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Function of the ABC Signature Sequences in the Human Multidrug Resistance Protein 1

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ABSTRACT

Human multidrug resistance protein 1 (MRP1) is a membrane ATP-binding cassette transporter that confers multidrug resistance to tumor cells by effluxing intracellular drugs in an ATP-dependent manner. The mechanisms by which transport occurs and by which ATP hydrolysis is coupled to drug transport are not fully elucidated. In particular, the function of the signature sequences in the nucleotide binding domains (NBDs) of MRP1 is unknown. We therefore investigated the effect of mutation of the signature sequences (G771D and G1433D) and of the Walker A motifs (K684M and K1333M) in the NBDs on the 8-azido-[α-^{32}P]ATP photolabeling and 8-azido-[α-^{32}P]ADP vanadate trapping of MRP1. Both mutations in the Walker A motif almost completely inhibited the labeling of the mutated NBD with 8-azido-[α-^{32}P]ATP but not the labeling of the other intact NBD. In contrast, the G771D mutation in the signature sequence of NBD1 enhanced the labeling of NBD1 but slightly decreased the labeling of NBD2. The G1433D mutation in the signature motif of NBD2 enhanced the labeling of NBD2 but did not affect the labeling of NBD1. These effects were all substrate-independent. Photolabeling of NBD2 and a very slight photolableing of NBD1 were detectable under vanadate trapping conditions with 8-azido-[α-^{32}P]ATP. Trapping at both NBD1 and NBD2 was almost completely inhibited by K684M and K1333M mutations and by the K684M/K1333M double mutation. The G771D mutation completely inhibited trapping at NBD2 and considerably inhibited trapping at NBD1. However, whereas the G1433D mutation also considerably inhibited trapping at NBD1, it only partially inhibited trapping of NBD2, and the trapping could still be enhanced by leukotriene C_4. Our findings suggest that both signature sequences of MRP1 are involved in ATP hydrolysis and must be intact for the ATP hydrolysis and the transport by MRP1.

Multidrug resistance protein (MRP1), a member of the ATP-binding cassette (ABC) transporter superfamily, has two nucleotide binding domains (NBDs) that are involved in binding and hydrolyzing ATP (Cole et al., 1992). The exact mechanism by which the NBDs function has not been completely worked out and is important for the understanding of MRP1 function and the design of drugs to modulate that function in disease states.

Previous reports indicated that the two NBDs of MRP1 are functionally distinct. Intact ATP is preferentially bound at NBD1, whereas trapping of the ATP hydrolysis product, ADP, occurs predominantly at NBD2 (Gao et al., 2000). ATP interaction with NBD1 consistently increased ATP or ADP binding at NBD2 (Hou et al., 2002). These findings suggested that the role of NBD1 is to regulate the efficiency of ATP binding and hydrolysis at NBD2. Functionally distinct NBDs have also been reported for other receptors such as the cystic fibrosis transmembrane conductance regulator (CFTR), which regulates CFTR ion channel gating (Szabo et al., 1999; Aleksandrov et al., 2001) and the sulfonylurea receptor (SUR) (Matsuo et al., 1999, 2000).

NBDs in all ABC transporter proteins have three conserved motifs: the Walker A, B, and C motifs (See Fig. 1). The C motif is known as the signature sequence. Studies carried out to determine the configuration of the two NBDs in ABC transporter proteins suggest that the NBDs in all ABC proteins adopt the same dimeric configuration. Thus, the NBDs of the ABC-transporter for vitamin B_{12} dimerize in a conformation that places the LSGGQ signature sequence motif of one NBD domain near the Walker A motif of the second NBD domain (Locher et al., 2002). A dimer interface between LSGGQ and Walker A motifs has also been implicated in that

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ABBREVIATIONS: MRP, multidrug resistance protein; ABC, ATP-binding cassette; NBD, nucleotide binding domain; CFTR, cystic fibrosis transmembrane conductance regulator; SUR, sulfonylurea receptor; wt, wild type; LTC_4, leukotriene C_4; AG-A, agosterol A; azido AG-A, azidophenyl agosterol A; PAGE, polyacrylamide gel electrophoresis; GSH, glutathione; VCR, vincristine; MDR, multidrug resistance.

the nucleotide binding site in the nucleotide binding subunits of both the maltose transporter complex of *Escherichia coli* and of Rad 50 (Hopfner et al., 2000; Borths et al., 2002; Smith et al., 2002; Verdon et al., 2003). Likewise, the signature sequences (LSGGQ) of P-glycoprotein have been shown to be localized adjacent to the apposing Walker A site. It was suggested that this conformation participated in forming the ATP-binding sites and that the conformation was disrupted upon ATP hydrolysis (Loo et al., 2002).

To more fully understand the role of the signature sequence in NBD function, mutation experiments have been carried out. Mutation of the signature sequence in bacterial ABC proteins suggest that the signature sequence is not involved in ATP binding, but rather in ATP hydrolysis (Shyamala et al., 1991; Koronakis et al., 1995; Schmees et al., 1999). Likewise, mutation of the CFTR signature sequence at G551D did not change the ATP-binding of purified NBD1 but reduced the ATPase activity (Li et al., 1996; Qu et al., 1997). Mutation of the MDR1 signature sequence at R538M also showed greatly decreased ATPase activity, although some amino acid replacements in the ABC signature region did not affect the ATPase function of MDR1 (Bakos et al., 1997).

