The ATP-binding cassette half-transporters ABCG5 (G5) and ABCG8 (G8) promote secretion of neutral sterols into bile, a major pathway for elimination of sterols. Mutations in either ABCG5 or ABCG8 cause sitosterolemia, a recessive disorder characterized by impaired biliary and intestinal sterol secretion, sterol accumulation, and premature atherosclerosis. The mechanism by which the G5G8 heterodimer couples ATP hydrolysis to sterol transport is not known. Here we examined the roles of the Walker A, Walker B, and signature motifs in the nucleotide-binding domains (NBD) of G5 and G8 using recombinant adenoviruses to reconstitute biliary sterol transport in G5G8-deficient mice. Mutant forms of each half-transporter were co-expressed with their wild-type partners. Mutations at crucial residues in the Walker A and Walker B domains of G5 prevented biliary sterol secretion, whereas mutations of the corresponding residues in G8 did not. The opposite result was obtained when mutations were introduced into the signature motif; mutations in the signature domain of G8 prevented sterol transport, but substitution of the corresponding residues in G5 did not. Taken together, these findings indicate that the NBDs of G5 and G8 are not functionally equivalent. The integrity of the canonical NBD formed by the Walker A and Walker B motifs of G5 and the signature motif of G8 is essential for G5G8-mediated sterol transport. In contrast, mutations in key residues of the NBD formed by the Walker A and B motifs of G8 and the signature sequence of G5 did not affect sterol secretion.

ABC transporters comprise a large family of polytopic membrane proteins that use the energy of ATP hydrolysis to translocate a wide variety of substrates across biological membranes (1). Most ABC transporters share a common architecture that includes two NBDs arranged in series with two membrane-spanning domains. The G family of ABC transporters includes five half-transporters that each contain a single hydrophilic NBD at the NH$_2$ terminus and a membrane-spanning domain consisting of six transmembrane $\alpha$-helices at the COOH terminus. Some of the G family members, such as ABCG2, function as homodimers, whereas other family members, such as ABCG5 (G5) and ABCG8 (G8), function as heterodimers (2).

G5 and G8 are expressed almost exclusively in hepatocytes and enterocytes, where they promote excretion of cholesterol and plant sterols into bile and into the gut lumen, respectively (3). Expression of the two proteins is coordinately regulated at the transcriptional level (3); ABCG5 and ABCG8 are juxtaposed on chromosome 2 and up-regulated by the nuclear hormone receptor liver X receptor (4), which promotes the expression of numerous other proteins involved in the centripetal movement of sterols from peripheral tissues to the liver (5). Expression of both G5 and G8 is required for either protein to be transported out of the endoplasmic reticulum (2, 6), and mutations in either ABCG5 or ABCG8 cause sitosterolemia, a rare autosomal recessive disorder characterized by sterol accumulation and premature coronary atherosclerosis (3, 7). Mice lacking G5 and G8 (G5G8$^{-/-}$) have a markedly reduced capacity to secrete sterols into bile (8), whereas overexpression of G5 and G8 in the liver dramatically increases biliary cholesterol levels (9).

The mechanism used by ABC transporters to couple ATP hydrolysis to sterol transport has not been fully elucidated. Three highly conserved sequence elements in the NBDs of ABC transporters: the Walker A motif, the Walker B motif, and a signature motif, play critical roles in nucleotide binding and hydrolysis (10). The x-ray crystallographic structures of several bacterial ABC transporters have revealed that the two NBDs of each transporter are formed by association between the Walker A and B motifs of one subunit and the signature motif of the other (11–14). ATP binds at the interface between the two subunits. A highly conserved basic residue (usually lysine) within the Walker A motif stabilizes the interaction between the NBD and the nucleotide (15), and the aspartate of the Walker B motif orients the magnesium ion in the nucleotide-binding site. The glutamate immediately downstream of the Walker B motif is highly conserved and plays a critical role in transport function (16–18), although the exact mechanistic role of this residue remains controversial. It has been suggested that the carboxylate group of the glutamate coordinates a water molecule that initiates ATP hydrolysis by promoting nucleophilic attack on the $\gamma$-phosphate of the bound ATP (15, 19). However, in ABCB1(P-glycoprotein), the conserved glutamate is not critical for the initial cleavage of the bond between the $\beta$ and $\gamma$ phosphates (17, 18). A conserved glycine at the fourth position of the signature motif interacts with the oxygen of the $\gamma$-phosphate of the ATP (11, 12, 14). Based on the available structural and functional data from other ABC transporters (11–15), we have developed a working model of the NBDs in the G5G8 heterodimer (Fig. 1).

