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Sequences in the Nonconsensus Nucleotide-binding Domain of ABCG5/ABCG8 Required for Sterol Transport*

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Jin Wang[‡], Nick Grishin[§], Lisa Kinch[§], Jonathan C. Cohen[‡¶], Helen H. Hobbs[‡§¶1], and Xiao-Song Xie[‡¶2]

From the [‡]Eugene McDermott Center for Human Growth and Development, the [§]Howard Hughes Medical Institute, and the [¶]Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas, 75390-8591

ATP-binding cassette transporters ABCG5 (G5) and ABCG8 (G8) form a heterodimer that transports cholesterol and plant sterols from hepatocytes into bile. Mutations that inactivate G5 or G8 cause hypercholesterolemia and premature atherosclerosis. We showed previously that the two nucleotide-binding domains (NBDs) in the heterodimer are not functionally equivalent; sterol transport is abolished by mutations in the consensus residues of NBD2 but not of NBD1. Here, we examined the structural requirements of NBD1 for sterol transport. Substitutions of the D-loop aspartate and Q-loop glutamine in either NBD did not impair sterol transport. The H-loop histidine of NBD2 (but not NBD1) was required for sterol transport. Exchange of the signature motifs between the NBDs did not interfere with sterol transport, whereas swapping the Walker A, Walker B, and signature motifs together resulted in failure to transport sterols. Selected substitutions within NBD1 altered substrate specificity: transport of plant sterols by the heterodimer was preserved, whereas transport of cholesterol was abolished. In summary, these data indicate that NBD1, although not required for ATP hydrolysis, is essential for normal function of G5G8 in sterol transport. Both the position and structural integrity of NBD2 are essential for sterol transport activity.

ATP-binding cassette (ABC)[3] transporters hydrolyze ATP to transport a wide variety of substrates across membranes (1). The members of the family share a common architecture that comprises two nucleotide-binding domains (NBDs), which mediate ATP binding and hydrolysis, and two transmembrane domains (TMDs), each of which typically contains six transmembrane helixes that together form a conduit through which the substrate is translocated across the membrane (2). The sequences of the TMDs are widely divergent among family members. In contrast, the NBDs share several characteristic motifs and are much more strongly conserved.

Canonical sequences found in the NBDs of ABC transporters include the Walker A motif (G*X*X*G*X*G*KST, where *X* is any amino acid), which contacts the α- and β-phosphates of ATP; the Walker B motif ($\varphi\varphi\varphi\varphi$DE, where φ is a hydrophobic amino acid), which positions a hydrolytic water molecule; the D-loop (SALD), which may contribute to the dimer interface; and a five-residue C-loop (alternatively called the signature sequence; LSGGQ) that packs against the γ-phosphate and closes off the active site (2). The NBDs also share conserved functional residues, including an A-loop aromatic residue that stacks with the adenine base of the bound nucleotide, a switch motif histidine that contacts the γ-phosphate, and a Q-loop glutamine that plays a role in mediating communication between the NBDs and TMDs (2).

Structural studies have indicated that the NBDs form dimers arranged in a head-to-tail orientation such that the Walker A and Walker B motifs of one NBD pair with the C-loop of the second NBD (2–7). In several ABC transporters, the two NBDs differ in ATP catalytic activity. These transporters invariably contain one NBD with canonical Walker A, Walker B, and C-loop sequences, whereas the other NBD may contain one or more degenerate motifs. At least 21 ABC transporters have an NBD with non-canonical motifs (8), and functional non-equivalence between the NBDs of some of these transporters has been well documented. For example, in the cystic fibrosis transmembrane conductance regulator (ABCC7), NBD2 binds and rapidly hydrolyzes ATP in a magnesium-dependent manner, whereas NBD1 binds ATP even in the absence of a divalent cation and hydrolyzes ATP very slowly with addition of Mg^{2+} (9). The NBDs of transporter associated with antigen processing (ABCB2/ABCB3) also differ in ATPase activity (10): the catalytic activity of NBD2 is essential for driving peptide transport, whereas inactivation of NBD1 results in only decreased transport activity.

In some ABC transporters, the two NBDs are encoded by separate transcripts, each of which contributes an NBD and a TMD to the mature complex. The hemitransporters ABCG5 (G5) and ABCG8 (G8) form heterodimers in the endoplasmic reticulum and are transported to the apical membranes of enterocytes and hepatocytes, where they limit the absorption and promote the excretion of neutral sterols (11–14). Mutations in either G5 or G8 cause the autosomal recessive disease sitosterolemia, which is characterized by accumulation of neutral sterols and premature coronary artery disease (15). We showed previously that G5G8 requires only one active ATPase to power sterol transport (3). Mutations in the Walker A or B motifs of G5 or in the C-loop of G8 disrupt sterol transport by purified reconstituted G5G8 *in vitro* and *in vivo* (3). In contrast, mutations in the Walker A and B motifs of G8 and the C-loop of G5 do not interfere with sterol transport.

