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Functional Interactions Between Nucleotide Binding Domains and Leukotriene C_4 Binding Sites of Multidrug Resistance Protein 1 (ABCC1)

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ABSTRACT

Multidrug resistance protein 1 (MRP1) is a member of the "C" branch of the ATP-binding cassette transporter superfamily. The NH_2-proximal nucleotide-binding domain (NBD1) of MRP1 differs functionally from its COOH-proximal domain (NBD2). NBD1 displays intrinsic high-affinity ATP binding and little ATPase activity. In contrast, ATP binding to NBD2 is strongly dependent on nucleotide binding by NBD1, and NBD2 is more hydrolytically active. We have demonstrated that occupancy of NBD2 by ATP or ADP markedly decreased substrate binding by MRP1. We have further explored the relationship between nucleotide and substrate binding by examining the effects of various ATP analogs and ADP trapping, as well as mutations in conserved functional elements in the NBDs, on the ability of MRP1 to bind the photoactivatable, high-affinity substrate cysteinyl leukotriene C_4 (LTC_4). Overall, the results support a model in which occupancy of both NBD1 and NBD2 by ATP results in the formation of a low-affinity conformation of the protein. However, nonhydrolyzable ATP analogs (β,γ-imidoadenosine 5′-triphosphate and adenylylmethylene diphosphonate) failed to substitute for ATP or adenosine 5′-*O*-(thiotriphosphate) (ATPγS) in decreasing LTC_4 photolabeling. Furthermore, mutations of the signature sequence in either NBD that had no apparent effect on azido-ATP binding abrogated the formation of a low-affinity substrate binding state in the presence of ATP or ATPγS. We suggest that the effect of these mutations, and possibly the failure of some ATP analogs to decrease LTC_4 binding, may be attributable to an inability to elicit a conformational change in the NBDs that involves interactions between the signature sequence and the γ-phosphate of the bound nucleotide.

Multidrug resistance protein (MRP) 1 confers resistance to a wide range of natural-product cytotoxic agents (Cole et al., 1992, 1994). The protein also transports structurally diverse conjugated organic anions, including the high-affinity physiological substrate cysteinyl leukotriene C_4 (LTC_4) (Leier et al., 1994). MRP1 is a member of the "C" branch of the ATP-binding cassette (ABC) transporter superfamily. Other C branch members include the cystic fibrosis transmembrane conductance regulator, the sulfonylurea receptors SUR1 and SUR2, and nine additional MRPs (Dean and Allikmets, 2001).

ABC proteins are generally composed of two hydrophilic nucleotide-binding domains (NBDs) located at the cytoplasmic surface of the membrane and two functionally linked hydrophobic membrane-spanning domains (MSDs), each of which typically has six transmembrane helices. However, a number of the C branch transporters, including MRP1, MRP2, MRP3, MRP6, and MRP7, as well as SUR1 and SUR2, are unusual in that they have an additional NH_2-terminal MSD that probably contains five transmembrane helices and has an extracellular NH_2 terminus (Hipfner et al., 1997; Kast and Gros, 1997). Furthermore, compared with many ABC transporters, the NBDs of the C branch proteins are relatively divergent, with some structural features that are characteristic of the ABCC proteins (Cole et al., 1992).

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ABBREVIATIONS: MRP, multidrug resistance protein; NBD, nucleotide binding domain; MSD, membrane-spanning domain; SUR, sulfonylurea receptor; ATPγS, adenosine 5′-*O*-(thiotriphosphate); AMP-PNP, β,γ-imidoadenosine 5′-triphosphate; AMP-PCP, adenylylmethylene diphosphonate; ABC, ATP-binding cassette; LTC_4, cysteinyl leukotriene C_4; SUR, sulfonylurea receptor; PAGE, polyacrylamide gel electrophoresis; P-gp, P-glycoprotein; mAb, monoclonal antibody; β-gus, β-glucuronidase.

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The NBDs of all ABC proteins contain three conserved sequence elements: the Walker A and B motifs, and the ABC signature sequence (LSGGQ) or C-motif. The X-ray crystal structures of NBDs of several ABC proteins have made major contributions to understanding the role of these motifs in ATP binding and hydrolysis. For example, the Walker A motif, or P-loop, seems to wrap around the phosphate chain of ATP with the nitrogens of the residues within this motif extensively hydrogen-bonding with the γ-phosphate of the bound nucleotide, whereas the Walker B motif contributes an aspartate residue that coordinates and stabilizes a magnesium ion, which is indispensable for ATP hydrolysis (Hung et al., 1998; Diederichs et al., 2000; Gaudet and Wiley, 2001). Crystal structures of the soluble ABC protein Rad50 (Hopfner and Tainer, 2003) and bacterial transporters such as vcMsbA (Chang, 2003) and BtuCD (Locher et al., 2002) indicate that Walker A and Walker B motifs of one NBD cooperate with the C motif of the other NBD, effectively sandwiching a nucleotide between the two NBDs. This observation has done much to explain the obligatory cooperativity between the two NBDs during ATP hydrolysis and substrate transport by proteins such as P-glycoprotein (P-gp) and MRP1 (Senior and Bhagat, 1998; Urbatsch et al., 1998; Gao et al., 2000; Hou et al., 2000, 2002; Payen et al., 2003).

The catalytic cycle of ABC transporters is believed to involve alternating conformational changes between high-affinity substrate-binding and low-affinity substrate-releasing states. In the original model proposed for P-gp, the alternating ATP binding/hydrolysis and subsequent release of ADP by either NBD drives a single cycle through high- to low- to high-affinity states, with the resultant transport of one molecule of substrate (Senior et al., 1995). More recently, it has been proposed that one ATP hydrolysis event results in conversion from the high- to low-affinity binding state and substrate transport, whereas hydrolysis of a second ATP is required to reset the protein in a high-affinity substrate-binding conformation (Sauna and Ambudkar, 2001). Whether each NBD has a distinct role in the transport process has not been firmly established. Studies of P-gp in which positions of NBDs were exchanged suggest that the location of the NBD in the protein may influence its ability to bind and hydrolyze nucleotide (Beaudet and Gros, 1995; Hrycyna et al., 1999).

We and others have shown that in MRP1, the NBDs behave very differently with respect to both ATP binding and hydrolysis. High-affinity binding of azido-ATP to NBD1 is readily demonstrable, whereas under hydrolytic conditions in the presence of vanadate, ADP is trapped predominantly by NBD2 (Gao et al., 2000; Hou et al., 2000). Furthermore, binding of azido-ATP and the trapping of ADP by NBD2 requires that NBD1 be able to bind and possibly to hydrolyze ATP. In contrast, binding of ATP by NBD1 remains readily detectable when NBD2 is inactivated by mutations that eliminate ATP binding or ATPase activity (Gao et al., 2000; Hou et al., 2000). Studies with soluble forms of the NBDs support the ability of NBD1 to bind ATP with relatively high affinity in the absence of NBD2 (Gao et al., 2000). Mutation of the Walker A motifs in each NBD also has different effects on transport activity (Gao et al., 2000; Hou et al., 2000). Mutation of the conserved Walker A lysine residue in NBD1 only partially inactivates the protein, whereas the comparable mutation in NBD2 essentially eliminates transport activity (Gao et al., 2000; Hou et al., 2000).

