH-proximal halves of MRP1 have been described (35). A two-step cloning strategy was used to introduce cDNA fragments encoding the MRP1 halves into the dual expression vector, pFASTBAC Dual (Life Technologies Inc.). The DNA fragment encoding amino acids 932–1531 was cloned first into pFASTBAC Dual. The previously described pFB-AN (MRP1 1–932) was linearized with Sall, made blunt-ended using Klenow fragment and then digested with the recovered SalI fragment. The fidelity of the PCR fragment was confirmed by dye sequencing. The cDNA fragment encoding the NH₂-proximal half-molecule, and the PCR fragment was cloned into pFASTBAC Dual, which had been digested with ScaI and KpnI to give pFBDual-MRP1 932–1531. The DNA fragment encoding amino acids 1–932 was then cloned into pFBDual-MRP1 932–1531. To achieve this, a SalI site was introduced by PCR immediately 3' of the stop codon in the sequence encoding the NH₂-proximal half-molecule, and the PCR fragment was cloned into pFASTBAC Dual. The previously described pFB-Dual-halves was then isolated and cloned into pFBDual-MRP1 932–1531, which had been digested with SalI, KpnI, and XhoI and ligated to pFBDual-MRP1 932–1531, in which the only amino acid introduced during the cloning was the initiator methionine.

To generate a similar construct expressing the second NBD of MRP1, a DNA fragment encoding amino acids 1294–1516 was amplified by PCR using YSMP-1-fc-ATG (35) as a template. The forward primer used was 5'-GAGCTGGAGAATTCGCCAGATCTGCGTCTGG-3', which includes a NheI site (underline). The PCR fragment was digested with NheI and ClaI, and the 180-bp NheI–Clal fragment was isolated. pFB-MRP1 was digested with ClaI and KpnI, and the 600-bp ClaI–KpnI fragment was also isolated. The NheI–ClaI and the Clal–KpnI fragments were ligated together to a prokaryotic expression vector pET-17b (Novagen), which had been digested with NheI and KpnI to create pET-MRP1 1294–1516. The fidelity of the PCR product was confirmed by dye sequencing. The PCR fragment was digested with NdeI, KpnI, and ClaI and cloned into Klenow fragment and then digested with NdeI. The recovered gene fragment was ligated to pFASTBAC1 and used to replace the equivalent region in pFB-Dual-MRP1 1296–1531 and three amino acids, methionine, alanine, and serine, were introduced before codon 1294 of MRP1.

The Walker A lysine mutants were generated by site-directed mutagenesis using the CLONTECH Transformer Kit. The coding sequence for NBD1 was included in a *BamHI–SpH1* fragment of MRP1. The frameshift was isolated from pBSMPR1-fc-ATG (35) and cloned into pGEM-3Zf(+) that had been digested with the same enzymes, to generate pGEM-NBD1. The coding sequence for NBD2 was in a *SacI–KpnI* fragment of MRP1, which was ligated into the vector EcoRI and KpnI to give pGEM-NBD2. The primers with the mismatched bases (boldface type) for K684M and K1333M were 5'-GGCGTCGGGATATGTCGTCGTCTGG-3' and 5'-GGCGTCGGGATATGTCGTCGTCTGG-3'. The mutations were selected by the selection primer provided in the kit, which changed an NdeI restriction site to an NsiI site in the vector backbone. The presence of the mutation and the fidelity of the sequence of the MRP1 coding region were confirmed by dye sequencing. The *BsU6I–SpH1* fragment bearing the K684M mutation was isolated from pFB-MRP1 and used to replace the same region in pFB-MRP1 (35) and pFBDual-halves to create pFB-MRP1/K684M and pFBDual-halves/K684M, respectively. The EcoRI–KpnI fragment with the K1333M mutation was isolated from pGEM-NBD2 and used to replace the equivalent region in pBSMPR1-fc-ATG to generate pBSMPR1-fc-ATG/K1333M. The SpH1–KpnI fragment was then isolated from the resulting plasmid and used to replace the same region in pFB-MRP1 and pFB-MRP1/K684M to give pFB-MRP1/K1333M and pFB-MRP1/Double KM, respectively. To generate pFBDual-halves/Doubl KM, the NcoI–KpnI fragment of pBSMPR1-fc-ATG/K1333M was ligated into pFB-MRP1 and used to replace the equivalent region in pFB-Dual-MRP1 932–1531. The SalI–XhoI fragment of pFBDual-halves/K684M was isolated and cloned into the resulting vector as described for pFB-Dual-halves. The missing 13 amino acids from human P-gp, GDIRTINVRFLREI, were introduced into MRP1 between amino acids 707 and 708 using a recombinant PCR technique (36). Two PCR products were amplified from MRP1 cDNA. The first contained MRP1 sequence (nucleotides 1924–2316 of MRP1 cDNA) and 27 bp at the 3'-end that encode the first 9 of the inserted amino acids (DIRTINVR). The primers for this PCR were as follows: primer 1, 5'-CTCGGTAGCAGCCAGACGACC3' (forward primer); primer 2, 5'-GACAAGGCTGAGGATGTCCTATA
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ATACGGAGCCCTGTAGACCC-3' (reverse primer). The underlined sequences are MRP1 sequences, while the remainder of primer 2 encodes the inserted amino acids. The second PCR product contained MRP1 sequence (nucleotides 2317–2979 of MRP1 cDNA) and 28 bp at the 5' end encoding the last 9 of the added amino acids (INVRF/PrL). Combination of the two PCR products by a 5' nested priming PCR (using the same sequence (encoding INVRF of the inserted amino acid sequence). The primers used for the second PCR were primer 3 (5'-CATCAACACGGC- GTCCTACGGAAATCTGTGGCATTGTCGCCAC-3') (forward primer) and primer 4 (5'-GTTGTCGTCCTGATGTTGTC-3') (reverse primer). Again, the MRP1 sequence is underlined, and the remainder of primer 3 encodes the inserted amino acids. The sequences that overlap in primer 2 and primer 3 are double underlined.