In some transporters, such as ABCB1 (MDR1 or P-glycoprotein), the binding of ATP to one NBD precedes the binding of a second nucleotide to the other NBD, and ATP hydrolysis at both sites is required for efficient substrate transport (20, 21). In other transporters, such as ABCC7 (cystic fibrosis transmembrane conductance regulator), ATP hydrolysis at only one NBD is required to energize transport (22). The precise roles of the two NBDs in G5 and G8 are not known. Develop-

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* This work was supported by National Institutes of Health Grants RO1HL-72304 and PO1HL-20948, the Perot Family Foundation, and the Donald W. Reynolds Clinical Cardiovascular Research Center at Dallas. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: ABC, ATP-binding cassette; G5, ABCG5; G8, ABCG8; TAP, antigen processing; NBD, nucleotide-binding domain; BeF$_6^-$, beryllium fluoride; AlF$_6^-$, aluminum fluoride; V$_o$, orthovanadate.
Functional Analysis of ABCG5 and ABCG8

FIGURE 1. A schematic model of the two catalytic sites in the ATP-sandwiched dimer of G5 and G8. The Walker A motifs (green), Walker B motifs (blue), and the signature motifs (yellow) of G5 and G8 are shown. The interaction between NBDs and phosphate groups of ATP are based on the crystal structures of HisP (15), MalK (11), Mu0796 (14), and Rad50 (12). Dashed lines symbolize hydrogen bonds. The residues that are the focus of study in this paper are highlighted in bold, and the amino acid position numbers are provided.

Experimental Procedures

Materials—Culture medium and fetal bovine serum were obtained from Invitrogen. 8-Azido-ATP, 8-azido-[α-32P]ATP (14.3 Ci/mmol), and 8-azido-[γ-32P]ADP (15.4 Ci/mmol) were purchased from Affinity Labeling Technologies, Inc. (Lexington, KY). BeCl2, AlCl3, NaF, and Vn were obtained from Sigma. Complete EDTA-free protease inhibitors were purchased from Roche Applied Science; Nonidet P-40 was purchased from Calbiochem (La Jolla, CA). All other chemicals and reagents were obtained from Sigma-Aldrich unless otherwise indicated.

Site-directed Mutagenesis—The mutations were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The template used was a shuttle vector, pACCMVpLpa(−)IoxP-SSP, containing G5 or G8 cDNAs. Oligonucleotides bearing mismatched bases at the residues to be mutated (underlined) were synthesized by IDT, Inc. (Coralville, IA). They are as follows: for G5, K93M-forward (5′-TCA GCC TCA GGG ATG ACC ACG CTG-3′), K93M-reverse (5′-CAG CGT GGT CAT CCC TGA GCC TGA-3′), K93R-forward (5′-TCA GCC TCA GGG AGG ACC ACG CTG-3′), K93R-reverse (5′-CAG CGT GGT CCT CCC TGA GCC TGA-3′), G197D-forward (5′-GGA ATT TCC AGT GAG CGG CGC CGA-3′), G197D-reverse (5′-TCA GCC TCA GGG ATG ACC ACG CTG-3′), 1194V5196G-reverse (5′-GCG CCG CTC GCC ACC GGA ACC TCC CCC AAA ATT-3′), E219D-forward (5′-ATG ATG CTA GAT GAC CCA ACC ACA GGA-3′), E219D-reverse (5′-TCC TGT TGG GTG ATC TAG CAT CAT-3′), E219Q-forward (5′-ATG ATG CTA GAT CAG CCA ACC ACA GGA-3′), E219Q-reverse (5′-TCC TGT TGG CTG ATC TAG CAT CAT-3′). For G8, R111M-forward (5′-TCA GCC TGC GGG ATG GCC TCA CTA CTC-3′), R111M-reverse (5′-GAG TAG TGC GGG CAT CCC GCA GCC TGA-3′), R111K-forward (5′-TCA GCC TGC GGG AGG GCC TCA CTA CTC-3′), R111K-reverse (5′-GAG TAG TGC GCC GTT CCC GCA GCC TGA-3′), G216D-forward (5′-GGG GTG TCC GGG GAT GAG CGG CGA CGA-3′), G216D-reverse (5′-TGG TCG GCC TCA CTC ACC CCC GGA FCC ACC CCC-3′), V214I-G215S-forward (5′-TAT GTA CGT GAT TCG GTG GAG CTC GAG CCA-3′), V214I-G215S-reverse (5′-TGG GTG GCC TCG TTC ACC CCC GGA FCC ACC CCC-3′), E238D-forward (5′-CTC ATT CTG GAT GAC CAT GCG ACC TCT GTC-3′), E238D-reverse (5′-GCC AGA AGT GGG GTT ATT GCG ACC TCT GTC GCG-3′), E238Q-forward (5′-CTC ATT CTG GAT CAG CAC ACC TCT GTC-3′), and E238D-reverse (5′-GCC AGA AGT GGG GTT ATT GCG ACC TCT GTC CAG-3′). The presence of the desired mutation and the integrity of each construct were verified by DNA sequencing.