We have demonstrated that the identity of the acidic residue COOH proximal to the conserved aspartic acid of the Walker B motif makes a critical contribution to the functional differences between the two NBDs (Payen et al., 2003). In NBD1 of MRP1, this residue is aspartic acid, and in NBD2 it is glutamic acid, which is the residue found in the majority of ABC NBDs. Interconversion of these two residues profoundly affects the ability of the mutated NBDs to bind, hydrolyze, and release nucleotides (Payen et al., 2003). A D793E mutation in NBD1 enhanced its hydrolytic capacity but caused occlusion of the resultant ADP by the mutant NBD1 in the absence of vanadate. The mutation also markedly decreased LTC_4 transport activity and resulted in an inability to shift from a high- to low-affinity LTC_4 binding state in the presence of ATP (Payen et al., 2003). The reciprocal E1455D mutation of NBD2 increased the affinity of NBD2 for both azido-ATP and -ADP, resulting in prolonged binding of both nucleotides. In the presence of ATP, this mutation effectively locked the protein in a low-affinity substrate-binding state. From these and other studies, we have proposed that transition from a high-affinity substrate-binding state to a low-affinity substrate-releasing state involves a conformational change that occurs after occupancy of NBD2 by ATP and persists as long as NBD2 is occupied by ADP. Furthermore, this transition apparently cannot occur when NBD1 is occupied by ADP rather than by ATP (Payen et al., 2003).

In this study, we investigated whether ATP hydrolysis by either NBD is essential for the conversion of MRP1 from a high- to low-affinity substrate-binding state. To do so, we examined the effects of various ATP analogs and the trapping of ADP in the presence of beryllium fluoride on LTC_4 binding. We also introduced mutations that have different effects on nucleotide binding and hydrolysis into each NBD and examined their influence on binding and transport of LTC_4. The results support a model in which the transition from high- to low-affinity substrate binding requires occupancy of both NBDs by ATP. However, we also found that the nonhydrolyzable ATP analogs, AMP-PNP and AMP-PCP, in contrast to ATPγS, are unable to substitute for ATP in driving the transition from high- to low-affinity binding. In addition, we show that mutations of the signature C sequence that have no apparent effect on ATP binding nevertheless result in loss of the ability to shift from high- to low-affinity states in the presence of ATP plus vanadate or of ATPγS. This observation suggests that the C signature mutations affect transduction of conformational changes that occur in the NBDs after ATP binding and which are required for the transition from high- to low-affinity substrate-binding states to occur.

Materials and Methods

Materials. 8-Azido-[α-^{32}P]ATP, 8-azido-[γ-^{32}P]N_3 ATP were purchased from Affinity Labeling Technologies, Inc. (Lexington, KY) (specific activity between 5 and 20 Ci/mmol). Beryllium, orthovanadate, ATP, AMP-PNP, AMP-PCP, ADP, and ATPγS compounds were from Sigma-Aldrich (St. Louis, MO). [^{3}H]LTC_4 was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA) (specific activity, 182 Ci mmol^{-1}).

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MRP1 Mutations. The pFBDual-MRP1 1–932/932-1531 construct (pFBDual-halves, pFBDual-Asp123/Asp45) was cloned into pFASTBAC Dual (Invitrogen, Carlsbad, CA). This construct, encoding the NH_2-$_{MSD1-MSD2-NBD1}$ and COOH-$_{MSD3-NBD2}$ proximal half-molecules of MRP1 has been described previously (Gao et al., 1996, 2000). The amino acid substitutions within NBDs of MRP1 were generated by site-directed mutagenesis using the Clontech Transformer Kit (BD Biosciences Clontech, Mississauga, ON, Canada). The templates used for site-directed mutagenesis, pGEM-NBD1 and pGEM-NBD2, were described previously (Gao et al., 2000). The forward primers for creating K684R, K684E, K1333R, and K1333E mutations of Walker A motifs were 5′-GGCTGCGGAAGGTCGTCCCTGC-3′, 5′-GGGCTGCGGAGAGTCGTCCCTGC-3′, 5′-GGGAGCTGGGAGGTCGTCCCTGA-3′, and 5′-GGGAGCTGGGGAGTCGTCCCTGA-3′, respectively. The forward primers for the G771A and G1433A mutations of signature sequences were 5′-CCTGTCTGGGGCCCAGAAGCAGC-3′ and 5′-CCTCAGTGTCGCGCAGCGCCAG-3′, respectively. The forward primers for the D792N and D1454N mutations of the Walker B motifs were 5′-CATTTACCTCTTCAATGATCCCCTC-3′ and 5′-ATCCTTGTGTTGAATGAGGCCACG-3′, respectively. The presence of the mutation and the fidelity of the sequence of the MRP1 coding region were confirmed by dideoxy sequencing (ACGT Corporation, Toronto, ON, Canada). The Bsu36I/SphI fragments bearing mutations at NBD1 were isolated from pGEM-NBD1 and were used to replace the same region in pFBDual-halves to create pFBDual-halves/MutNBD1. The EcoRI/KpnI fragments with mutations at NBD2 were isolated from pGEM-NBD2 and were used to replace the equivalent region in pBS-Asp45 to generate pBS-Asp45/MutNBD2. Then, pBS-Asp45/MutNBD2 was digested with NcoI and KpnI and the NcoI/KpnI fragment was used to replace the equivalent region of pFBDual-Asp45 to give pFBDual-Asp45/MutNBD2. Finally, the SalI/XbaI fragment of pFBDual-halves was isolated and cloned into pFBDual-Asp45/MutNBD2, which had been digested with the same enzymes, to generate pFBDual-halves/MutNBD2.

Viral Infection and Membrane Vesicle Preparation. Viral infection of Sf21 cells was carried out as described previously (Gao et al., 1996, 2000). To generate membrane vesicles, Sf21 cells were disrupted by nitrogen cavitation, and vesicles were subsequently isolated by discontinuous sucrose-gradient density centrifugation (Leier et al., 1994; Loe et al., 1996).

Immunoblotting and Quantification of MRP1 Polypeptides. Membrane vesicle proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using 7 to 15% gradient gels. Proteins were transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA) using 25 mM Tris-base, 192 mM glycine, and 20% methanol buffer. MRP1 polypeptides were detected using an enhanced chemiluminescence kit (Amersham Biosciences Inc., Quebec, Canada) and the murine mAb MRPm6 and the rat mAb MRPr1 (Pierce, Rockford, IL) (Flens et al., 1994; Hipfner et al., 1998). The relative levels of various mutant MRP1 polypeptides were estimated by comparison with vesicles containing wt MRP1. Densitometry of film images was performed using a ChemiImager 4000 (Alpha Innotech, San Leandro, CA). The relative protein expression levels were calculated by dividing the integrated densitometry values obtained for 0.5, 1, and 2 μg of total membrane protein from infected cells expressing the mutant proteins by the integrated densitometry values obtained for the comparable amounts of total membrane proteins from infected cells expressing the wt protein.

Transport of [^{3}H]LTC$_4$ into Insect Membrane Vesicles. Uptake of [^{3}H]LTC$_4$ (50 nM, 182 Ci/mmol; PerkinElmer) into membrane vesicles was measured at 23°C in the presence of ATP (4 mM) or AMP (4 mM) using a rapid filtration technique as described previously (Loe et al., 1996).

Photoaffinity Labeling of MRP1 with [^{3}H]LTC$_4$. Unless otherwise indicated in the figure legend, insect membrane vesicles (50 μg of total protein in 20 μl) were incubated with [^{3}H]LTC$_4$ (0.13 μCi, 200 nM) at room temperature for 20 min. The vesicle samples were then frozen in liquid nitrogen (1 min) and subjected to UV cross-linking at 312 nm (1 min) in a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). This process was repeated 10 times with each sample (Qian et al., 2001). Radiolabeled vesicles were then analyzed on SDS-PAGE (7–15%). Proteins were fixed by 25% isopropanol, 65% water, and 10% acetic acid for 30 min. Gels were then soaked in Amplify (Amersham Biosciences) for 30 min and dried at 80°C for 2 h before autoradiography using Kodak BioMax MS films (Eastman Kodak, Rochester, NY) (Qian et al., 2001).