Gel-purified PCR products were mixed, denatured, and slowly cooled to allow annealing of the overlapping sequences and then made double-stranded using Klenow fragment. Approximately 10% of the product from the Klenow reaction was used as template for a third PCR in which only primer 1 and primer 4 were used. The product contained nucleotides 1924–2979 of MRP1 cDNA with a 39-bp insertion after nucleotide 2316 encoding the 13 amino acids from human P-gp. Digestion of the fragment with NcoI and SpH1 yielded a 940-bp product that was used to replace the region of MRP1 cDNA between nucleotides 1157 and 3066. After sequencing of this region, a BglIII fragment was excised and cloned into the equivalent region of the MRP1 mammalian expression vector pCEBV7. In this way, the construct pCEBV7-Ins708 was produced. A SpH1 fragment was isolated from pCEBV7-Insa708 and used to replace the equivalent region in either pFB-MRP1-Ins708 or pFB-MRP1-Ins13100 to produce pFB-Insa708-Ins292 or pFB-Dual-halves to create pFB-Dual-Ins708halves. A vector expressing the Ins708 NBD1 as a soluble polypeptide was also generated from pFB-Insa708-Ins292 as described for the wild-type form.

Viral Infection, Membrane Vesicle, and Crude Cytosol Preparation—Recombinant bacmid and baculoviruses were generated as described (35, 37). The conditions used for viral infection were similar to those described previously. For membrane vesicle preparation, cells were disrupted by nitrogen cavitation, which were centrifuged at 15,000 rpm at 4 °C for 20 min to remove cell debris. The supernatant was used without further purification.

Immunoblotting and Quantification of MRP1 Polypeptides—SDS-polyacrylamide gel electrophoresis (PAGE) of membrane vesicle proteins was performed essentially as described (3, 39, 40) using 5–15% gradient gels. Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) using 25 mM Tris base, 192 mM glycine, and 9.5% methanol buffer, and MRP1 polypeptides were detected using an antibody against MRP1 (39, 49, 50). Immunocomplexes were solubilized in Laemmli buffer and subjected to SDSPAGE and autoradiography in a PhosphorImager (Molecular Dynamics, Inc.). For 8-azido-ATP binding studies with soluble NBDs expressed in Sf21 cells, a crude cytosolic fraction (15 μl) was used under comparable conditions. For immunoprecipitation, 8-azido-ATP binding studies were carried out using 40 μg of membrane protein (obtained from UV irradiation, membrane proteins were dispersed in 20% methanol buffer, and MRP1 polypeptides were detected using an antibody against MRP1 (35) as described in the legends to Figs. 3, 6, 8, and 10B. The reactions were stopped by the addition of 0.5 ml of ice-cold Tris-EGTA buffer (50 mM Tris-Cl, pH 7.4, 0.1 mM EGTA, 5 mM MgCl2), and the membranes were centrifuged at 14,000 rpm for 15 min at 4 °C. The pellets were washed again and resuspended in 20 μl of the same buffer. The samples were transferred to a 96-well plate and treated as described in the binding procedure above. The ImageQuaNT program (Molecular Dynamics, Inc., Sunnyvale, CA) was used to quantify the relative amounts of labeling of MRP1 and MRP1 polypeptides under various conditions.