Although these reports suggest that the signature sequence of ABC proteins does not participate in ATP binding, its role in ATPase activity and transduction is ambiguous. In most previous studies, the effects of signature sequence mutations on nucleotide binding were examined in isolated NBDs. The structural and functional properties of isolated NBDs may differ from those of NBDs in intact or reconstituted MRP1.

In this study, we have therefore investigated the role of the signature sequences and Walker motifs in NBD function in reconstituted MRP1. It has been previously shown that it is possible to reconstitute an active transporter by infecting *Spodoptera frugiperda* Sf21 cells with a virus with a dual-expression cassette that allows the coexpression of the N and C terminal fragments of MRP1. This model system has already proven its usefulness in analysis of NBD function in a previous study which indicated that the two NBDs of MRP1 differ markedly with respect to their ability to bind 8-azido-[^{32}P]ATP and to trap 8-azido-[^{32}P]ADP (Gao et al., 2000). In this study, we examined the effect of mutations in the signature sequences or Walker motifs of the NBD domains on the binding of 8-azido-[^{32}P]ATP and trapping of 8-azido-[^{32}P]ADP by each NBD when they are expressed in combination. This is the first study of the effect of signature sequence mutations on NBD function in reconstituted MRP1.

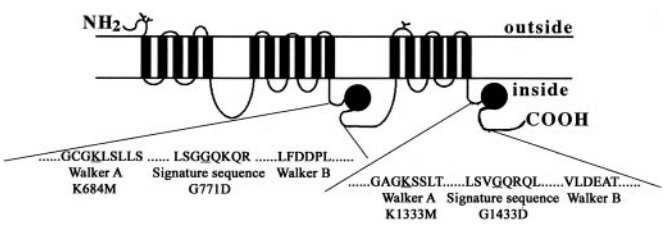

Fig. 1. A schematic diagram depicting the membrane topology of MRP1 and the sequences of the conserved Walker A, B, and C (signature sequence) motifs in NBD1 and NBD2. Residues in the Walker A and C motifs that were mutated for this study are underlined.

Materials and Methods

Materials. [^{125}I]NaI [3.7 GBq (100 μCi)/ml] and [14,15,19,20-^{3}H(N)]leukotriene (LT) C_4 (146 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). 8-Azido-[α-^{32}P]ATP (20 Ci/mmol) was from ICN Biomedicals (St. Laurent, PQ, Canada). Unlabeled LTC_4 was from Calbiochem (La Jolla, CA). The synthesis and use of [^{125}I]11-azidophenyl agosterol A ([^{125}I]azido AG-A) was described in our previous article (Ren et al., 2001). Cellfectamine and competent DH10Bac *Escherichia coli* cells were purchased from Invitrogen (Carlsbad, CA). Anti-MRP1 monoclonal antibodies, MRPr1 (epitope amino acids 229–281) and MRPm6 (epitope amino acids 1389–1351) were obtained from Progen Biotechnick (Heidelberg, Germany).

Cell Culture and Membrane Vesicle Preparation. Sf21 insect cells were cultured in serum-free Sf-900 II SFM medium (Invitrogen). Membrane vesicles were prepared from Sf21 insect cells infected with various recombinant baculoviruses as described previously (Ren et al., 2001, 2002). Membrane vesicles were suspended in a buffer containing 10 mM Tris-HCl, pH 7.5, and 250 mM sucrose. Protein concentrations were determined by the method of Bradford (1976).

Generation of Constructs and Viral Infection. The construction of pFastBac1 for dual expression of both N- and C-terminal halves of MRP1 and the strategy for mutagenesis have been described previously (Ren et al., 2001). The MRP1 construct encoding the K684M mutation was generated by site-directed mutagenesis using the reverse primer 5′-GAGAGCAGGGACAGC**A**TTCCG-CAGCCC-3′ (bold indicates a mismatched base encoding the K684M mutation; underlining indicates a silent mutation). The MRP1 K1333M mutant was constructed using the oligonucleotide DNA 5′ CGGGAGCTGGGA**T**GTCGTCCCTGAC3′ (bold indicates a mismatched base) and the Gene Editor in vitro site-directed mutagenesis system (Promega, Madison, WI) according to the manufacturer's protocol. The MRP1 K684M/K1333M double mutant was generated by exchanging DNA fragments from the single mutations.

The strategies employed for site-directed mutagenesis of G771D and G1433D in MRP1 cDNA were described previously (Ren et al., 2001). The primer used to generate G771D and G1433D mutations were forward primers 5′-CTGGGGACCAGAAGCAGCGCGTGAG-3′ and 5′-AGTGTCGATCAGCGCCAGCTTGTG-3′ (underlining indicate mismatched bases encoding G771D and G1433D mutations, respectively). Baculoviruses expressing the wild-type and mutant MRP1s described above were generated using the Bac-to-Bac expression system (Invitrogen) as described previously (Gao et al., 2000; Ren et al., 2001).

Immunoblotting of MRP1 Fragments. Immunoblotting was performed as described previously (Ren et al., 2000). Anti-MRP1 monoclonal antibodies MRPr1 (epitope amino acids 229–281) and MRPm6 (epitope amino acids 1389–1351) were used for the immunoblotting of the MRP1 fragments. We used the image analysis software Quantity One, version 4.2.0 (Bio-Rad, Hercules, CA) to quantify the densities of the bands and normalized the data in the figures.