Photolabeling of NBD1 and NBD2 of MRP1 with 8-Azido-[^{32}P]ATP. 8-Azido-[γ-^{32}P]ATP or 8-azido-[α-^{32}P]ATP photoaffinity labeling was performed as described previously (Gao et al., 2000). Membrane vesicles (20 μg of total protein) were resuspended in transport buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 0.02% Na_3N) containing 5 mM $MgCl_2$ and 5 μM 8-azido-[^{32}P]ATP. After 5 min at 4°C in a 96-well plate, the membranes were irradiated for 7 min on ice in a Stratalinker UV cross-linker (λ = 312 nm; Stratagene). After the addition of 300 μl of ice-cold buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, and 5 mM $MgCl_2$), the membranes were centrifuged at 14,000 rpm for 15 min at 4°C. A second wash was performed, and the pellets were resuspended in 14 μl of ice-cold buffer. After the addition of Laemmli buffer (4×) containing dithiothreitol (100 mM final concentration), vesicle proteins were separated by SDS-PAGE using 7 to 15% gradient gels. After drying for 2 h at 80°C, gels were subjected to autoradiography either using a PhosphorImager (Amersham Biosciences) and/or by exposure to Kodak BioMax MS films.

Vanadate and Beryllium Fluoride-Induced Trapping of 8-Azido-[α-^{32}P]ADP by MRP1. Membrane vesicles (20 μg of protein) were resuspended in transport buffer containing 5 mM $MgCl_2$ and 15 μM 8-azido-[α-^{32}P]ATP. The 15-min incubation at 37°C was performed in the presence or absence of 1 mM vanadate or 200 μM beryllium fluoride. The reaction was started by the addition of 8-azido-[α-^{32}P]ATP and stopped by transfer on ice and addition of ice-cold buffer as described previously. Unreacted nucleotides were then removed (2×) by the addition of 300 μl of ice-cold buffer followed by centrifugation. Pellets were resuspended in 14 μl of ice-cold buffer, and vesicle membranes were irradiated for 7 min on ice in a Stratalinker UV cross-linker (λ = 312 nm) as described previously (Gao et al., 2000). After the addition of Laemmli buffer (4×) containing dithiothreitol (100 mM final concentration), membrane vesicles were separated by gradient SDS-PAGE (7–15%). After drying for 2 h at 80°C, gels were processed as described above.

Results

Effect of Nucleotide Analogs on Photolabeling of MRP1 by [^{3}H]LTC$_4$. We have shown that LTC$_4$ photolabels MRP1 predominantly in MSD2 and to a lesser extent in MSD3 (Gao et al., 1998; Qian et al., 2001). The extent of photolabeling, particularly of the site in MSD2, is also moderately attenuated in the presence of ATP and is strongly attenuated in the presence of ATP plus vanadate (Qian et al., 2001; Payen et al., 2003). From these and more recent studies with mutant proteins, we proposed that it is the occupancy of NBD2 by either ATP or ADP that maintains MRP1 in a low-affinity state. To further investigate this proposal, we have examined the effect of the nonhydrolyzable ATP analogs AMP-PNP and AMP-PCP, as well as beryllium-induced ADP trapping, on the binding of LTC$_4$.

At 23°C, in the presence of ATP or ATP plus vanadate, LTC$_4$ photolabeling by wt MRP1 was decreased by approximately 55 and 75%, respectively (Fig. 1A). As observed previously, ATPγS, a poorly hydrolysable analog of ATP, also caused a moderate (43%) decrease in LTC$_4$ binding similar to that of ATP in the absence of vanadate (Fig. 1A). We also

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examined the effect of beryllium fluoride on LTC_4 photolabeling, because ADP trapping in the presence of beryllium fluoride results in an NBD conformation closely resembling that of an ATP binding ground state rather than the posthydrolytic transition state formed in the presence of orthovanadate (Fisher et al., 1995; Smith and Rayment, 1996; Sankaran et al., 1997). The combination of ATP and beryllium fluoride reduced LTC_4 labeling by approximately 80% and thus was at least as effective as ATP plus vanadate (Fig. 1A). These results, combined with those obtained with ATPγS, strongly support the suggestion that the decrease in LTC_4 binding occurs upon ATP binding. However, because ATPγS can be slowly hydrolyzed, we examined the ability of two completely nonhydrolyzable analogs, AMP-PNP and AMP-PCP, to shift MRP1 from a high- to low-affinity state. Neither analog decreased labeling by LTC_4 (Fig. 1A). In view of this result, we compared the ability of ATPγS and AMP-PNP to compete with azido-ATP for binding to the NBDs of MRP1 (Fig. 1B). ATPγS competed for photolabeling of both NBDs by 8-azido-[γ-^{32}P]ATP (5 μM), with competition being detectable at the lowest concentration used (5 μM). In contrast, AMP-PNP was at least 20-fold less effective, particularly at competing for binding to NBD2, where no competition was evident at concentrations of up to 100 μM (Fig. 1B). However, at a concentration of 1 mM, like that used in the LTC_4-labeling studies, AMP-PNP decreased 8-azido-[γ-^{32}P]ATP photolabeling of both NBDs by more than 90%. Thus, the lack of an effect on labeling of MRP1 by LTC_4 cannot simply be attributed to a failure of the analog to bind to the protein at the concentration used.

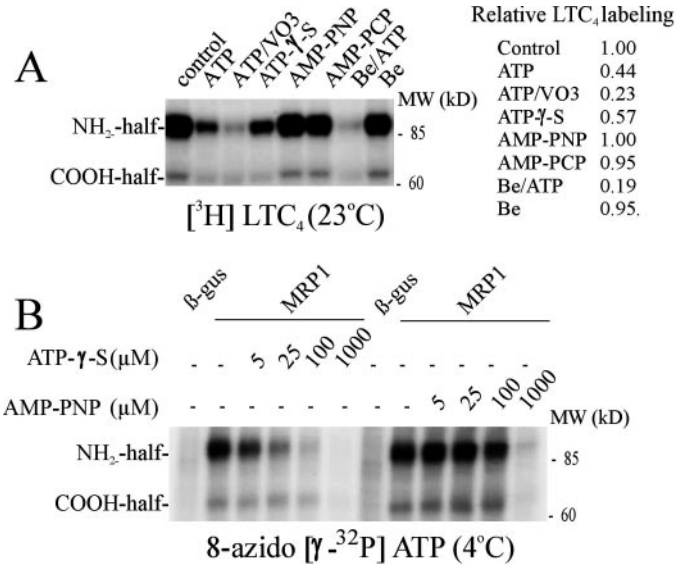

Fig. 1. Photolabeling of MRP1 with LTC_4 and 8-azido-[γ-^{32}P]ATP. A, effect of ATP analogs and ADP trapping on [^{3}H]LTC_4 photolabeling of wt MRP1. Membrane vesicles containing wt MRP1 (50 μg of total protein) were incubated in transport buffer alone at 23°C for 20 min or transport buffer containing one of the following: ATPγS (4 mM), AMP-PNP (4 mM), AMP-PCP (4 mM), ATP (1 mM), or ATP (1 mM), plus either vanadate (1 mM) or BeF (200 μM), before the addition of [^{3}H]LTC_4 (200 nM, 0.13 μCi). The [^{3}H]LTC_4 photolabeling was performed as described under *Materials and Methods*. Similar results were obtained in at least three additional independent experiments. The relative levels of photolabeling by LTC_4 as determined by densitometry are indicated. B, effect of ATP analogs on photolabeling of MRP1 by 8-azido-[γ-^{32}P]ATP. Membrane vesicles (20 μg of total protein) were incubated with 8-azido-[γ-^{32}P]ATP (5 μM) for 5 min at 4°C in transport buffer in the absence (−) or presence of various concentrations (5 μM to 1 mM) of ATPγS or AMP-PNP. The samples were photo–cross-linked, and unincorporated nucleotides were removed as described under *Materials and Methods*. Similar results were obtained in at least two independent experiments. The positions of the labeled NH_2- and COOH-halves of MRP1 containing NBD1 and NBD2, respectively, are indicated.