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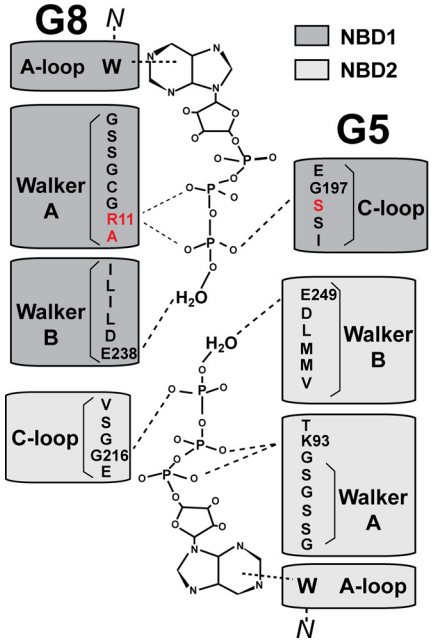
[1] To whom correspondence may be addressed: McDermott Center of Human Growth and Development, MC8591, University of Texas Southwestern Medical Center, Dallas, TX 75390-8591. E-mail: helen.hobbs@utsouthwestern.edu.

[2] To whom correspondence may be addressed: McDermott Center of Human Growth and Development MC8591, University of Texas Southwestern Medical Center, Dallas, TX 75390-8591. E-mail: xiao-song.xie@utsouthwestern.edu.

[3] The abbreviations used are: ABC, ATP-binding cassette; NBD, nucleotide-binding domain; TMD, transmembrane domain; G5, ABCG5; G8, ABCG8.



	Walker A	Q-loop	C-loop	Walker B	D-loop	Switch	
	G8	G8	G5	G8	G5	G8	
Human	GSSGCG**RA**	VRQHN	IS**T**GE	ILILDE	TGLD	SLHQP	
Mouse	GSSGCG**RA**	VRQHD	IS**S**GE	ILILDE	TGLD	SLHQP	
Cow	GSSGCG**RA**	VRQHD	IS**H**GE	ILILDE	TGLD	SIHQP	**NBD1**
Opossum	GSSGYGKS	VRQHD	IS**N**GE	ILILDE	TGLD	SLHQP	
Chicken	GSTAGGKT	VRQDD	ISGGE	ILILDE	TGLD	SLHQP	
Frog	GNTGCGKT	VRQDD	IS**S**GE	ILILDE	TGLD	SIHQP	
Zebrafish	GSSGCGKT	VRQDD	ISGGE	ILILDE	TGLD	SVHQP	
	G5	G5	G8	G5	G8	G5	
Human	GSSGSGKT	VLQSD	LSGGE	VMLFDE	SGLD	TIHQP	
Mouse	GSSGSGKT	VLQSD	VSGGE	VMMLDE	SGLD	TIHQP	
Cow	GSSGSGKT	VLQSD	VSGGE	VMLFDE	SGLD	TIHQP	**NBD2**
Opossum	GSSGSGKT	ALQNE	VSGGE	VMLFDE	SGLD	TIHQP	
Chicken	GNSGSGKT	VPQND	ISGGE	VMLLDE	SGLD	TIHQP	
Frog	GNSGSGKT	VLQHD	VSGGE	IILLDE	SGLD	SIHQP	
Zebrafish	GNSGSGKT	VLQSD	VSGGE	VILLDE	SGLD	TIHQP	
CONSENSUS	GXXGXGKS/T	XXQXX	φSGGQ	φφφφD	S/TXLD	XXHXX	

FIGURE 1. **Evolutionary conservation of key functional motifs in the NBDs of G5 and G8.** The schematic shows mouse NBD1 (*dark gray*) and NBD2 (*light gray*) bound to ATP. The alignment shows the Walker A, Q-loop, C-loop, Walker B, D-loop, and switch (H-loop) motifs from seven species. Residues that do not conform to the consensus sequence are shown in *red*.

We previously arbitrarily referred to the active NBD of G5G8 as NBD1 and the inactive NBD as NBD2 (3, 16, 17). In this work, we have switched the numbering of the NBDs to be consistent with terminology used to describe asymmetric full ABC transporters where the inactive ATPase is N-terminal to the active ATPase and is therefore designated NBD1 (9). To investigate the determinants and the consequences of functional asymmetry in the G5G8 NBDs, we used an *in vivo* sterol transport assay. The assay takes advantage of the fact that mice lacking G5 or G8 cannot secrete sterols into bile (11) and that biliary sterol transport can be restored in these animals by using recombinant adenoviruses to coexpress G5 and G8 in the liver.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Mutations were introduced into G5 or G8 cDNAs using QuikChange™ II site-directed mutagenesis kits (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The sequences of the oligonucleotides used to make the mutant constructs are available on request. The presence of the desired mutation and the integrity of each construct were verified by DNA sequencing.