RESULTS

LTC4 Transport by MRP1 Half-molecules Expressed either by Co-infection with Two Vectors or by Infection with a Dual Expression Vector—The vectors used for co-infection have been described previously and encoded either amino acids 1–932 or 932–1531 of MRP1 (35). Construction of the dual expression vector and conditions used for infection of Sf21 cells were as described under “Materials and Methods.” Membrane vesicles were prepared from co-infected cells and cells infected with the dual expression vector. The levels of expression of NH2- and COOH-proximal halves of MRP1 were then determined by immunoblotting with mAbs QCRL-1 (epitope amino acids 918–924) (41) and MRPm6 (epitope amino acids 1511–1520) (43), respectively, as described (35). To provide an indication of the relative levels of the NH2- and COOH-proximal half-molecules, vesicles containing full-length MRP1 were also analyzed on the same immunoblots.

Under the conditions used for infection, the levels of the half-molecules were similar in membrane vesicles prepared from either the co-infected cells or cells infected with the dual expression vector (Fig. 1A). However, the rate of ATP-dependent LTC4 transport by membranes from cells infected with the dual expression vector was 2.5–3.0-fold higher than obtained with vesicles from the co-infected cells and was approximately 80% of the rate obtained with vesicles containing the intact protein (Fig. 1B). Thus, reconstitution of a functional transporter in cells infected with the dual expression vector is extremely efficient, supporting our previous suggestion that population heterogeneity limited the extent of reconstitution obtained in co-transfection experiments.

8-Azido-ATP Supports Active Transport by MRP1 as Efficiently as ATP—Radioactive photoactivatable analogues of ATP have been used to study the characteristics of binding and/or hydrolysis of ABC proteins, such as P-gp, CFTR, and SUR1 (31, 44–48). It has also been shown that 8-azido-α-32P]ATP in the absence or presence of vanadate can be used to photolabel MRP1 (39, 49, 50). However, the efficiency with which MRP1 is able to hydrolyze this ATP analogue has not been reported. To examine the ability of 8-azido-ATP to support active transport by MRP1, LTC4 uptake assays were carried out in the presence of 4 mM ATP or 8-azido-ATP using membrane vesicles from cells infected with the dual expression vector. As shown in Fig. 2A, the uptake of LTC4 by the co-expressed dual halves of MRP1 was almost identical with both ATP and 8-azido-ATP. Initial rates of LTC4 uptake by the dual halves of MRP1 were determined at several ATP or 8-azido-ATP concentrations. Double-reciprocal plots of the data yielded an apparent Km of 23 μM for ATP and an apparent Km of 13 μM for 8-azido-ATP (Fig. 2B). Comparable Km values were also obtained when full-length MRP1 was used.

Photoaffinity Labeling of MRP1 by 8-Azido-ADP Occurs Primarily at NBD2—It has been shown previously that the photoaffinity labeling of full-length MRP1 by 8-azido-α-32P]ATP is...
enhanced in the presence of orthovanadate, presumably as a result of the trapping of 8-azido-α-[32P]ADP (49). To assess the distribution of photolabeling between the two NBDs, membranes from Sf21 cells expressing both half-molecules of MRP1 from the dual expression vector or from cells infected with vectors expressing either the NH2- or COOH-terminal half-molecules were prepared. Immunoblotting of these membranes, together with membranes containing full-length MRP1 expressed by co-infection and infection from the dual expression vector or from cells infected with a control vector (data not shown). No labeling of either NBD1 or NBD2 could be detected with membranes from cells expressing one or the other half-molecule. In contrast, photolabeling of NBD2 was readily detectable in membranes from cells infected with the dual expression vector, but very little labeling of NBD1 was observed. The extent of labeling of both NBDs was also modestly increased in the presence of the high affinity MRP1 substrate LTC4 (1 μM) (Fig. 3B).

Photoaffinity Labeling with 8-Azido-ATP Occurs Preferentially at NBD1 of MRP1—The preferential photoaffinity labeling of MRP1 NBD2 following vanadate trapping prompted us to investigate whether a similar labeling profile was observed under conditions designed to minimize hydrolysis of the 8-azido-ATP. Membranes expressing either the NH2- or COOH-proximal half-molecules were incubated on ice, rather than at 37 °C, for 5 min with 5 μM 8-azido-α-[32P]ATP in the presence and absence of LTC4. The samples were then photocross-linked, and membrane proteins were analyzed by SDS-PAGE. As shown in Fig. 4A, weak labeling of the NH2-proximal half was observed, but no labeling of the COOH-proximal half could be detected when either total (Fig. 4A) or immunoprecipitated proteins (data not shown) were analyzed. The addition of 1 μM LTC4 during the 5-min incubation on ice had no effect on
the labeling of either NBD.