Effect of Conservative and Nonconservative Mutations of the Walker A Lysine Residue in NBD1 and NBD2 on LTC_4 Transport. Mutation of the conserved Walker A Lys684 in NBD1 (Fig. 2) to methionine substantially reduces but does not eliminate MRP1 transport activity, whereas the comparable mutation in NBD2, K1333M, essentially inactivates the protein (Gao et al., 2000; Hou et al., 2000). Despite the retention of ~30% of wt LTC_4 transport activity by the K684M mutant protein, we were unable to detect photolabeling of either NBD with 8-azido-ATP (Gao et al., 2000). Comparable mutations in other ABC transporters uniformly eliminate ATPase activity at the mutated NBD but have variable effects on ATP binding depending on the nature of the substitution (Shyamala et al., 1991; Schneider et al., 1994; Urbatsch et al., 1998). Therefore, we compared the effect of introducing conservative lysine to arginine and opposite-charge lysine-to-glutamic acid mutations at positions 684 and 1333 on both transport activity and ATP binding. Mutant half-molecules containing these substitutions were expressed with the appropriate wt half-molecule using dual-expression vectors.

Densitometry of immunoblots of vesicle proteins indicated that levels of the K684R, K684E, K1333R, and K1333E MRP1 mutants ranged from 30 to 60% those of wt MRP1 (Fig. 3A). Therefore, ATP-dependent transport of LTC_4 was normalized to the expression level of wt MRP1. It is noteworthy that the K684R substitution in NBD1 decreased ATP-dependent LTC_4 uptake by only 40%, whereas the K1333R mutation in NBD2 reduced transport by approximately 80% (Fig. 2B). In contrast, the nonconservative Walker A lysine-to-glutamic acid mutation in either NBD decreased ATP-dependent LTC_4 uptake by >90% (Fig. 3B).

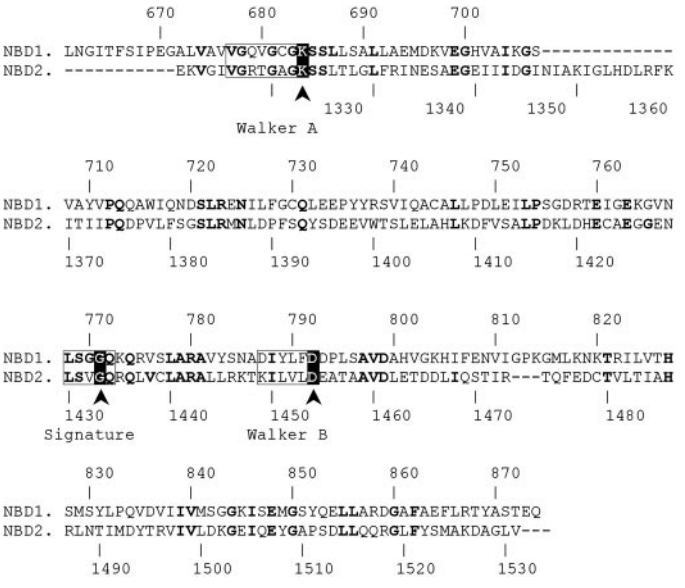

Fig. 2. Alignment of the amino acid sequences of NBD1 and NBD2 of MRP1. The sequence alignment was generated using ClustalW. Amino acids that are identical in both NBDs are shown in boldface type, and conserved motifs are boxed. Amino acids mutated in the studies described are shown in white on a black background and are indicated by arrowheads.

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Effect of Mutations of Walker A Lysine on ATP Binding and Vanadate-Induced ADP Trapping. The membrane vesicles used in the transport assays described above were also used for binding and photolabeling studies with 8-azido-[γ-^{32}P]ATP (Fig. 3C). For binding experiments, the levels of wt MRP1, which were 2- to 3-fold higher than in membranes containing the mutant proteins, were adjusted by dilution with β-gus control vesicles so that close to comparable amounts of total protein and MRP1 half-molecules (wt or mutant) were subjected to photolabeling (Fig. 3, C and D). The K684E mutation markedly decreased binding at both the mutant NBD1 and the wt NBD2, as observed previously