Adenovirus-mediated Expression of G5 and G8 in Mice and in Cultured Cells—Recombinant adenoviruses expressing WT or mutant G5 and G8 were generated using a commercial system (AdenoVator, Qbiogene). WT mice (C57BL/6J, The Jackson Laboratory, Bar Harbor, ME) and *G5G8*$^{-/-}$ mice (11) maintained on a regular chow diet (Harlan Teklad, Madison, WI) were injected with recombinant adenoviruses (5×10^{12} particles/kg) via the tail vein. After 72 h, mice were fasted for 4 h, anesthetized with halothane, and killed by exsanguination. Livers were removed, and liver membrane vesicles were prepared as described previously (17). Bile samples were collected, and biliary neutral sterol levels were measured using gas chromatography/mass spectroscopy (18).

Cultured rat hepatoma cells (CRL-1601) were maintained in DMEM (glucose, 1 g/liter) containing 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were infected with recombinant adenoviruses encoding WT or mutant G5 and/or G8 as described (13). After 60 h, the cells were harvested, and the plasma membrane-enriched fraction was prepared (3). Protein concentrations of the membrane fractions were determined using a colorimetric assay (DC protein assay kit, Bio-Rad), and aliquots of the membrane preparations were stored at −80 °C.

SDS-PAGE and Immunoblot Analysis—Membrane proteins were subjected to SDS-PAGE and Western blotting (16) using rabbit polyclonal antibodies against mouse G5 and G8, respectively (11, 12) and an anti-G8 monoclonal antibody (1B10A5) raised against a recombinant peptide corresponding to amino acids 1–350 of G8 (14).

RESULTS

Canonical Amino Acids of the Q- and D-loops of NBD1 and NBD2, although Highly Conserved, Are Not Required for Sterol Transport—Alignment of the protein sequences of G5 and G8 from several animal species revealed that the canonical Walker A, Walker B, and signature motifs of NBD2 are highly conserved, whereas the Walker A motif and the signature motif of NBD1 are degenerate in higher mammals. Residues corresponding to the Q-loop glutamine, the D-loop aspartate, and the switch-loop histidine (Fig. 1) are conserved in both NBDs.

To determine whether the Q-, D-, and switch-loop residues of NBD1 and NBD2 are required for G5G8 function, we generated a series of recombinant adenoviruses expressing mutant forms of G5 and G8 in which each canonical residue was replaced with alanine. Each mutant protein was then expressed in mice together with its WT partner. The levels of protein and proportions of the mature G5G8 complex were comparable in