To determine whether the extent of labeling was altered when both halves of the protein were co-expressed, we carried out similar analyses with membranes from cells infected with the dual expression vector. Despite the fact that the levels of the half-molecules in membranes prepared from these cells were similar to those obtained when the half-molecules were expressed individually (Fig. 3A), the labeling of the NH$_2$-proximal half of MRP1 was greatly enhanced, and weak labeling of the COOH-proximal half-molecule (Fig. 4A), and it was confirmed by immunoprecipitation (Fig. 4B, top panel). In addition, very weak labeling of the COOH-proximal half-molecule was also observed but was not stimulated by the presence of LTC$_4$ for comparison (Fig. 4B, bottom panel).

A comparable series of experiments was carried out with 8-azido-$\gamma$-$^{32}$P]ATP to confirm that the nucleotide bound by each of the NBDs was being detected in its triphosphate form. Labeling of the individually expressed NH$_2$-proximal half of the molecule (Fig. 4B) with 8-azido-$\gamma$-$^{32}$P]ATP was less than that obtained with 8-azido-$\alpha$-$^{32}$P]ATP (Fig. 4A) and was barely detectable on total membranes (Fig. 4B, top panel). However, it was detectable following immunoprecipitation (Fig. 4B, bottom panel). Again, no labeling of the individually expressed COOH-proximal half of the protein could be detected (Fig. 4B, bottom panel), even after immunoprecipitation (data not shown).

When the two halves were co-expressed, labeling of the NH$_2$-proximal half of the protein was again readily detectable and strongly enhanced by 1 $\mu$m LTC$_4$ (Fig. 4B, top panel). In addition, very weak labeling of the COOH-proximal half-molecule was also observed but was not stimulated by the presence of LTC$_4$ (Fig. 4B, top panel), and it was confirmed by immunoprecipitation (Fig. 4B, bottom panel).

**Fig. 3.** Vanadate-induced nucleotide trapping by MRP1 half-molecules expressed either individually or co-expressed by the dual expression vector. A, immunoblots of membrane proteins from SF21 cells expressing either the NH$_2$-proximal half-molecule (N-half) or the COOH-proximal half-molecule (C-half) of MRP1 or both halves of MRP1 by the dual expression vector (Dual-halves). Left, detection of the NH$_2$-proximal half-molecule of MRP1 by MRP1-specific mAb QCRL-1; right, detection of the COOH-proximal half-molecule of MRP1 by MRP1-specific mAb MRPm6. The sizes of protein standards are indicated in kilodaltons. B, membrane proteins (20 $\mu$g) used in A were incubated with 5 $\mu$m 8-azido-$\alpha$-$^{32}$P]ATP, 1 mM sodium orthovanadate in the presence or absence of 1 $\mu$m LTC$_4$, at 37 °C, following the trapping procedure as described under “Materials and Methods.” The position of the labeled MRP1 N-half and C-half polypeptides are indicated, and an endogenous protein labeled is indicated by a star.

**Fig. 4.** Nucleotide binding in individually expressed MRP1 half-molecules and dual expression vector-expressed MRP1 half-molecules. A, membrane proteins (20 $\mu$g) from SF21 cells expressing either the NH$_2$-proximal half-molecule (N-half) or the COOH-proximal half-molecule (C-half) of MRP1 or both halves of MRP1 (Dual-halves) were incubated on ice with 5 $\mu$m 8-azido-$\alpha$-$^{32}$P]ATP in the presence or absence of 1 $\mu$m LTC$_4$ following the trapping procedure as described under “Materials and Methods.” Membranes from SF21 cells expressing $\beta$-gus were incubated in the presence of LTC$_4$ for comparison. An endogenous protein labeled is indicated by a star. B, top, experiments comparable with those described in A were performed using 5 $\mu$m 8-azido-$\gamma$-$^{32}$P]ATP instead of 8-azido-$\alpha$-$^{32}$P]ATP; bottom, phosphor image of immunoprecipitates of 8-azido-$\gamma$-$^{32}$P]ATP-labeled membrane proteins (40 $\mu$g) from SF21 cells infected with the control vector ($\beta$-gus), the vector encoding the NH$_2$-proximal half-molecule (N-half), and the vector encoding both halves of MRP1 (Dual-halves).
The NBDs of MRP1 were incubated with 5 μl of crude cytosol from Sf21 cells infected with baculoviruses coding for the individual NBDs of MRP1, which were then loaded on a 5–15% gradient gel and immunoblotted as described under “Materials and Methods.” Membranes (4 μg) from Sf21 cells expressing full-length MRP1 were included for comparison. Left, expression of MRP1NBD1 (amino acids 617–932) was detected by MRP1-specific mAb QCRL-1; right, expression of MRP1NBD2 (amino acids 1295–1531) was detected with MRP1-specific mAb MRPm6. The sizes of protein standards are indicated in kilodaltons. A, crude cytosolic fractions (15 μl) from Sf21 cells expressing either MRP1NBD1 or MRP1NBD2 were incubated with 5 μM 8-azido-α-[32P]ATP following the binding procedure as described under “Materials and Methods.”