[^{3}H]LTC_4 Uptake by Membrane Vesicles. The extent of [^{3}H]LTC_4 uptake was measured using a rapid filtration technique as described previously (Ren et al., 2001). In brief, isolated membrane vesicles (25 μg of protein) were incubated in the presence or absence of 4 mM ATP in 50 μl of transport buffer [0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM phosphocreatine, and 100 μg/ml creatine phosphokinase] with 100 nM [^{3}H]LTC_4 for the indicated times at 37°C. The reaction was stopped with 3 ml of ice-cold stop solution (0.25 M sucrose, 0.1 M NaCl, and 10 mM Tris-HCl, pH 7.5). The samples were passed through Millipore filters (0.22-μm pore size) under a light vacuum. After three rinses with 3 ml of ice-cold stop solution, the filters were immersed in liquid scintillation fluid, and their radioactivity was measured.

Photoaffinity Labeling of MRP1. [^{125}I]Azido AG-A (7.2 μCi/nmol) was used for photolabeling studies that were carried out as described previously (Ren et al., 2001). For photolabeling of NBD1 and NBD2 of MRP1 by 8-azido-[α-^{32}P]ATP, membrane vesicles (50 μg of protein) were incubated for 5 min on ice with 5 μM of 8-azido-[α-^{32}P]ATP in the presence of 5 mM $MgCl_2$ and 1 μM LTC_4 as indicated. After continuous irradiation of the samples with a short wavelength (366 nm) UV lamp for 10 min on ice, the samples were solubilized in SDS sample buffer as described by Debenham et al. (1982) and subjected to SDS-PAGE. Autoradiograms were developed after 6 to 24 h exposure at room temperature.

Orthovanadate Trapping of 8-Azido-[α-^{32}P]ADP by MRP1. Membrane vesicles (100 μg of protein) were incubated in 20 μl of transport buffer containing 5 μM 8-azido-[α-^{32}P]ATP, 5 mM $MgCl_2$, and 1 mM sodium orthovanadate at 37°C for 15 min in the presence and absence of 1 μM LTC_4. Trapping in the absence of orthovanadate was used as a negative control. The reactions were stopped by the addition of 1 ml of ice-cold Tris-EGTA buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, and 5 mM $MgCl_2$). The membranes were collected by centrifugation at 25,600*g* for 30 min at 4°C. The pellets were washed again with the same buffer and resuspended in 20 μl of transport buffer. The samples were then irradiated with UV and subjected to SDS-PAGE as described above. Autoradiograms were developed after 10 to 24 h of exposure at −70°C. Gel slices corresponding to specific bands on the autoradiogram were cut and the radioactivity (counts per minute) was measured with an Auto Well α,β counter (Aloka, Tokyo, Japan). Background radioactivity in equal areas of the gel in each lane was measured and subtracted from the radioactivity of the band.

Results

Expression of Mutant N- and C-Terminal NBDs of MRP1. To investigate the function of the NBD domains in MRP1, we constructed a panel of mutants in the NBD signature sequence and Walker A motifs. The location and sequence of the mutations in the N- and C- terminal NBD1 and NBD2 domains of MRP1 are summarized in Fig. 1. The N- and C-terminal halves of MRP1 encoding wild-type or mutant combinations as indicated were simultaneously expressed in insect cells using a dual expression vector and expression levels of the N- and C-terminal halves were examined by immunoblot analysis (Fig. 2). The expression levels of the N-terminal halves of the mutated dual N+C fragments relative to that for wt N+C were 0.98, 0.55, 0.41, 1.58, and 0.84 for N K684M+C, N+C K1333M, N K684M+C K1333M, N G771D+C, and N+C G1433D, respectively. The expression levels of the C-terminal halves of the mutated dual N+C fragments relative to that for wt N+C were 0.85, 0.49, 0.37, 1.21, and 0.51 for N K684M+C, N+C K1333M, N K684M+C K1333M, N G771D+C ,and N+C G1433D, respectively. The expression levels of N and C halves of the wild type dual N+C fragments were higher than those of the mutated dual N+C fragments except for NG771D+C.

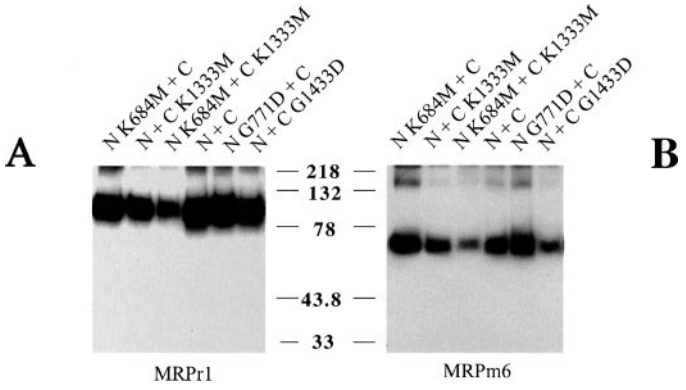

Fig. 2. Membrane vesicles (20 μg of protein) from insect cells infected with recombinant baculovirus dual expression vectors encoding both N- and C-termini of either wild-type or mutant MRP1 in the indicated combinations, were separated by 8.5% SDS-PAGE and immunoblotted with either the anti-N-terminal antibody r1 (A) or the anti-C-terminal antibody m6 (B), respectively. Molecular size markers are shown in the middle.

Effect of NBD Mutations on ATP-Dependent LTC_4 Uptake. To determine the effect of the NBD mutations on drug transport, membrane vesicles were prepared from insect cells coexpressing N- and C-wild type or mutant halves of MRP1, and ATP-dependent LTC_4 uptake into the vesicles was examined (Fig. 3). Although the expression levels of dual NK684M+C and dual NG771D+C were very near and higher, respectively, than that of the wild type N+C, the LTC_4 uptake activities of the membrane vesicles expressing these two mutant N+C fragments were considerably decreased compared with that expressing nonmutated N+C fragments. Furthermore, G771D mutation in signature sequence of NBD1 more effectively lowered LTC_4 uptake activity than K684M in Walker A motif of the same NBD, suggesting that the signature sequence has a more important role than the Walker A motif in the transport of the substrate.