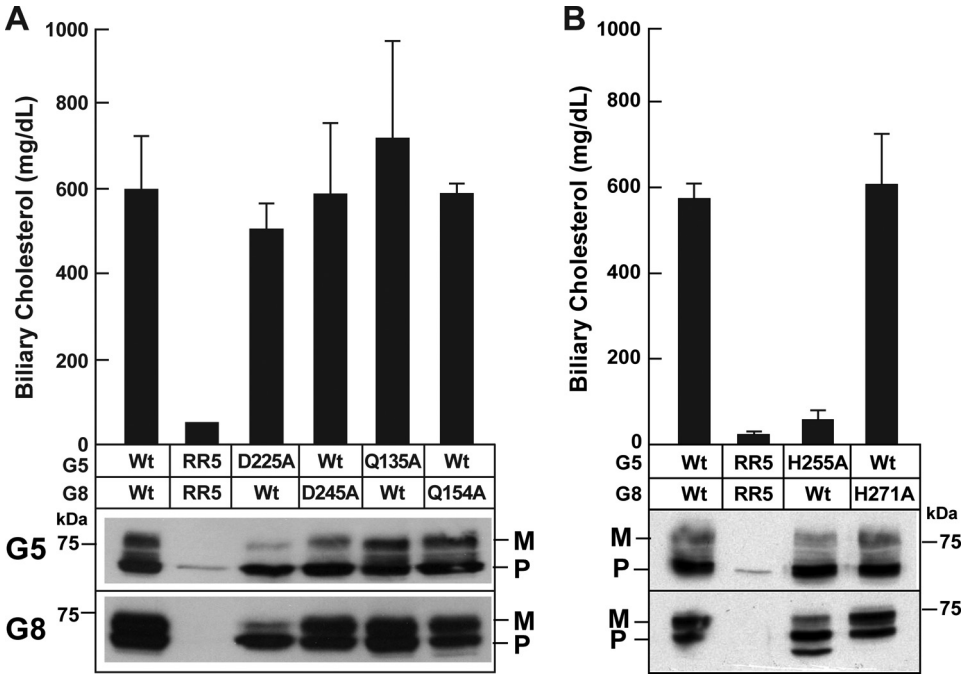


FIGURE 2. **Functional impact of substituting the D-loop aspartate, Q-loop glutamine, and H-loop histidine in G5 or G8 with alanine on sterol transport *in vivo*.** *A*, recombinant adenoviruses expressing G5 and G8 containing alanine substitutions in the D-loop aspartate and Q-loop glutamine were expressed in the livers of wild-type mice (four mice per group). After 3 days, the biliary cholesterol levels were measured using gas chromatography/mass spectroscopy as described under "Experimental Procedures." *B*, parallel experiments were performed in *G5G8*$^{-/-}$ mice using recombinant adenoviruses expressing G5 and G8 containing alanine substitutions in the H-loop (switch). Immunoblot analysis of hepatic membrane proteins was performed as described under "Experimental Procedures." The precursor (*P*) and mature (*M*) glycosylated forms of G5 and G8 are shown. All experiments were repeated twice, and the results were similar.

the livers of the five groups of mice, except for lower levels of expression in mice expressing G5-D225A together with WT G8 (Fig. 2*A*). Introduction of recombinant viruses expressing WT G5 and G8 into WT mice caused a 10-fold increase in biliary cholesterol. Expression of G5 and G8 with mutations in the D-loop aspartate (D225A and D245A, respectively) and Q-loop glutamine (Q135A and Q154A, respectively) led to comparable increases in biliary cholesterol. Thus, the Q- and D-loops of NBD1 and NBD2, although highly conserved, are not required for G5G8 function.

The Switch-loop Histidine in G5 but Not G8 Is Required for Sterol Transport—We injected the NBD1 and NBD2 switch-loop histidine mutants together with their WT G8 counterparts into *G5G8*$^{-/-}$ mice. Mutation of the switch-loop histidine in NBD1 did not impair sterol transport (Fig. 2*B*). In contrast, mutation of the switch-loop histidine of NBD2 (G5) to alanine prevented the G5G8-mediated increase in biliary cholesterol levels (Fig. 2*B*).

Thus, these results, together with our previous findings (3), indicate that none of the canonical ABC motifs of NBD1 are required for sterol transport. The H-loop of NBD2 is essential for G5G8 function, whereas the Q-loop and the D-loop of this domain do not play a significant role in G5G8-mediated sterol transport.

Mutations in the A-loops of G5 and G8 Do Not Interfere with Sterol Transport of G5G8—In other ABC transporters, an aromatic residue (tyrosine, tryptophan, or phenylalanine) 25–30 residues upstream of the Walker A motif engages the adenyl group of ATP. Substitution of the aromatic residue at this position inactivates transport in other ABC transporters (19, 20). To investigate the role of the A-loops, we generated constructs

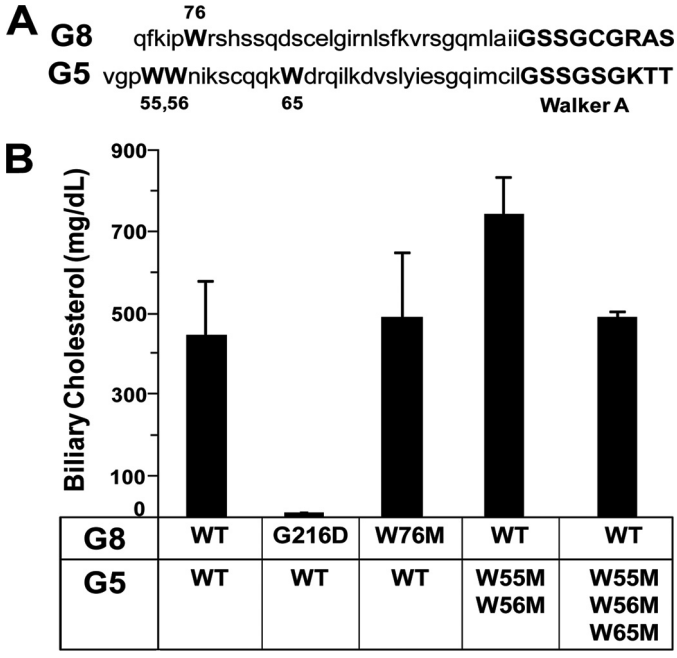


FIGURE 3. **The aromatic amino acids in the putative "A-loops" of G5 and G8 are not required for sterol transport.** Recombinant adenoviruses expressing G5 and G8 containing substitutions in the tryptophan residues predicted to compose the A-loops were expressed in *G5G8*$^{-/-}$ mice, and biliary cholesterol levels were measured as described in the legend to Fig. 2 and under "Experimental Procedures." Experiments were repeated twice, and the results were similar.