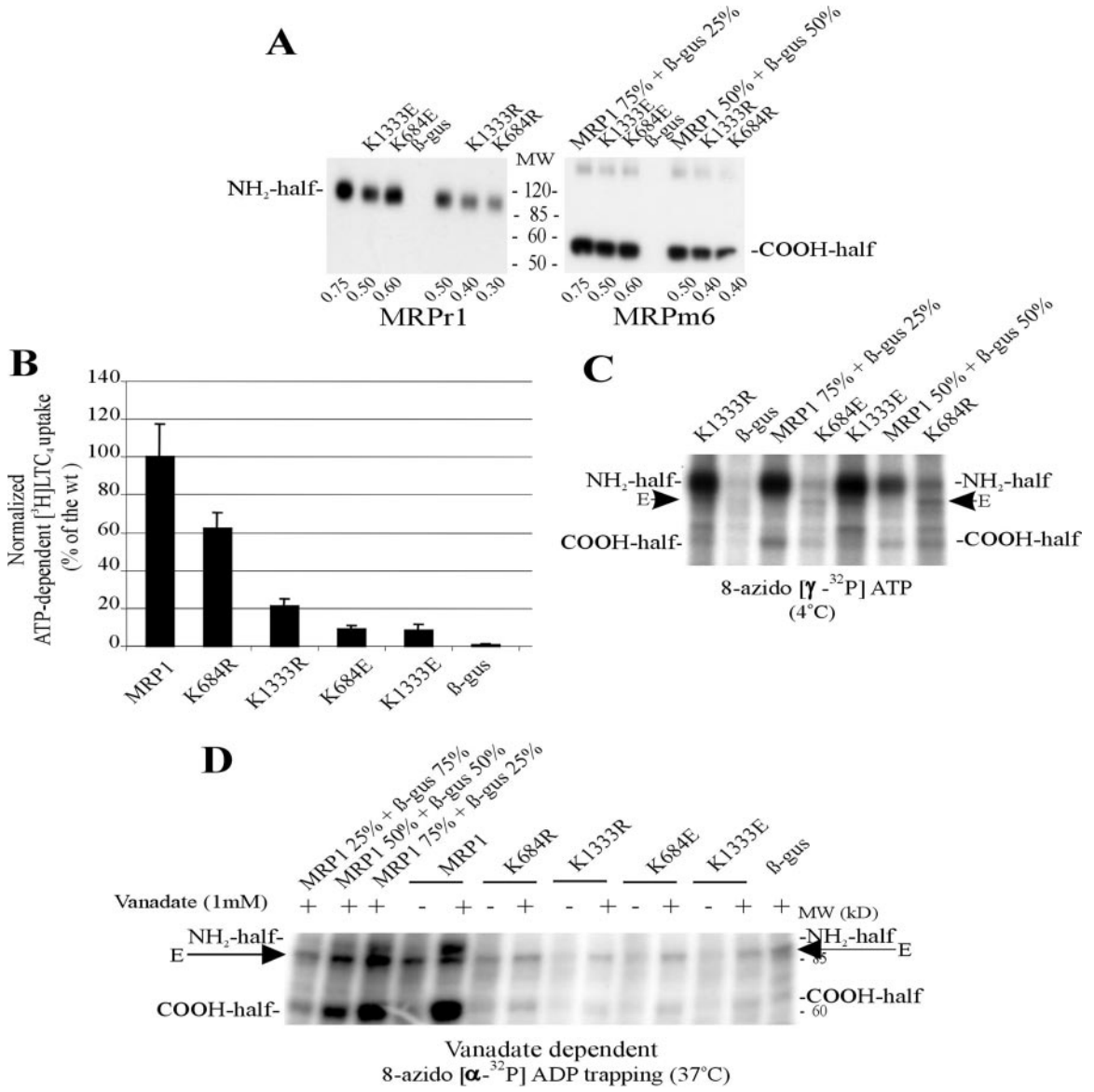

Fig. 3. Effects of conservative and nonconservative mutations of Walker A Lys residues. A, expression levels of wt and mutant half-molecules. Membrane vesicles (1 μg of total protein) prepared from Sf21 cells expressing a combination of a wt and mutant half-molecule containing a K684E, K684R, K1333E, or K1333R mutation were separated by SDS-PAGE on gradient gels and transferred to Immobilon-P membranes. Membrane vesicles expressing both wt type halves of MRP1 were diluted with control membranes from cells expressing β-gus, as described under *Materials and Methods* and indicated in the figure, and 1 μg of total protein was subjected to SDS-PAGE. Left, detection of the NH_2-proximal half-molecule by rat MRP1-specific mAb MRPr1. Right, detection of COOH-proximal half-molecule by murine MRP1-specific mAb MRPm6. Minor amounts of high-molecular-weight species present in COOH-proximal half-molecule blot are oligomers of the half-molecules. The relative expression levels of wt and mutant proteins evaluated by densitometry are indicated in the figure. B, effect of K684E, K684R, K1333E, and K1333R mutations on ATP-dependent LTC_4 transport activity. Membrane vesicles (2 μg of total protein) containing either both wt or a combination of wt and mutant MRP1 half-molecules, together with control vesicles from cells expressing β-gus vector, were assayed for ATP-dependent LTC_4 transport activity as described. Results shown are means ± S.D. of triplicate determinations in a single experiment. Similar results were obtained in at least three additional independent experiments. C, comparison of nucleotide binding by wt and mutant MRP1 half-molecules. Membrane vesicles (20 μg of total protein) containing either both wt or a combination of wt and mutant MRP1 half-molecules were incubated with 8-azido-[γ-^{32}P]ATP (5 μM) for 5 min at 4°C in transport buffer before photo–cross-linking and removal of unincorporated nucleotides, as described. Similar results were obtained in at least three independent experiments. The positions of NH_2- and COOH-halves of MRP1 and labeled endogenous proteins (E▶) are indicated. D, vanadate-dependent ADP trapping by wt and mutant MRP1 half-molecules. Samples of membrane vesicles used in C were incubated in transport buffer containing 8-azido-[α-^{32}P]ATP (15 μM) for 15 min at 37°C in the absence (−) or presence (+) of 1 mM vanadate. Unbound nucleotides were removed before cross-linking and analysis by SDS-PAGE as described. The positions of NH_2- and COOH-halves of MRP1 and endogenous labeled proteins (E ▶) are indicated. Similar results were obtained in at least two independent experiments.

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with the methionine mutation (Gao et al., 2000). The conservative K684R mutation also decreased photolabeling of both NBDs but to a lesser extent than either the aspartic acid or methionine mutations (Fig. 3C). In contrast, both the K1333R and the K1333E mutations essentially eliminated binding at NBD2 but had little or no effect on the labeling of NBD1 (Fig. 3C).

Under vanadate trapping conditions, the majority of ADP is trapped at NBD2 of the wt protein (Fig. 3D). Both the conservative and nonconservative Lys684 mutations markedly reduced the trapping at the associated wt NBD2 and no difference between them was apparent. Likewise, both the K1333R and K1333E mutations eliminated trapping by the mutant NBD2 (Fig. 3D). Thus, despite the difference in the extent to which the conservative and nonconservative mutations compromise LTC_4 transport, all mutations essentially eliminate the ability to detect vanadate-dependent ADP trapping by NBD2.

Effect of Mutations of Walker A Lys on LTC_4 Photolabeling. As an alternative approach to assessing the effect of the Walker A mutations on the function of MRP1, we examined LTC_4 binding in the presence and absence of ATP, ATP/vanadate, and ATPγS (Fig. 4). This approach enabled much higher nucleotide concentrations to be used than is feasible for nucleotide-photolabeling studies with ^{32}P-labeled derivatives. In the absence of nucleotides, the mutant proteins displayed an LTC_4-labeling profile very similar to that of wt protein. However, in the presence of ATP, ATP/vanadate, and ATPγS, none of the Walker A Lys mutants displayed the reduction in LTC_4 labeling observed with wt MRP1 (Fig. 4).

Effect of Mutations of Walker B Asp on LTC_4 Transport, ATP Photolabeling, Vanadate-Induced ADP Trapping, and LTC_4 Binding. Substitution of the conserved aspartic acid residue in the Walker B motif (Fig. 2) has been shown in several ABC proteins to result in the loss of both ATP binding and hydrolysis (Shyamala et al., 1991; Ueda et al., 1997; Hrycyna et al., 1999). This residue in both NBDs was converted to polar asparagine, and the mutant half-molecules were coexpressed with the appropriate wt partner (Fig. 5). The D792N mutation diminished transport activity by approximately 65%, whereas the D1454N mutation essentially inactivated the protein (Fig. 5B). As observed with the NBD1 Walker A mutations, the D792N mutation drastically decreased binding by NBD1 and, to a lesser extent, binding by NBD2. In contrast, the D1454N mutation eliminated photolabeling of only NBD2 and had no effect on photolabeling of NBD1 (Fig. 5C). The D792N mutation also strongly decreased ADP trapping by both NBD1 and NBD2, although the D1454N mutation eliminated trapping by NBD2 but only modestly decreased trapping by NBD1 (Fig. 6B). Despite the partial retention of vanadate-dependent trapping at NBD2 of the D792N mutant, we were unable to detect an ATP/vanadate-dependent decrease in LTC_4 binding with either mutant (Fig. 6C).

Effect of Mutation of the Invariant Glycine in the ABC Signature Sequences of NBD1 and NBD2. The invariant glycine at the fourth position of the ABC signature sequence in each of the NBDs was mutated to alanine to minimize changes in the bulk and chemical characteristics of the amino acid (Fig. 2). The G771A and G1433A mutants were expressed at 90 and 50%, respectively, of the level of wt MRP1. The levels of ATP-dependent LTC_4 transport activity and the MRP1 content of proteins samples used for nucleotide photolabeling studies were normalized to that of wt MRP1 as described above (Fig. 7A).

In contrast to the results obtained with the Walker A and B mutants, the NBD1 ABC signature mutation G771A eliminated LTC_4 transport, whereas the NBD2 G1433A mutant retained approximately 30% of the activity of the wt protein (Fig. 7B). In addition, unlike the Walker A and B mutations, neither of the NBD1 or NBD2 signature mutations decreased the binding and photolabeling of the protein by 8-azido-[γ-^{32}P]ATP (Fig. 7C). Under vanadate-induced trapping conditions, both of the G771A and G1433A mutations markedly decreased the trapping of ADP at NBD2 but had relatively little effect on the low level of trapping typically observed at NBD1 (Fig. 7D). However, like the Walker A and B mutations, the signature sequence mutations eliminated the decrease in LTC_4 photolabeling observed in the presence of ATPγS and ATP/vanadate (Fig. 8).