617 to 932 and 1295 to 1531, respectively. Almost all of the NBD was recovered in a cytosolic, membrane-free protein fraction, which was used without further purification. Immunoblotting indicated that both NBDs accumulated to similar levels (Fig. 5A). We have shown previously that the NBDs expressed as soluble polypeptides in Sf21 cells can be immunoprecipitated by MRP1 conformational mAbs (51), thus providing supporting evidence that they are properly folded. SDS-PAGE of total cytosolic proteins following incubation with 8-azido-α-[32P]ATP was precluded by the presence of high levels of phosphatase and protein kinase activity in the crude cytosol. However, a qualitatively similar profile of labeling was obtained with 8-azido-α-[32P]ATP using bacterially expressed, highly purified glutathione S-transferase fusion proteins corresponding to NBD1 and NBD2 (data not shown). Thus, at least qualitatively, the difference in ability to bind and be photolabeled by 8-azido-ATP observed when the two halves of MRP1 were expressed either individually or together, is retained when the NBDs are produced as soluble polypeptides.

Effect of Mutations of Walker A Lysine Residues in NBD1 and NBD2 on LTC4 Transport and Vanadate Trapping by Full-length MRP1—To further characterize the roles of each NBD of MRP1 in ATP binding and hydrolysis, mutations were introduced into the Walker A lysine residues in both NBDs. Vectors encoding full-length MRP1 in which the Walker A lysine was mutated to methionine in either or both NBDs were generated, and membrane vesicles were prepared. Immunoblots of these vesicles revealed that all three mutants proteins accumulate to levels comparable with that of wild-type MRP1 (Fig. 6A). To determine the effect of the mutations on transport activity, ATP-dependent LTC4 uptake was measured using membrane vesicles containing the three mutant proteins. The rate of ATP/MPR1-dependent LTC4 uptake by vesicles from cells expressing the NBD1 mutant, MRP1/K684M, was approximately 25% (at 1 min and in the presence of 50 mM LTC4) of that obtained with vesicles containing the wild-type protein (Fig. 6B). In contrast, the rates of ATP-dependent LTC4 uptake by membranes containing either MRP1/K1333M or MRP1/Doub KM were less than 5% of that of the membranes expressing the wild-type protein.

Vanadate-induced trapping of ADP by these full-length mutant proteins was also examined. Membranes from Sf21 cells expressing wild-type MRP1 or from cells infected with a control vector were included for comparison. In the presence of 1 mM vanadate, weak labeling of an endogenous protein with a sim-
lar electrophoretic mobility to full-length MRP1 could be detected (indicated by a star in Fig. 6C). The intensity of labeling at this position was greatly enhanced with proteins from vesicles containing wild-type MRP1 and was further increased in the presence of 1 μM LTC4. However, no increase in labeling above the background of the endogenous protein could be detected in membranes containing any of the mutant proteins. Thus, despite the transport activity observed with the K684M mutant, no trapping of 8-azido-ADP was detectable.

**LTC4 Transport by Co-expressed Half-molecules of MRP1 Containing Walker A Lysine Mutations**—To further characterize the effect of the Walker A K684M and K1333M substitutions on the ability to photolabel each NBD with 8-azido-α-[32P]ATP, these mutations were introduced into each of the half-molecules, which were then expressed either together, or with the appropriate wild-type half-molecule, using dual expression vectors. Immunoblotting of membranes prepared from cells infected with the dual expression vectors revealed very similar levels of wild-type and mutant MRP1 half-molecules (Fig. 7A). The ATP/MPR1-dependent uptake of LTC4 by membrane vesicles prepared from the various populations of cells was then compared. The rate of LTC4 uptake by vesicles containing the K684M mutation was approximately 35% (at 1 min and in the presence of 50 nM LTC4) of that obtained with vesicles containing both halves of the wild-type protein (Fig. 7B). The rates of LTC4 uptake by both halves of MRP1/K1333M and MRP1/Double KM were 8 and 5% that of the wild-type protein, respectively. Thus, the results with the dual expression vectors were in good agreement with those obtained with full-length mutant proteins.