in which a tryptophan located 30 residues upstream of the Walker A motif of G8 (Trp-76) and three tryptophans in the corresponding region of G5 (Trp-55, Trp-56, and Trp-65) (Fig. 3) were replaced with methionine. The mutant proteins were

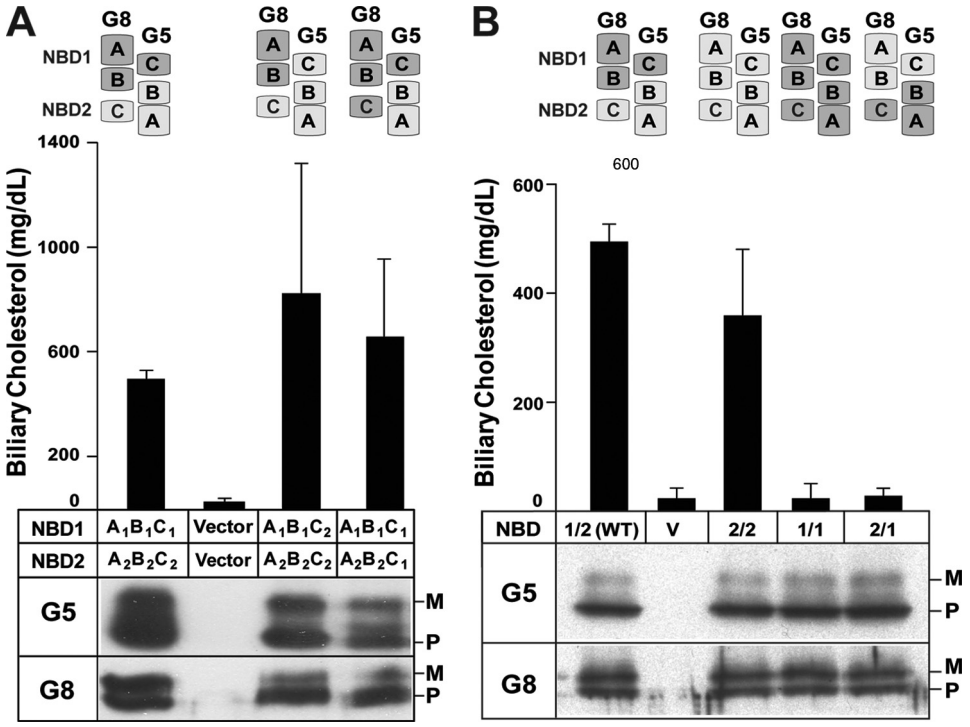


FIGURE 4. **Exchange of the C-loop between NBDs does not impair sterol transport (*A*), whereas swapping the Walker A, Walker B, and C-loop motifs together between the NBDs abolishes sterol transport (*B*).** Recombinant WT and mutant G5 and G8 were expressed in the livers of *G5G8*$^{-/-}$ mice using recombinant adenoviruses, and biliary cholesterol levels were measured as described in the legend to Fig. 2 and under "Experimental Procedures." Experiments were repeated six times, and the results were similar. *P*, precursor; *M*, mature; *V*, vector.

expressed together with the WT partners in the livers of *G5G8*$^{-/-}$ mice. All of the mutant proteins supported sterol transport into bile (Fig. 3). Thus, the aromatic residues in G5G8 that correspond to the A-loop sequences of other transporters are not required for sterol transport.

The Degenerate Walker C Motif of NBD1 Is Competent for Sterol Transport—To determine whether the degenerate C motif in NBD1 is sufficient for G5G8-mediated sterol transport, we exchanged the C-loops of NBD1 and NBD2 and expressed the recombinant proteins in *G5G8*$^{-/-}$ mice (Fig. 4*A*). Substitution of the C-loop of NBD2 with the degenerate sequence from NBD1 did not compromise biliary sterol transport, consistent with our previous finding (3). Thus, the C-loop of NBD1, although degenerate, can support ATP hydrolysis when placed in an appropriate context. Similarly, replacing the C-loop of NBD1 with the canonical C-loop of NBD2 did not adversely affect biliary cholesterol excretion. The levels of protein and the proportions of the mature G5G8 complex were comparable in the livers of the three groups of mice. Therefore, the degenerate C-loop of NBD1 is sufficient but not necessary for G5G8 function.

Transposition of the Consensus Walker Motifs in NBD1 and NBD2 Abolishes Sterol Transport—To further examine the role of the degenerate motifs in NBD1, we replaced Walker A, Walker B, and the C-loop of NBD1 with those of NBD2 (Fig. 4*B*). Expression of the recombinant constructs in *G5G8*$^{-/-}$ mice fully restored biliary sterol excretion, indicating that the constructs were functional. This finding indicates that G5G8 does not require degenerate motifs to affect sterol transport.