Mechanism of Inhibition of Drug Transport by NBD Mutations. Because mutations in the Walker A motifs, or the signature sequence, inhibited drug transport, we determined whether these mutations were modulating drug transport by affecting drug binding and/or ATP binding to MRP1. Drug binding of the membrane vesicles was assayed by photolabeling of the vesicles with [^{125}I]azido AG-A in the pres-

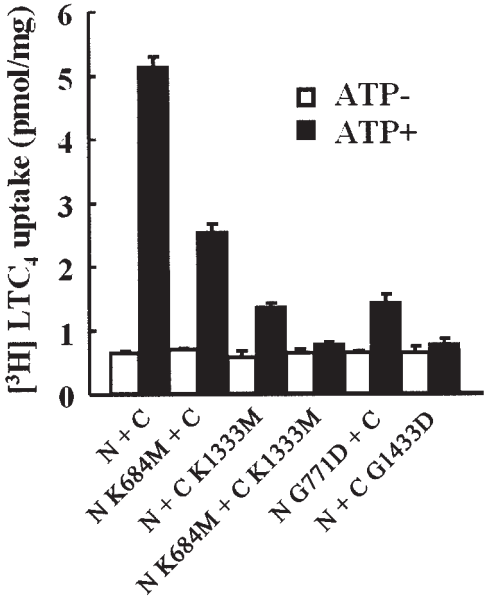

Fig. 3. Effect of NBD mutations on ATP-dependent uptake of [^{3}H]LTC_4 by membrane vesicles. Membrane vesicles (25 μg of protein) coexpressing both N- and C-terminal wt or mutant fragments of MRP1 were incubated with 50 nM [^{3}H]LTC_4 in 50 μl of transport buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM phosphocreatine, and 100 μg/ml creatine phosphokinase) in the absence (□) or presence (■) of 4 mM ATP at 37°C for 5 min. Uptake in 5 min was calculated and expressed as picomoles per milligram of protein. The data represent the means ± S.E. from three separate experiments.

ence of 10 mM GSH. As shown in Fig. 4, drug binding by the wild-type or mutated MRP1 was comparable or increased, indicating that mutation of the NBD domains in the N- or C-halves of MRP1 did not abrogate drug binding.

ATP binding to the membrane vesicles was investigated by photoaffinity labeling of the membrane vesicles on ice using 8-azido-[α-^{32}P]ATP. Consistent with previous reports (Gao et al., 2000), the NH_2-proximal half of wild-type MRP1 was intensely labeled with 8-azido-[α-^{32}P]ATP, and weak labeling of the COOH-proximal half of the protein was detected (Fig. 5). However, in contrast to previous reports, LTC_4 enhanced labeling of the C-terminal half with 8-azido-[α-^{32}P]ATP but not that of the N-terminal half (Gao et al., 2000). Mutation of the Walker A motif in the N-terminal (K684M) NBD1 or the C-terminal (K1333M) NBD2 almost completely inhibited the labeling of the NBD in their respective fragments. However, mutation of the Walker A motif in NBD1 also slightly attenuated the labeling of NBD2. In contrast, mutation of the Walker A motif in NBD2 did not affect the labeling of NBD1.

Unlike the situation observed with the Walker A motifs, mutation of the signature sequence in the N (G771D) or the C (G1433D) - terminal half enhanced the labeling of the NBD in their respective fragments. However, similar to the Walker A motifs, mutation of the NBD1 signature sequence slightly inhibited labeling of NBD2, whereas mutation of the NBD2 signature sequence did not affect the labeling of NBD1. These effects of the mutations were observed both in the presence and absence of LTC_4 indicating that they were drug independent (Fig. 5).

In summary, mutations in either the Walker A motif or in the signature sequence in NBD1 can modulate NBD2-ATP binding function, whereas NBD2 mutations are unable to modulate the function of NBD1. Furthermore, mutations in the Walker A or the signature sequences seem to have opposite effects on ATP binding that are unaffected by LTC_4 binding.

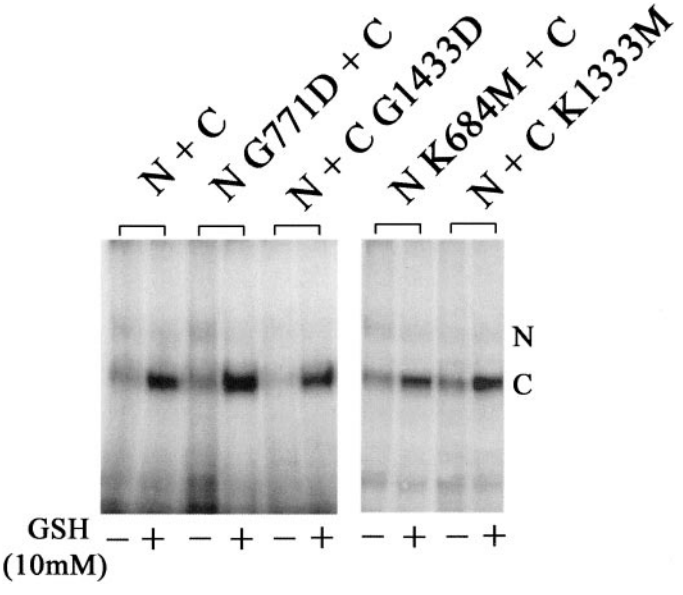

Fig. 4. Effect of NBD mutations on MRP1 drug binding activity. Membrane vesicles (50 μg of protein) coexpressing both N- and C-terminal wt or mutant fragments of MRP1, as indicated at right, were incubated with 5 μM [^{125}I]azido AG-A in the absence (−) or presence (+) of GSH (10 mM). The samples were then separated by 8.5% SDS-PAGE. Autoradiograms were developed after 8-h exposure at room temperature.