**Effect of Walker A Lysine Mutations on Photolabeling with 8-Azido-ATP**—Membrane vesicles used in the transport assays described above were also used for binding and photolabeling studies with both 8-azido-γ-[32P]ATP (Fig. 8A) and 8-azido-α-[32P]ATP (Fig. 8B). Despite similar levels of the NH2-proximal half-molecules in the membrane vesicles used (Fig. 7A), labeling of the NH2-proximal half-molecule containing the K684M mutation (for both Dual-halves/K684M and Dual-halves/Dual KM) was not detectable with either 8-azido-α- (Fig. 8D) or 8-azido-γ-[32P]ATP (Fig. 8A), regardless of whether it was expressed with a wild-type or mutant COOH-proximal half-molecule. In addition, the K684M mutation eliminated any LTC4 enhancement of the photolabeling of NBD1 and all labeling of a co-expressed wild-type COOH-proximal half-molecule. Weak labeling of a protein with a mobility similar to that of the COOH-half molecule was detected, but this was present at the same level in all membrane preparations used and thus is clearly an endogenous protein. As expected, the K1333M mutation also eliminated all labeling of NBD2. However, labeling of the NH2-proximal half-molecules with either 8-azido-γ-[32P]ATP or 8-azido-α-[32P]ATP was only slightly reduced and could still be enhanced by LTC4 (Fig. 8A and B).

We also examined photolabeling of each NBD of the mutant half-molecules with 8-azido-α-[32P]ATP under vanadate trapping conditions, with and without the addition of 1 μM LTC4 (Fig. 8C). As described above, when the dual expression vector was used to express both halves of the wild-type protein as a positive control, NBD2 was preferentially labeled, and labeling could be stimulated by LTC4 (Fig. 8C). Weak labeling of NBD1 could also be detected. Consistent with the results obtained with the full-length protein, no labeling of either NBD was observed when either the K1333M or K684M mutant half-molecules were co-expressed with the appropriate wild-type half of the protein despite the demonstrable transport activity of the latter combination.

**Effect of Increasing the Spacing between Walker A and B Motifs of MRP1 NBDM1 on LTC4 Transport**—As noted previously, the spacing between the Walker A and B motifs in the NH2-proximal NBDs of the MRP-related proteins and CFTR is shorter than the spacing in their COOH-proximal NBDs and both NBDs of the P-gps. This difference is primarily attributable to a relative deletion of 13 amino acids at a conserved location in these proteins, which in MRP1 is between amino acids 707 and 708. To investigate the possible functional significance of the deletion, the corresponding sequence from NBDM1 of human P-gp was introduced into the NH2-proximal half-molecule between amino acids 707 and 708 by recombinant PCR to generate an NH2-proximal Ins708 half-molecule, which was then expressed together with the wild-type COOH-proximal half-molecule using the dual expression vector. Immunoblotting of membranes prepared from cells expressing both halves of the wild-type protein or the Ins708 mutation
plus the wild-type COOH-proximal half of the protein revealed very similar levels of wild-type and mutant MRP1 polypeptides (Fig. 9A). The ATP-dependent uptake of LTC₄ by membrane vesicles prepared from both populations of cells was then compared. The rate of ATP/MRP1-dependent LTC₄ uptake by vesicles containing the Ins708 mutation was approximately 30% (at 1 min and in the presence of 50 nM LTC₄) of that obtained with vesicles containing both halves of the wild-type protein (Fig. 9B). Thus, the results of LTC₄ transport studies were very similar to those obtained with K684M mutation.