To determine whether G5G8 function requires an active ATPase positioned specifically at NBD2, we transposed the Walker A, Walker B, and signature motifs of NBD1 and NBD2. Immunoblot analysis indicated that levels of mature G5 and G8 in livers of mice reconstituted with the mutant proteins were comparable with those in animals injected with the WT constructs (Fig. 4*B*). These data show that the mutant proteins heterodimerized and were transported out of the endoplasmic reticulum as efficiently as was WT G5G8. However, the chimeric G5G8 transporter in which the motifs from NBD1 and NBD2 were transposed did not support sterol transport. Thus, the location of the active ATPase in NBD2 is essential for G5G8 function.

The Walker A Motif of NBD1 Is Not Required for Sterol Transport—If NBD1 catalytic activity is not essential for sterol transport, what is the role of this domain in G5G8 function? Alignment of G5 and G8 sequences from diverse vertebrates indicates that NBD1 has been highly conserved through vertebrate evolution (Fig. 1) and that the degeneracy of the Walker A motif occurred relatively recently. The critical lysine in Walker A is present in zebrafish, frog, chicken, and opossum but is replaced by arginine in placental mammals. The evolutionary conservation of NBD1 strongly suggests that NBD1 plays an essential role in G5G8 function. In the cystic fibrosis transmembrane conductance regulator, inactive NBD1 binds ATP in a divalent cation-independent manner to activate the chloride channel (21). This finding suggests that ATP binding by NBD1, but not hydrolysis, is essential for chloride transport.

To further evaluate whether ATP binding to NBD1 is required for sterol transport by G5G8, we used a structural model to design three G8 constructs with mutations in the Walker A motif that were predicted to disrupt ATP binding but not to interfere with folding and heterodimerization (Fig. 5*A*).

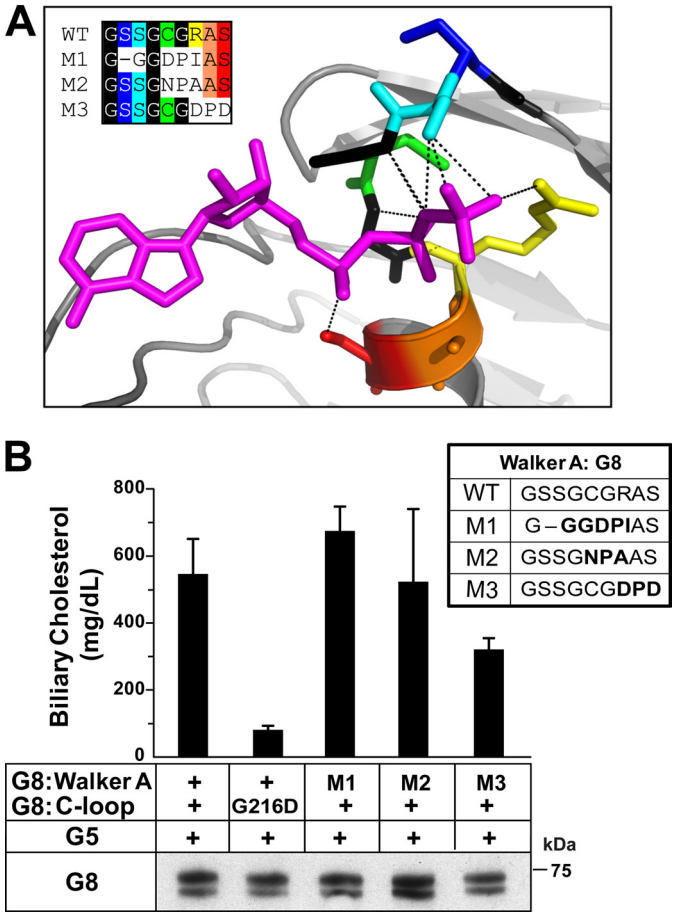


FIGURE 5. **Disruption of the Walker A motif of NBD1 in G5G8 does not impair sterol transport.** *A*, structural model of NBD1 of G5G8. Mutations that are designed to disrupt ATP binding but not to interfere with folding and heterodimerization are shown. *B*, biliary cholesterol levels in mice infected with recombinant adenoviruses encoding three mutant forms of G8 together with WT G5 were measured as described in the legend to Fig. 2 and under "Experimental Procedures." Experiments were repeated three times, and the results were similar. *M1*, mutant 1; *M2*, mutant 2; *M3*, mutant 3.