Effect of NBD Mutations on Vanadate Trapping of 8-Azido-[α-^{32}P]ATP. Vanadate trapping is an indirect measure of the ATPase activity of ABC transporters. We therefore wished to examine the effect of the NBD mutations on trapping with 8-azido-[α-^{32}P]ATP of MRP1 fragments at 37°C in the presence of vanadate. We first characterized vanadate trapping of the wild-type N + C coexpressed fragments. No trapping was detected in the absence of vanadate from cells expressing both half molecules (Fig. 6). Photolabeling of NBD2, and a weak photolabeling of NBD1, were detectable under vanadate-trapping conditions with 8-azido-[α-^{32}P]ATP. The trapping at NBD2 was enhanced by LTC_4, GSH, AG-A, GSH+AG-A, VCR, and GSH+VCR, with GSH+AG-A showing the highest enhancement of almost 3-fold.

Either single or double mutation of the Walker A motifs in NBD1 and/or NBD2 (K684M, K1333M, or K684M/K1333M double mutations) almost completely inhibited trapping by both NBD1 and NBD2 domains (Fig. 7A). Single mutations of the signature sequence in either NBD1 or NBD2 also considerably decreased trapping at NBD1 (Fig. 7B). However, whereas mutation of the signature sequence of NBD1 (G771D) completely inhibited the trapping at NBD2, mutation of the signature sequence of NBD2 (G1433D) only partially inhibited

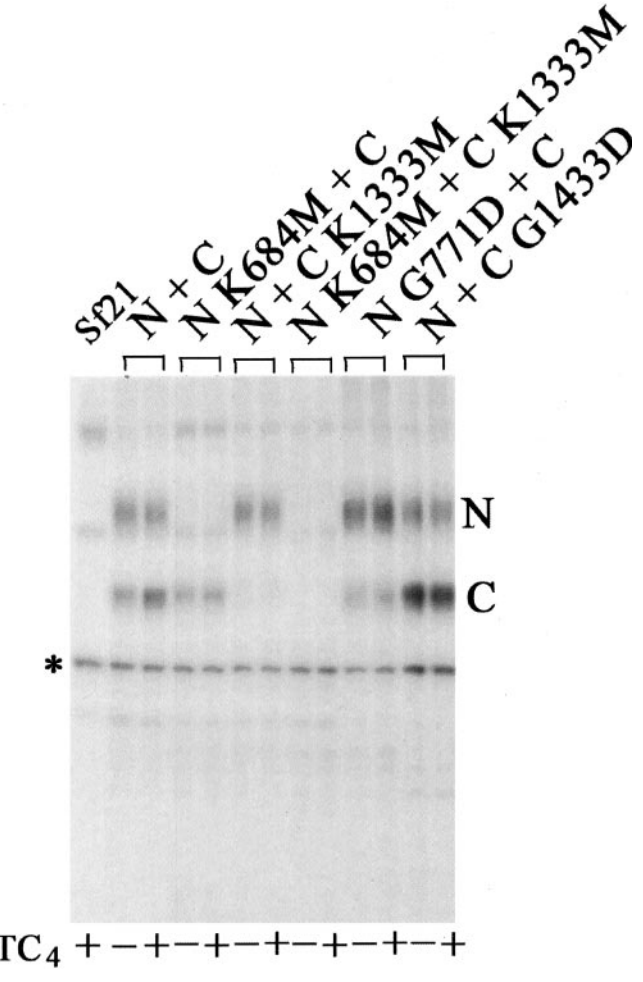

Fig. 5. Effect of NBD mutations on MRP1-ATP binding activity. Membrane vesicles (50 μg of protein) from insect cells coexpressing both N- and C-terminal wt or mutant fragments of MRP1 were incubated on ice with 5 μM 8-azido-[α-^{32}P]ATP and 5 mM $MgCl_2$ in the presence or absence of 1 μM LTC_4 as described under *Materials and Methods*.

the trapping at NBD2, and the trapping could still be enhanced by LTC_4 (Fig. 7C). This data indicates that Walker A and signature sequence play different roles in NBD function.

Discussion

We investigated the role of signature sequences in the NBDs of MRP1 in a system whereby the two NBDs could be coexpressed in a reconstituted MRP1 molecule.

This system involved the coexpression of N and C terminal fragments of MRP1 encoding various combinations of wild-type or mutant NBDs using a virus with a dual expression cassette. The dual expression vector used in this study has been described previously and results in the reconstitution of a functional receptor (Gao et al., 2000). This system has been used to demonstrate the functionally distinct properties of NBD1 and NBD2. Our data are consistent with the previous findings that NBD1 is preferentially labeled with 8-azido-[α-^{32}P]ATP and that trapping of 8-azido-[^{32}P]ADP occurs predominantly at NBD2. Mutation of the conserved Walker A lysine 684 or 1333 eliminated binding by NBD1 or NBD2, respectively, and all detectable trapping of 8-azido-ADP at both NBD1 and NBD2.