**Effect of the Ins708 Mutation on Labeling with 8-Azido-ATP and -ADP**—To determine whether the nucleotide binding characteristics of NBD1 were affected by the Ins708 mutation, membranes containing the Ins708 half-molecule and the wild-type COOH-proximal half-molecule were incubated with 5 μM 8-azido-α-[³²P]ATP on ice for 5 min. Membranes containing both halves of the wild-type protein were also included for comparison. Despite similar levels of the two NH₂-proximal half-molecules in the membrane vesicles used (Fig. 9A), labeling of the Ins708 half-molecule could not be detected even in the presence of LTC₄ (Fig. 10A). In addition, labeling of the COOH-proximal half of the protein with 8-azido-α-[³²P]ATP when expressed with the Ins708 half-molecule was lost (Fig. 10B). As described above, when the dual expression vector was used to express both halves of the wild-type protein, NBD2 was preferentially labeled in the presence of orthovanadate, and labeling could be stimulated by LTC₄ (Fig. 10B). Under identical conditions, labeling of NBD2 could not be detected when co-expressed with the Ins708 half-molecule (Fig. 10B). Thus, the effect of the Ins708 mutation was similar to that of the K684M mutation both with respect to LTC₄ transport activity and labeling by 8-azido-ATP and -ADP.
MRP1 appears to be dependent on the presence of NBD1. A strikingly different nucleotide labeling profile was observed when experiments were carried out in the absence of vanadate at reduced temperatures to minimize hydrolysis of the 8-azo-ATP. Under these conditions, co-expression of the two MRP1 half-molecules revealed strong labeling of NBD1 with relatively weak labeling of NBD2. This difference was observed whether 8-azo-α- or 8-azo-γ-[32P]ATP was used, confirming the ability of NBD1 to bind the triphosphate form of the nucleotide relatively strongly. Furthermore, binding to NBD1 but not NBD2 could be detected when these domains were expressed in individual half-MRP1 molecules or as soluble polypeptides. However, it was also clear from these experiments that binding of nucleotide by NBD1 was strongly enhanced by the presence of NBD2. When the two NBDs were co-expressed as soluble polypeptides, no increase in binding by NBD1 could be detected, suggesting that the associated MSDs are required to stabilize, or transduce the effects of interactions between the NBDs. Overall, the experiments with the dual expression vector support the existence of cooperativity between the NBDs with respect to both nucleotide binding and hydrolysis. They also indicate that this cooperativity is dependent on the presence of the MSDs.

The effect of substrate on 8-azo-ATP binding by each of the NBDs under nonhydrolysis conditions also differed from that observed under vanadate trapping conditions. In the presence of LTC4, trapping of 8-azo-α-[32P]ADP increased moderately at NBD2, but the presence of substrate had little effect on trapping by NBD1. In contrast, LTC4 increased the binding of 8-azo-ATP to NBD1 severalfold with little effect on binding of nucleotide by NBD2. These data differ from those obtained previously with P-gp, which suggest that substrate stimulates the hydrolysis of ATP by the protein but does not enhance nucleotide binding (59–61). One possible explanation for the observed differences between MRP1 and P-gp may be the relative lipophilicity of their substrates. There is considerable evidence that P-gp binds its hydrophobic substrates in the lipid environment of the membrane (reviewed in Ref. 62). MRP1, on the other hand, is likely to bind at least some of its comparatively hydrophilic substrates from the cytoplasm, suggesting that cytoplasmic regions of MRP1, possibly the NBDs them-
selves, may be important for initial binding of some transported substrates. Consistent with this possibility, we have shown previously that QCRL-3, an MRPI-specific mAb that recognizes a conformational epitope in NBD1, inhibits LTC₄ transport and decreases LTC₄ binding to intact MRPI (12, 40, 51). LTC₄-dependent stimulation of ATP binding by NBD1 did not occur when this domain was expressed alone as part of the NH₂-proximal half of MRPI, indicating that interaction between both half-molecules is required. Whether this is because of an obligatory interaction between the two NBDs or because LTC₄ binding requires elements in the COOH-proximal half of the protein is presently not known.

The differences detected between the two NBDs of MRPI with respect to labeling with 8-azido-ATP and vanadate trapping of 8-azido-ADP prompted us to examine the functional consequences of inactivating one or both NBDs by mutation of the conserved lysine residue in the Walker A motifs in methionine. In other ABC transporters, comparable mutations have been shown to eliminate ATP binding and hydrolysis. In P-gp, such a mutation in either NBD completely inactivates the protein (63–65), while in CFTR, mutation of the conserved lysine in NBD1 impairs channel opening and mutation of the comparable residue in NBD2 impairs channel closing (66, 67).