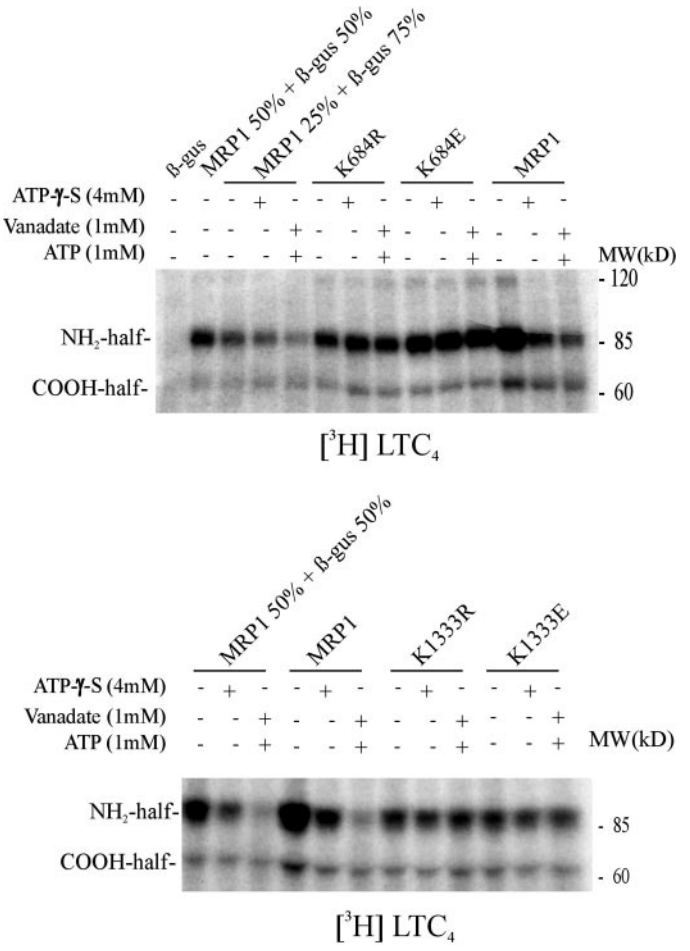

Fig. 4. Effect of conservative and nonconservative mutations of Walker A lysine residues on [^3H]LTC_4 photolabeling in the presence of either ATP plus vanadate or ATPγS. Membrane vesicles (50 μg of total protein) containing wt and the K684R, K684E, K1333R, and K1333E mutant MRP1 half-molecules were incubated in transport buffer at 23°C for 20 min in the absence or presence of ATPγS (4 mM) or ATP (1 mM) plus vanadate (1 mM) before the addition of [^3H]LTC_4 (200 nM, 0.13 μCi). [^3H]LTC_4 photolabeling was performed as described under *Materials and Methods*. Similar results were obtained in four additional independent experiments.

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Discussion

Crystal structures of ABC NBDs indicate that the generally conserved glutamic acid residue following the Walker B motif interacts with bound Mg^{2+} and a water molecule that are essential for cleavage of the $\beta\gamma$-phosphodiester bond of ATP (Moody et al., 2002; Smith et al., 2002; Verdon et al., 2003). Although the COOH-proximal NBDs of ABCC proteins contain the conserved glutamic acid, the NH_2-proximal NBDs do not. In MRP1 NBD1, the corresponding residue is aspartic acid and in other ABCC proteins is an uncharged residue. We have shown recently that the lack of a glutamic acid residue at this location in NBD1 is a major contributor to differences in functional characteristics of the two NBDs of ABCC proteins (Payen et al., 2003). A glutamic acid-to-aspartic acid mutation in NBD2 drastically decreases ATP hydrolysis and release of both ATP and ADP (Payen et al., 2003). Because this mutation strongly potentiates the decrease in LTC_4 photolabeling observed in the presence of either ATP or ATPγS, we proposed that MRP1 exists in a low-affinity substrate-binding state when NBD2 is occupied by either ATP or ADP (Payen et al., 2003). That fact that beryllium fluoride and orthovanadate are equally effective in potentiating the shift to a low-affinity LTC_4-binding state strongly supports our suggestion that the decrease in substrate affinity occurs upon binding of ATP to NBD2 and persists in the transition state after cleavage of the γ-phosphate (Fig. 1A).

To confirm that ATP hydrolysis by NBD2 was not required to shift the protein to a low-affinity state, we used two nonhydrolyzable analogs, AMP-PNP and AMP-PCP. However, neither AMP-PNP nor AMP-PCP caused a detectable decrease in photolabeling of MRP1 with LTC_4. Although this might suggest that ATP hydrolysis is required for the decrease in LTC_4 binding to occur, the extent to which these analogs fully mimic ATP seems to be transporter-dependent. AMP-PNP has been reported to decrease affinity of Chinese hamster P-gp for vinblastine (Martin et al., 2001), but this analog failed to decrease binding of [^{125}I]iodoarylazidoprazosine by the human protein (Sauna and Ambudkar, 2000). Likewise, AMP-PNP does not promote homodimerization of the bacterial ABC NBDs MJ0796 and MJ1267 (Moody et al., 2002), nor does it elicit an ATP-dependent, SecA-coupled conformational change in SecYEG, the bacterial complex involved in preprotein extrusion. In this case, only one AMP-PNP binding site could be detected, suggesting that the NBD dimer formed an abnormal interface that did not generate a second binding site for the ortholog (Tziatzios et al., 2004).

AMP-PNP and AMP-PCP contain a nitrogen and carbon atom, respectively, between the β- and γ-phosphate moieties. In contrast, the $\beta\gamma$-phosphodiester bond of ATP is unchanged in ATPγS. This may be critical for the formation of the correct dimer interface between NBDs of some ABC proteins. ATPγS clearly competes much more effectively than AMP-PNP for the binding of azido-ATP by MRP1. ATPγS also stimulates ADP trapping at NBD2 of MRP1 and thus mimics ATP binding by NBD1, but analogs such as AMP-PNP do not (Hou et al., 2002). Therefore, the failure of AMP-PNP to decrease the affinity of MRP1 for LTC_4 could be attributable to the fact that it fails to accurately simulate ATP when interacting with one or both binding sites on the NBD dimer.

To further characterize the influence of nucleotide interactions on changes in substrate binding by MRP1, we created mutations in conserved elements in each NBD that had different effects on transport activity, ATP binding, and ADP trapping. Any substitution of the invariant Walker A lysine in ABC NBDs generally abolishes ATP hydrolysis (Urbatsch