For this study, the signature sequences were mutated to alter the conserved glycine at G771 or G1433 to aspartic acid. Although ATP-dependent LTC_4 transport by G771D and G1433D MRP1 mutants, as well as transport by the K684M and K1333M mutants in the Walker A motifs, were considerably decreased, GSH-dependent photolabeling with azido AG-A of these MRP1 mutants was retained. These findings suggest that the drug binding site (s) is (are) conserved and that gross conformational changes were not produced by the mutations, indicating that these mutations are suitable for elucidation of the function of the signature sequences of MRP1.

Based on studies of bacterial ABC proteins and some mammalian ABC transporters, the signature sequence is believed to be involved not in ATP binding but in ATP hydrolysis or transduction (Shyamala et al., 1991; Koronakis et al., 1995; Li et al., 1996; Qu et al., 1997; Schmees et al., 1999). Our studies provide supportive evidence for this hypothesis, in that ATP binding to the NBDs with the mutated signature sequence was not abolished.

Our results also support the hypothesis that the signature sequences play a role in ATPase activity. However, whereas previous studies suggested a preferential role for NBD2 in ATP hydrolysis, our studies suggest that NBD1 may also play a role, albeit a lesser one than NBD2. Thus NBD2 was preferentially photolabeled with 8-azido-[α-^{32}P]ATP under vanadate-trapping conditions (Fig. 6) as previously reported (Gao et al., 2000). However, NBD1 was also slightly photolabeled under the same conditions, and LTC4, GSH, AG-A, and VCR enhanced the labeling. Furthermore, in the presence of both GSH and AG-A, considerably enhanced trapping at NBD1 was observed. No labeling of either NBD with 8-azido-[α-^{32}P]ATP under vanadate-trapping conditions was observed when the G771D mutant half molecule was coexpressed with the wild-type COOH-proximal half-molecule.

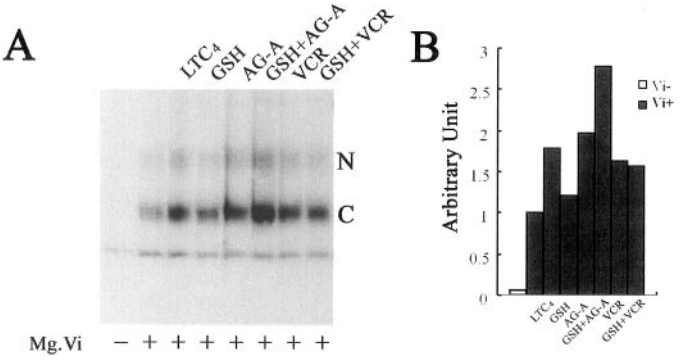

Fig. 6. Characterization of vanadate trapping of wt N+C coexpressed fragments of MRP1. Membrane vesicles (100 μg of protein) from insect cells coexpressing wt MRP1 N- and C- terminal half molecules were incubated with 5 μM 8-azido-[α-^{32}P]ATP and 1 mM sodium orthovanadate in the absence or presence of 1 μM LTC_4, 10 mM GSH, 100 μM AG-A, 10 mM GSH + 100 μM AG-A, 100 μM VCR, or 10 mM GSH + 100 μM VCR following trapping procedures as described under *Materials and Methods* (+) or without trapping (−) (A). Trapping was quantified by excision of the radioactive C-terminal halves of MRP1 and measurement of the bound ^{32}P (B).