In full-length MRPI, mutation of lysine 684 in NBD1 decreased LTC₄ transport activity by approximately 70%, while the comparable mutation of lysine 1333 in NBD2 essentially inactivated the protein. The K684M mutation had a similar effect on the activity of the reconstituted transporter, and we confirmed that binding of 8-azido-ATP by the mutated NBD1 had been abolished. However, despite the retention of 30% of the wild-type level of ATP-dependent transport activity, the K684M mutation also eliminated detectable trapping of 8-azido-ADP by NBD2. Consequently, to ensure that the transport activity detected in the K684M mutation was indeed dependent on ATP hydrolysis, assays were carried out with the nonhydrolyzable ATP analogue, ATPγS, and no transport activity could be detected (data not shown).

In addition to mutating the Walker A motif of NBD1, we also examined the consequences of increasing the spacing between the Walker A and B motifs by inserting 13 amino acids from NBD1 of human P-gp between amino acids 707 and 708. This insertion creates an NBD with a spacing between Walker motifs similar to that in NBD2 of MRP1 and both NBDs of P-gp. The mutation eliminated the ability to label NBD1 with 8-azido-ATP, either when the NBD was expressed as part of a reconstituted transporter or as a soluble polypeptide, in the presence or absence of LTC₄. The Ins708 mutation also failed to enhance the ability to trap 8-azido-ADP at NBD1 and eliminated the ability to detect trapping at NBD2. Furthermore, like the K684M mutation, it reduced LTC₄ transport by approximately 70%. Thus, the Ins708 mutation behaved in a manner indistinguishable from the K684M mutation with respect to nucleotide binding, vanadate-induced trapping, and transport activity.

No major differences could be detected between the ATP dependence of the initial rates of transport of the K684M and Ins708 mutations when compared with the wild-type protein. As observed with the wild-type protein, initial rates of LTC₄ transport of both the K684M and Ins708 mutations reached a maximum between 0.5 and 1 mM ATP (data not shown). This observation, coupled with the inability to detect 8-azido-ATP binding by NBD1 following introduction of these two quite different mutations, argues strongly that ATP hydrolysis at NBD2 is sufficient to support transport of LTC₄, albeit with decreased maximal efficiency relative to the wild-type protein. This behavior is similar to that observed with histidine permease mutants in which inactivation of either NBD reduces the activity of the protein by approximately 50% rather than completely eliminating transport as it does in P-glycoprotein (28). The inability to trap nucleotide at NBD2 in the K684M and the Ins708 mutant proteins suggests that in the absence of ATP binding and possibly hydrolysis at NBD1, either 8-azido-ADP is released rapidly from NBD2 even in the presence of vanadate or that the conformation in which NBD2 binds the 8-azido-ADP vanadate complex cannot be efficiently photoaffinity-labeled. In contrast, the K1333M mutation essentially eliminated transport and abolished 8-azido-ADP trapping by NBD2 and the low level of trapping detectable at NBD1. However, it had no effect on binding of 8-azido-ATP at NBD1; nor did it affect the ability of LTC₄ to enhance the binding. The combined results of LTC₄ transport and photoaffinity labeling studies with the K684M, Ins708, and K1333M mutants are consistent with a model in which ATP hydrolysis at NBD1 is obligatorily coupled to hydrolysis at NBD2 but not vice versa. This lack of reciprocity between the coupling of the two NBDs could explain the relatively low level of trapping of 8-azido-ADP observed at NBD1 despite the high level of photoaffinity labeling with 8-azido-ATP. Under these circumstances, trapping of 8-azido-ADP at NBD2 would be expected to prevent trapping at NBD1, while trapping at NBD1 would not necessarily eliminate trapping at NBD2.

The lack of reciprocal coupling between the two NBDs of MRPI and the different consequences of the K684M and K1333M mutations raise an important question with respect to the role played by NBD1 in substrate transport. In P-glycoprotein and histidine permease, there is compelling evidence that both NBDs contribute directly to substrate transport (28–32). However, it is not possible to determine with the data presently available for MRPI whether the decrease in LTC₄ transport efficiency seen with the K684M mutation is a direct consequence of the inactivation of NBD1 or the result of a decrease in the efficiency of ATP hydrolysis at NBD2. Several observations, including (i) the LTC₄-dependent stimulation of 8-azido-ATP binding by NBD1, (ii) the retention of partial transport activity following inactivation of NBD1 but not NBD2 and, (iii) loss of the ability to trap and photolabel NBD2 in the K684M and Ins708 mutants with 8-azido-ADP, are equally compatible with a mechanism in which the role of NBD1 is to regulate, in a substrate-responsive manner, the efficiency of ATP binding and hydrolysis at NBD2. It may be possible to distinguish between these two possibilities by determining the molar stoichiometry between ATP hydrolysis and substrate transport. However, this will require establishment of a reconstituted MRPI transport system with highly purified wild-type and mutant proteins.

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