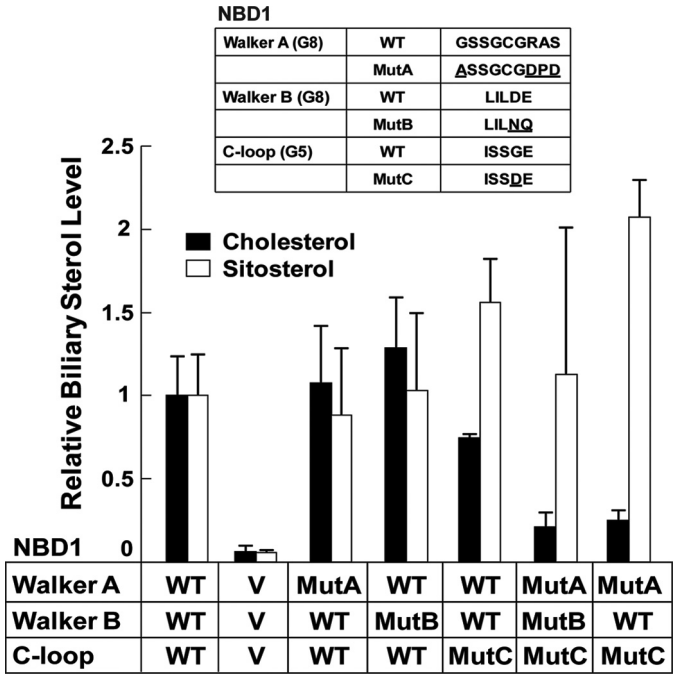


FIGURE 6. **Combinatorial mutations of Walker A and the C-loop of NBD1 alter substrate selectivity of G5G8.** Adenoviruses expressing G5 and G8 with mutations in Walker A, Walker B, and the C-loop of NBD1 were expressed in the livers of *G5G8*$^{-/-}$ mice. The experiment was performed as described in the legend to Fig. 2. The experiment was repeated five times, and the results were similar. *V*, vector.

In mutant 1 (*M1*), the first serine in the loop was deleted to narrow the binding pocket. In addition, the serine and arginine residues that are predicted to form hydrogen bonds with the β- and γ-phosphates of ATP were replaced with glycine and isoleucine, respectively. A proline and an aspartate were introduced to further restrict the size of the binding pocket and provide charge-charge repulsion. In mutant 2 (*M2*), the arginine was replaced with alanine, and an asparagine-proline pair was introduced to cap the helix that begins at the N-terminal portion of the Walker A domain. In the third mutant (*M3*), the arginine-alanine-serine triplet was replaced with aspartate-proline-aspartate to occlude the binding pocket and to introduce negative charges at the phosphate-binding loci.

All three mutant G8 proteins supported sterol transport into bile when expressed together with WT G5. Biliary cholesterol levels were lower in mice reconstituted with one of the mutant proteins (*M3*), although this appears to be due to lower expression, rather than lower activity, of the mutant protein (Fig. 5*B*). Thus, the Walker A sequence of NBD1 is not required for sterol transport by G5G8.

Combinatorial Mutations in NBD1 Alter Substrate Specificity of G5G8—We showed previously that substitutions of key residues in Walker A, Walker B, and the C-loop of NBD1 do not significantly impair sterol transport when introduced individually (3). Accordingly, we examined the combinatorial effect of mutations in NBD1 on sterol transport. For these experiments, we coexpressed forms of G5 and G8 that preserved the integrity of NBD2 while systematically altering the Walker A, Walker B, and signature motifs of NBD1 (Fig. 6). Mutations in either the Walker A motif or the signature sequence of NBD1 had little to no effect on sterol transport into bile, consistent with our previous findings (3). However, simultaneous mutation of both the Walker A motif and the signature sequence caused a striking change in substrate selectivity. The transport of cholesterol was profoundly impaired, whereas sitosterol transport was preserved (Fig. 6). Transport of campesterol, another plant sterol that contains a methyl rather than an ethyl group at C24, was also preserved (data not shown).

DISCUSSION

In this work, we examined the role of NBD1 of G5G8 in sterol transport. We showed previously that mutations in the Walker A motif that are predicted to interfere with ATP hydrolysis abolish sterol transport when introduced into NBD2 but do not impair sterol transport *in vivo* and sterol transfer *in vitro* when introduced into NBD1 (3, 16). A major finding of this study is that NBD1, although functionally degenerate, is not dispensable for sterol transport. Although disruption of individual motifs and canonical residues of NBD1 had no appreciable effect on sterol transport *in vivo*, mutation of the Walker A motif and the C-loop of NBD1 together markedly reduced cho-

lesterol transport while preserving efflux of non-cholesterol sterols. These data are consistent with a model in which NBD1 plays a structural rather than a catalytic role in G5G8-mediated sterol transport.

The mechanism by which alterations in NBD1 affect substrate specificity is not known. A similar alteration in substrate selectivity was seen when the highly conserved lysine in the Walker A motif of NBD2 was replaced with arginine (K93R) (3). Thus, it is possible that the mutations in NBD1 alter substrate specificity by altering the rate of ATP hydrolysis in NBD2. The transport of cholesterol by G5G8 may be more energetically costly than that of the plant sterols and thus be more susceptible to mutations that reduce the efficiency of ATP hydrolysis.