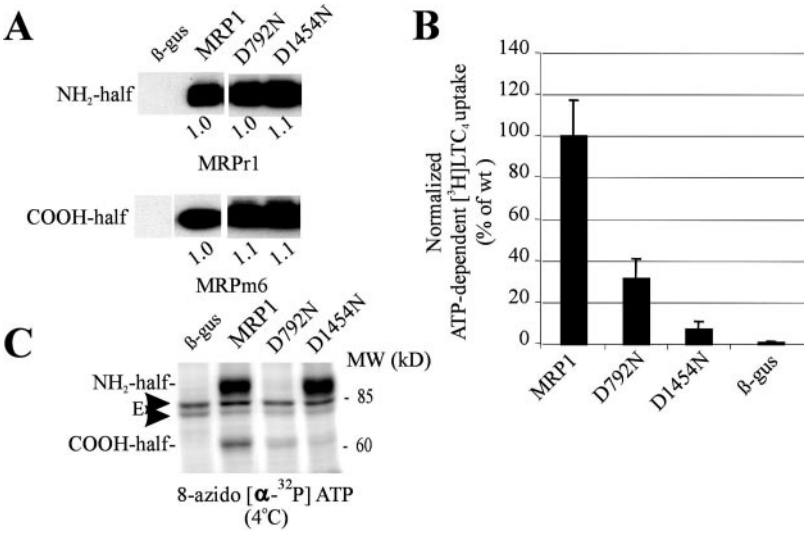

Fig. 5. Effect of Walker B aspartic acid mutations on transport activity and nucleotide binding. A, expression levels of wt and mutant half-molecules of MRP1. Vesicle proteins were separated by SDS-PAGE and immunoblotted as described above. The relative expression levels of wt and mutant half-molecules were evaluated by densitometry and are indicated on the figure. B, effect of D792N and D1454N mutations on ATP-dependent LTC_4 transport activity. Membrane vesicles (2 μg) containing wt and the D792N and D1454N mutant MRP1 half-molecules or control β-gus were assayed for ATP-dependent LTC_4 transport activity by incubation in transport buffer containing [^{3}H]LTC_4 (50 nM, 0.13 μCi) at 23°C for 2 min in the presence and absence of ATP (4 mM) as described under *Materials and Methods*. Results shown are means ± S.D. of triplicate determinations in a single experiment. Similar results were obtained in three additional independent experiments. C, effect of D792N and D1454N mutations on photolabeling with 8-azido-[α-^{32}P]ATP. Membrane vesicles (20 μg) were incubated in transport buffer containing 8-azido-[α-^{32}P]ATP (5 μM) for 5 min at 4°C. Samples were photo–cross-linked, unincorporated nucleotides were removed, and membrane proteins were separated by SDS-PAGE as described under *Materials and Methods*. Similar results were obtained in three independent experiments. The positions of the labeled NH_2- and COOH-halves of MRP1 are shown, and endogenous proteins that are also labeled are indicated (E▶).

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et al., 1998; Gao et al., 2000; Hou et al., 2000), but the effect on nucleotide binding depends on whether the mutation is conservative or nonconservative (Shyamala et al., 1991; Schneider et al., 1994). In several ABC NBDs, substitutions of the conserved Walker B aspartic acid residue that eliminate the negatively charged side chain have been shown to abolish both ATP hydrolysis and to strongly decrease nucleotide binding (Shyamala et al., 1991; Ueda et al., 1997; Hrycyna et al., 1999). Consistent with the retention of partial activity by NBD1 Walker A lysine mutations being attributable to a greater or lesser ability to bind ATP, LTC_4 transport was decreased only 40% by a conservative arginine mutation, whereas the opposite-charge glutamic acid mutation decreased activity by more than 90%. In comparison, the lysine-to-methionine mutation in NBD1 described previously resulted in a 70% decrease in LTC_4 transport. These results support the suggestion that some level of transport activity can be retained, providing that NBD1 is capable of binding but not necessarily hydrolyzing ATP. In contrast, both conservative and nonconservative mutations in NBD2 decreased transport by at least 80%. Despite differences in the level of transport activity, conservative and nonconservative NBD1 Walker A mutations drastically reduced ATP binding and vanadate-dependent trapping of ADP by NBD2. In contrast, the comparable NBD2 mutations had little effect on ATP binding by the coexpressed wt NBD1, although they eliminated azido-ATP binding and ADP trapping by the mutant NBD2. The effect of the MRP1 Walker B D792N and D1454N mutations on ATP binding and ADP trapping was similar to that of the Walker A lysine mutations; the level of transport activity of the NBD1 aspartic acid-to-asparagine mutation was comparable with that of the Walker A lysine-to-methionine mutation (Gao et al., 2000).

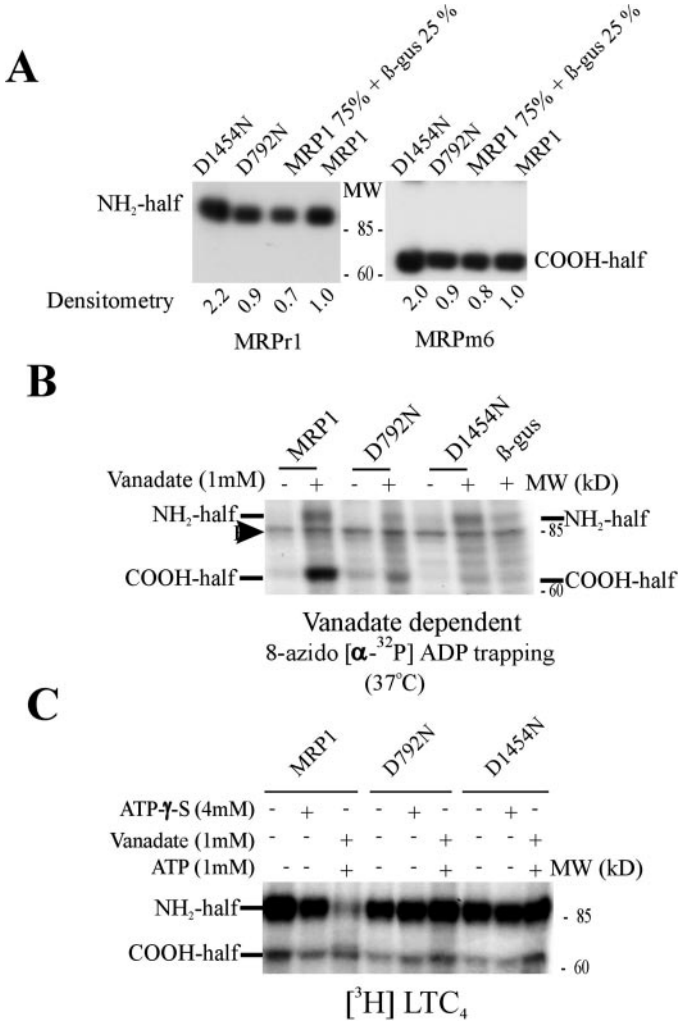

Fig. 6. Effect of Walker B aspartic acid mutations on vanadate-induced ADP trapping and [^{3}H]LTC_4 photolabeling in the presence and absence of nucleotide. A, expression levels of wt and mutant half-molecules of MRP1. Vesicle proteins were separated by SDS-PAGE and immunoblotted as described above. The relative expression levels of wt and mutant proteins were evaluated by densitometry and are indicated in the figure. Densitometry indicated that the level of the D1454N mutant protein in the vesicle preparation used was approximately 2-fold higher than in control vesicles (A). Therefore, the MRP1 Asp1454 vesicle preparation was adjusted by dilution with control vesicles before use in photolabeling studies, as described under *Materials and Methods*. B, effect of D792N and D1454N mutations on vanadate-dependent nucleotide trapping. Membrane vesicles (20 μg) were incubated for 15 min at 37°C in transport buffer containing 8-azido-[α-^{32}P]ATP (15 μM) in the absence (−) or presence (+) of 1 mM vanadate. After removal of unbound nucleotide, samples were photo–cross-linked and analyzed by SDS-PAGE. Similar results were obtained in two independent experiments. The positions of the labeled NH_2- and COOH-halves of MRP1 and an endogenous protein (E▶) that is also labeled are indicated. C, effect of D792N and D1454N mutations on LTC4 photolabeling in the presence or absence of nucleotide. Membrane vesicles (50 μg of total protein) containing wt and the D792N and D1454N mutant MRP1 half-molecules were incubated in transport buffer at 23°C for 20 min in the absence or presence of ATPγS (4 mM) or ATP (1 mM) plus vanadate (1 mM) before the addition of [^{3}H]LTC_4 (200 nM, 0.13 μCi). [^{3}H]LTC_4 photolabeling was performed as described under *Materials and Methods*. Similar results were obtained in four additional independent experiments.

In contrast to Walker A and B mutations, substitution of conserved residues in the signature sequences of a number of ABC proteins has been found to have little effect on ATP binding (Shyamala et al., 1991; Schmees et al., 1999; Tombline et al., 2004). Recent studies of MRP1 in which conserved signature glycine residues were mutated to glutamic acid indicated that ATP binding by the mutant proteins was apparently normal. However, the proteins were inactive and failed to trap ADP in the presence of vanadate, suggesting that the glycine residues are essential for the formation of a posthydrolytic complex (Szentpetery et al., 2004). Therefore, we investigated whether such mutant proteins differed from the Walker A and B mutants in their ability to shift from a high- to low-affinity binding state in the presence of ATP or ATPγS.