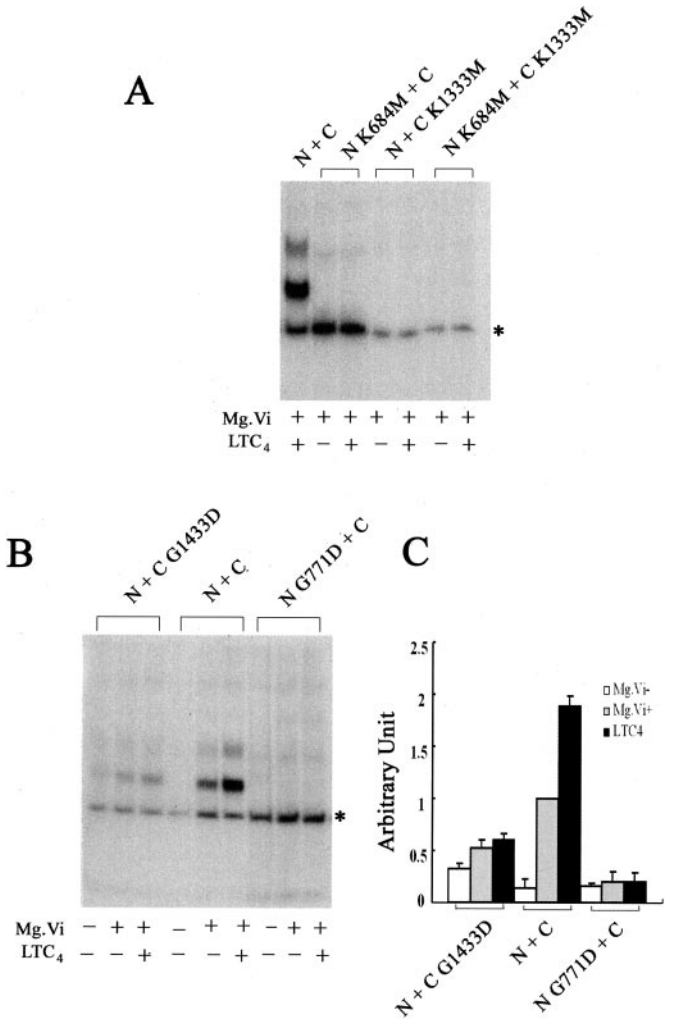

Fig. 7. Effect of NBD mutations on vanadate trapping of MRP1. Membrane vesicles (100 μg of protein) from insect cells coexpressing both N- and C-terminal wt or mutant fragments of MRP1 were incubated with 5 μM 8-azido-[α-^{32}P]ATP and 1 mM sodium orthovanadate in the absence and presence of 1 μM LTC_4, following trapping procedures as described under *Materials and Methods* (Mg.Vi+) or without trapping (Mg.Vi-) (A and B). Mutated Walker A motifs are shown in A; mutated signature sequences are shown in B. Quantification of the effect of mutations in the signature sequences on trapping is shown in C. Quantification was carried out as in Fig. 6. The data represent the mean ± S.E. from three separate experiments.

However, weak labeling of NBD2 and no trapping at NBD1 were observed when the G1433D mutant half molecule was coexpressed with the wild-type NH_2-proximal half-molecule. These findings suggest that each signature sequence is involved in the ATP hydrolysis of MRP1 and that mutation in either signature sequence affects the ATPase activity of both NBDs. It seems that mutants in the signature sequence have a more pronounced effect on nucleotide hydrolysis at the opposing NBD. This may suggest also that in MRP1, a dimmer interface between LSGGQ and Walker A motifs from the opposite NBDs may be implicated as the nucleotide hydrolysis site.

Mutation of the signature sequence in NBD1 (G771D) considerably inhibited the transport of LTC_4 by the reconstituted MRP1. This result also suggests the possibility that NBD1 has ATPase activity and that this activity is involved in MRP1 substrate transport, although ATPase activity of NBD2 seems to be more critical for the transport of LTC_4 than that of NBD1.

Our findings suggest that the intact signature sequence of NBD1 is indispensable for the ATPase function of NBD2, because mutation of the conserved glycine to aspartic acid in the signature sequence in NBD1 of MRP1 completely abolished the trapping of 8-azido-ATP at NBD2 of MRP1. These data are in contrast to data on SUR1, in which it was reported that the NBD1 of SUR1 had no ATPase activity and that mutation of the conserved serine to arginine of the signature sequence in NBD1 of SUR1 did not abolish ATP hydrolysis at NBD2 of SUR1 (Matsuo et al., 2002). It was suggested that the mutation of SUR1 reduced MgATP activation by impairing the transduction of nucleotide binding into channel activation. However, this study could not completely exclude the possibility that the mutation in SUR1 reduced hydrolysis at NBD2, because their method of detecting ATP hydrolysis was not quantitative (Matsuo et al., 2002). The dissimilarity between the data on SUR1 and our data on MRP1 may perhaps be attributed to the different nature of the two transporters. On the other hand, it may occur because different amino acids in the signature sequence were mutated, leading to different functional effects (Bakos et al., 1997).

The structural basis underlying the inhibition of ATPase activity by mutations in the signature sequence has not been completely elucidated, and various models have been proposed. One model suggests that the LSGGQ motif might contact the γ phosphate of ATP (Manavalan et al., 1995). In support of this model, mutations in the LSGGQ motif have been shown to inhibit ATP hydrolysis (Schmees et al., 1999; Loo et al., 2003). A second model proposes that dimerization of His P, the well studied NBD of the bacterial histidine permease, aligns the LSGGQ motif of one subunit with the Walker A motif of the second subunit, thereby creating two nucleotide-binding sites between these two sets of motifs along the dimer interface (Jones and George, 2002). This dimer architecture was observed in the structure of the catalytic domain of Rad 50 that is an ABC protein but belongs to a subfamily that functions in DNA repair rather than transport (Hopfner et al., 2000). It has also been shown that the signature sequences of P-glycoprotein are adjacent to the opposing Walker A site, suggesting that these sites might participate in forming the ATP-binding sites and are displaced upon ATP hydrolysis. This might induce a conformational change that could be the signal responsible for coupling ATP hydrolysis to drug transport by inducing conformational changes in the transmembrane segments (Loo et al., 2002). Our results are consistent with these models. However, we need to create some other mutations in signature sequences of MRP1 and perform similar experiments to confirm the important roles of the sequences in ATP hydrolysis. Further study is also required to elucidate whether the signature sequence has some role in the transmission of conformational changes in NBDs to transmembrane domains of MRP1.

This is the first report in which the function of signature sequences of MRP1 has been studied. Our data provide supportive evidence for previous studies but also strongly suggest that both signature sequences of MRP1 are involved in ATP hydrolysis and must be intact for ATP hydrolysis and substrate transport by MRP1.

References

Aleksandrov L, Mengos A, Chang X, Aleksandrov A, and Riordan JR (2001) Differential interactions of nucleotides at the two nucleotide binding domains of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem* **276:**12918–12923.

Bakos E, Klein I, Welker E, Szabo K, Muller M, Sarkadi B, and Varadi A (1997) Characterization of the human multidrug resistance protein containing mutations in the ATP-binding cassette signature region. *Biochem J* **323:**777–783.

Borths EL, Locher KP, Lee AT, and Rees DC (2002) The structure of Escherichia coli BtuF and binding to its cognate ATP binding cassette transporter. *Proc Natl Acad Sci USA* **99:**16642–16647.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.

Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, and Deeley RG (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science (Wash DC)* **258:**1650–1654.