Alternatively, mutations in NBD1 and NBD2 of G5G8 may alter the substrate specificity of the transporter by changing the kinetics of specific steps of the catalytic cycle, rather than the overall rate of ATP hydrolysis. Ernst *et al.* (22) showed that mutation of the switch-loop histidine (H1068A) in the yeast multidrug ABC transporter Pdr5 does not influence ATP hydrolysis but abolishes the transport of rhodamine while retaining the ability to transport other substrates. Those authors proposed that by specifically altering nucleotide binding, hydrolysis, or release, the H1068A mutation alters the equilibration time of transport substrates with the inward-facing drug-binding site. For substrates with different on- or off-rates (K_{on} or K_{off}), changes in equilibration time could lead to differential changes in transport efficiency, thereby altering substrate specificity. Thus, changes in the Walker A motif of NBD2 or in Walker A and the C-loop of NBD1 of G5G8 could alter substrate specificity by affecting the ATP catalytic cycle, rather than by changing the substrate-binding architecture of the transporter.

A third possibility is that mutations in the NBDs could alter substrate specificity by altering the interactions between the NBDs and TMDs. Beaudet *et al.* (23) found that selected mutations around the Walker B motif of NBD1 in P-glycoprotein alter substrate specificity of the transporter. The mutations did not influence substrate binding but selectively altered substrate-induced ATPase activity. Accordingly, the authors proposed that the residues involved may mediate communication between the NBDs and TMDs. Although structural studies suggest that the Walker A lysine is not exposed to the TMDs during the catalytic cycle, we cannot exclude the possibility that mutations in NBD1 and NBD2 that alter substrate specificity act by altering a signaling relay between the TMDs and NBDs.

Degeneracy of NBD1 is not required for G5G8 function, as substitution of the degenerate motifs with the canonical sequences neither impaired nor augmented G5G8-mediated sterol transport. This finding indicates that the predicted loss of ATPase activity that results from the degenerate motifs in NBD1 has little direct impact on G5G8 function. If NBD1 is not required to catalyze ATP hydrolysis, and if the degenerate motifs do not serve a specific purpose, what is the role of NBD1 in G5G8 function? In other asymmetric ABC transporters such as the cystic fibrosis transmembrane conductance regulator, the degenerate NBD retains the ability to bind ATP (21). To test the hypothesis that ATP binding to NBD1 of G5G8 is required for transporter function, we designed a series of mutations in the Walker A motif of G8 that would be expected to disrupt ATP binding. None of these mutations interfered with sterol transport activity (Fig. 6). However, we were unable to confirm that the mutations completely disrupted ATP binding because all of the mutant proteins bound 8-azido-[α-^{32}P]ATP in the presence of an inhibitor, beryllium fluoride (data not shown). It is possible that the ATP that is covalently linked to G8 in these experiments bound to the C-loop of NBD2 (which is contributed by G8) rather than to NBD1. Alternatively, the mutant NBD1 sites in these proteins may bind ATP with sufficient affinity to affect sterol transport even when the sequence of the Walker A motif is disrupted.

To further investigate possible determinants of ATP binding to NBD1, we mutated the aromatic residues corresponding to the A-loops in NBD1 and NBD2. These mutations did not alter sterol transport *in vivo*, suggesting that the A-loop residues do not play a critical role in ATP binding to G5G8. It remains possible that other amino acids in G5G8 coordinate binding to the nucleotide ring, as they do in members of the ABCD family, where isoleucine or leucine residues serve this function (20).

In contrast to NBD1, preservation of the ATPase activity of NBD2 of G5G8 is both necessary and sufficient for sterol transport. Mutating key residues predicted to disrupt ATP hydrolysis by this domain abolishes G5G8 function. The observation that the switch-loop histidine is essential for sterol transport indicates a critical role for this residue in ATP hydrolysis. This finding is consistent with the model proposed by Schmitt and co-workers (24) in which the glutamate of Walker B and the switch-loop histidine form a catalytic dyad. Interestingly, neither the D-loop aspartate nor the Q-loop glutamine of NBD2 is required for G5G8-mediated sterol transport, suggesting that neither residue contributes significantly to ATP hydrolysis by this transporter.

The location of ATPase activity in NBD2 is also critical. Transposing the consensus sequences of the two NBDs preserved expression and trafficking of the transporter but eliminated all sterol transport. Among 19 full transporters with degenerate ATPase motifs, the non-canonical sequences are invariably in NBD1 (2). In summary, these findings suggest that ATPase activity in NBD2 is essential for substrate transport by ABC transporters.

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