Despite the more conservative glycine-alanine, as opposed

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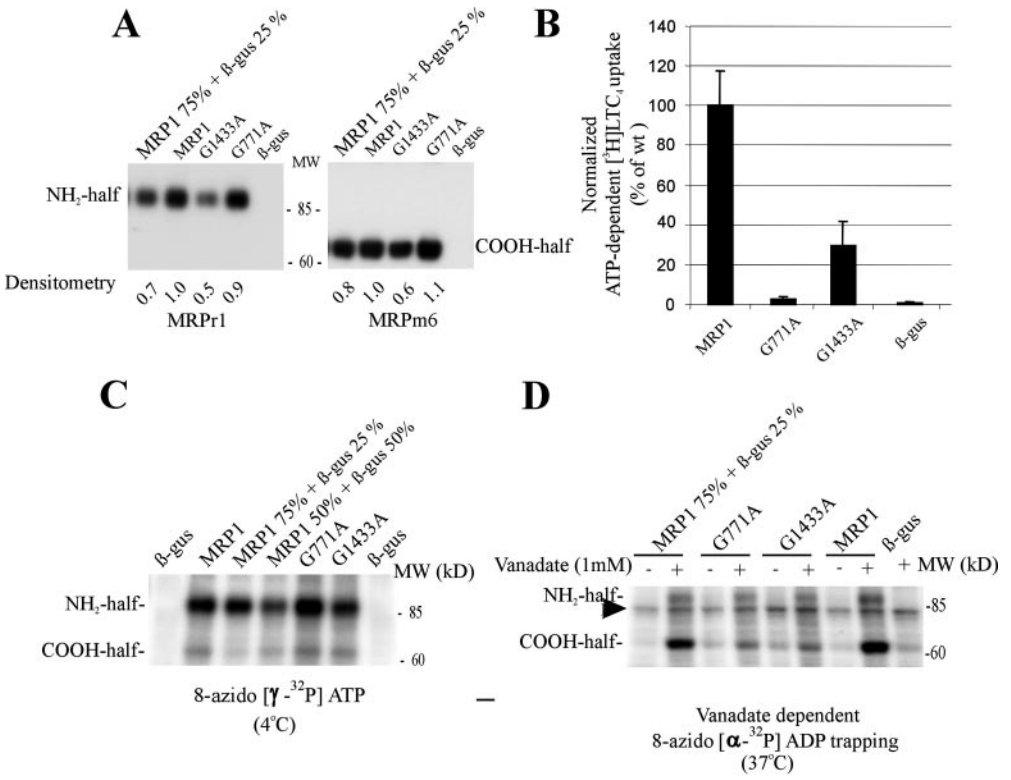

Fig. 7. Effect of mutating the conserved glycine residue in the signature motif on ATP-dependent transport activity, nucleotide binding, and vanadate-dependent ADP trapping. A, expression levels of wt and G771A and G1433A mutant MRP1 half-molecules. Vesicle proteins were separated by SDS-PAGE and immunoblotted as described above. The relative expression levels of wt and mutant proteins were evaluated by densitometry and are indicated in the figure. B, effect of G771A and G1433A mutations on ATP-dependent LTC_4 transport activity. Membrane vesicles (2 μg) containing wt and the G771A and G1433A mutant MRP1 half-molecules or control β-gus were assayed for ATP-dependent LTC_4 transport activity by incubation in transport buffer containing [^{3}H]LTC_4 (50 nM, 0.13 μCi) at 23°C for 2 min in the presence and absence of ATP (4 mM) as described under *Materials and Methods*. Results shown are means ± S.D. of triplicate determinations in a single experiment. Similar results were obtained in three additional independent experiments. C, effect of G771A and G1433A mutations on photolabeling with 8-azido-[γ-^{32}P]ATP. Membrane vesicles (20 μg) were incubated in transport buffer containing 8-azido-[γ-^{32}P]ATP (5 μM) for 5 min at 4°C. Samples were photo–cross-linked, unincorporated nucleotides were removed, and membrane proteins were separated by SDS-PAGE as described. Similar results were obtained in three independent experiments. The positions of the labeled NH_2- and COOH-halves of MRP1 are shown, and endogenous proteins that are also labeled are indicated (E▶). D, effect of G771A and G1433A mutations on vanadate-dependent nucleotide trapping. Membrane vesicles (20 μg) were incubated for 15 min at 37°C in transport buffer containing 8-azido-[α-^{32}P]ATP (15 μM) in the absence (−) or presence (+) of 1 mM vanadate. After removal of unbound nucleotide, samples were photo–cross-linked and analyzed by SDS-PAGE. Similar results were obtained in two independent experiments. The positions of the labeled NH_2- and COOH-halves of MRP1 and that of an endogenous protein that is also labeled are indicated (E▶).

to glycine-glutamic acid, substitutions used in the present study, the NBD1 mutation essentially inactivated the protein, whereas the mutation in NBD2 decreased LTC_4 transport activity by approximately 70%. Thus the relative effect of signature sequence mutations NBD1 and NBD2 on transport is the converse of the Walker A and B mutations, as might be expected if the signature sequence contributes to ATP hydrolysis by the apposed NBD. As observed with the signature glycine to glutamic acid mutations in MRP1 (Szentpetery et al., 2004), the glycine-alanine mutations had little or no effect on binding of ATP by either the NBD containing the mutation or the apposing wt NBD. However, both NBD mutations strongly decreased but did not eliminate vanadate-dependent trapping at NBD2, whereas the low level of trapping observed at NBD1 in the wt protein was relatively unaffected. Although nucleotide binding at NBD1 and NBD2 and vanadate-dependent trapping at NBD1 were apparently unaffected, both signature glycine mutations eliminated the shift to a low-affinity substrate-binding state, not only in the presence of ATP and vanadate, but also in the presence of ATPγS. These observations suggest that the formation of a closed dimer as a result of ATP binding to both NBD1 and NBD2 may not be sufficient to mediate the conformational shift from high to low affinity. They also raise the possibility that other prehydrolytic conformational

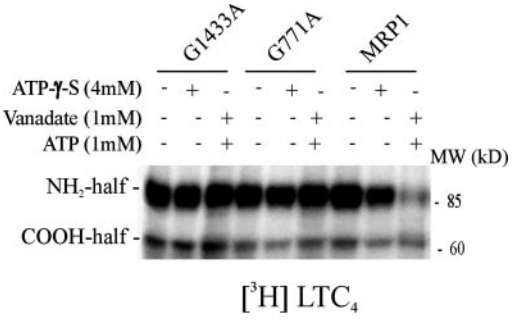

Fig. 8. Effect of signature glycine mutations on [^{3}H]LTC_4 photolabeling in the presence and absence of nucleotide. Membrane vesicles (50 μg of total protein) containing wt and the G771A and G1433A mutant MRP1 half-molecules were incubated in transport buffer at 23°C for 20 min in the absence or presence of ATPγS (4 mM) or ATP (1 mM) plus vanadate (1 mM) before the addition of [^{3}H]LTC_4 (200 nM, 0.13 μCi). [^{3}H]LTC_4 photolabeling was performed as described under *Materials and Methods*. Similar results were obtained in four additional independent experiments.

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changes involving interaction of the signature glycine residue with the γ-phosphate of ATP are required for the shift in affinity for substrate to occur. If so, the failure of ATP analogs such as AMP-PNP to induce changes in substrate binding may also be related to an inability to establish the necessary interactions with highly conserved residues in the signature sequence.

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