Debenham PG, Kartner N, Siminovitch L, Riordan JR, and Ling V (1982) DNA-mediated transfer of multiple drug resistance and plasma membrane glycoprotein expression. *Mol Cell Biol* **2:**881–889.

Gao M, Cui HR, Loe DW, Grant CE, Almquist KC, Cole SP, and Deeley RG (2000) Comparison of the functional characteristics of the nucleotide binding domains of multidrug resistance protein 1. *J Biol Chem* **275:**13098–13108.

Hopfner KP, Karcher A, Shin DS, Craig L, Arthur LM, Carney JP, and Tainer JA (2000) Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. *Cell* **101:**789–800.

Hou YX, Cui L, Riordan JR, and Chang XB (2002) ATP binding to the first nucleotide-binding domain of multidrug resistance protein MRP1 increases binding and hydrolysis of ATP and trapping of ADP at the second domain. *J Biol Chem* **277:**5110–5119.

Jones PM and George AM (2002) Mechanism of ABC transporters: a molecular dynamics simulation of a well characterized nucleotide-binding subunit. *Proc Natl Acad Sci USA* **99:**12639–12644.

Koronakis E, Hughes C, Milisav I, and Koronakis V (1995) Protein exporter function and in vitro ATPase activity are correlated in ABC-domain mutants of HlyB. *Mol. Microbiol.* **16:**87–96.

Li C, Ramjeesingh M, Wang W, Garami E, Hewryk M, Lee D, Rommens JM, Galley K, and Bear CE (1996) ATPase activity of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem* **271:**28463–28468.

Locher KP, Lee AT, and Rees DC (2002) The E. coli BtuCD structure: a framework for ABC transporter architecture and mechanism. *Science (Wash DC)* **296:**1091–1098.

Loo TW, Bartlett MC, and Clarke DM (2002) The "LSGGQ" motif in each nucleotide-binding domain of human P-glycoprotein is adjacent to the opposing walker A sequence. *J Biol Chem* **277:**41303–41306.

Loo TW, Bartlett MC, and Clarke DM (2003) Drug binding in human P-glycoprotein causes conformational changes in both nucleotide-binding domains. *J Biol Chem* **278:**1575–1578.

Manavalan P, Dearborn DG, McPherson JM, and Smith AE (1995) Sequence homologies between nucleotide binding regions of CFTR and G-proteins suggest structural and functional similarities. *FEBS Lett* **366:**87–91.

Matsuo M, Kioka N, Amachi T, and Ueda K (1999) ATP binding properties of the nucleotide-binding folds of SUR1. *J Biol Chem* **274:**37479–37482.

Matsuo M, Tanabe K, Kioka N, Amachi T, and Ueda K (2000) Different binding properties and affinities for ATP and ADP among sulfonylurea receptor subtypes, SUR1, SUR2A and SUR2B. *J Biol Chem* **275:**28757–28763.

Matsuo M, Dabrowski M, Ueda K, and Ashcroft FM (2002) Mutations in the linker domain of NBD2 of SUR inhibit transduction but not nucleotide binding. *EMBO (Eur Mol Biol Organ) J* **21:**4250–4258.

Qu BH, Strickland EH, and Thomas PJ (1997) Localization and suppression of a kinetic defect in cystic fibrosis transmembrane conductance regulator folding. *J Biol Chem* **272:**15739–15744.

Ren XQ, Furukawa T, Aoki S, Nakajima T, Sumizawa T, Haraguchi M, Chen ZS,

Kobayashi M, and Akiyama S (2001) Glutathione-dependent binding of a photoaffinity analog of agosterol A to the C-terminal half of human multidrug resistance protein. *J Biol Chem* **276:**23197–23206.
Ren XQ, Furukawa T, Aoki S, Sumizawa T, Haraguchi M, Nakajima Y, Ikeda R, Kobayashi M, and Akiyama S (2002) A positively charged amino acid proximal to the C-terminus of TM17 of MRP1 is indispensable for GSH-dependent binding of substrates and for transport of LTC_4. *Biochemistry* **41:**14132–14140.
Ren XQ, Furukawa T, Chen ZS, Okumura H, Aoki S, Sumizawa T, Tani A, Komatsu M, Mei XD, and Akiyama S (2000) Functional comparison between YCF1 and MRP1exprssed in Sf21 insect cell. *Biochem Biophys Res Commun* **270:**608–615.
Schmees G, Stein A, Hunke S, Landmesser H, and Schneider E (1999) Functional consequences of mutations in the conserved 'signature sequence' of the ATP-binding-cassette protein MalK. *Eur J Biochem* **266:**420–430.
Shyamala V, Baichwal V, Beall E, and Ames GF (1991) Structure-function analysis of the histidine permease and comparison with cystic fibrosis mutations. *J Biol Chem* **266:**18714–18719.
Smith PC, Karpowich N, Millen L, Moody JE, Rosen J, Thomas PJ, and Hunt JF (2002) ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer. *Mol Cell* **10:**139–149.
Szabo K, Szakacs G, Hegeds T, and Sarkadi B (1999) Nucleotide occlusion in the human cystic fibrosis transmembrane conductance regulator. Different patterns in the two nucleotide binding domains. *J Biol Chem* **274:**12209–12212.
Verdon G, Albers SV, van Oosterwijk N, Dijkstra BW, Driessen AJ, and Thunnissen AM (2003) Formation of the productive ATP-Mg^{2+}-bound dimer of GlcV, an ABC-ATPase from *Sulfolobus solfataricus*. *J Mol Biol* **334:**255–267.

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