Infection of CRL-1601 Cells with Recombinant Adenoviral Vectors—Recombinant adenoviral vectors containing cDNAs for wild-type and mutant G5 and G8 were generated by in vitro cre-lox recombination as described previously (2). Cultured rat hepatoma cells (CRL-1601) were maintained in Dulbecco’s modified Eagle’s medium (glucose, 1 g/liter) containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. On day one, the cells were seeded at 5 × 106 cells/150-mm plate. After 24 h, the cells were infected with recombinant adenoviral vectors containing cDNAs for wild-type or mutant G5 and G8 (8 × 1010 particles/plate). After 60 h, the cells were harvested, and a plasma membrane-enriched fraction was isolated by centrifugation through sucrose, as described (23). Protein concentrations were determined using the BCA assay (Pierce) according to the manufacturer’s protocol, and the membrane fractions were aliquotted and stored at −80 °C.

Expression of Recombinant G5 and G8 in Mice Using Adenovirus—Mice homozygous for disrupted alleles at Abcg5 and Abcg8 (G5G8−/−) were generated as described (8) and maintained on a regular chow diet (Harlan Teklad, Madison, WI). Adenoviral particles (5 × 1013 particles/kg) were injected into the tail veins of the mice. After 72 h, the mice were fasted for 4 h, anesthetized with halothane, and killed by exsanguination. Blood was collected, and neutral salt levels were measured using gas liquid chromatography and mass spectrometry as described (6). Liver tissue was snap frozen in liquid nitrogen and stored at −80 °C.

SDS-PAGE and Immunoblot Analysis of G5 and G8—The membrane proteins were subjected to SDS-PAGE and then transferred to nitrocellulose membranes (Amersham Biosciences) by electroblotting. Immunoblotting was performed using an anti-G8 monoclonal antibody (1B10A5) or a rabbit polyclonal antibody (5161) directed against a recombinant peptide corresponding to amino acids 1–350 of G8 (6). Antibody binding was detected using horseradish peroxidase-conjugated goat anti-mouse or with donkey anti-rabbit IgG (Amersham Biosciences), followed by enhanced chemiluminescence detection (Pierce). The membranes were then exposed to F-BX8101™ Blue X-Ray films (Phoenix Research Products, Hayward, CA).
**Immunoprecipitation of G5 and G8**—The membrane fractions of cells were solubilized in 400 µl of lysis buffer (50 mM HEPES, 100 mM NaCl, 1.5 mM MgCl₂, 5 mM dithiothreitol, 1% (v/v) Nonidet P-40, 0.1% SDS, 5 mM EDTA) containing 1X protease inhibitors (Complete EDTA-free protease inhibitors; Roche Applied Science) for 2 h at 4 °C. The cell lysates were precleared by adding 10 µl of a 50% (v/v) suspension of protein A-agarose (RepliGen Corp., Waltham, MA) and 10 µl of a 50% (v/v) suspension of protein G-agarose (Upstate, Lake Placid, NY), and the samples were centrifuged at 16,000 × g for 5 min. The pre-cleared lysates were incubated with an anti-G8 monoclonal antibody (20 µg) and 20 µl of a 50% (v/v) suspension of protein G-agarose overnight at 4 °C. After centrifugation at 3,000 × g for 5 min, the pellets were washed in lysis buffer three times for 10 min at 4 °C, and then the proteins were eluted using 1 × SDS loading buffer (31 mM Tris-HCl, pH 6.8, 1% SDS, 12.5% glycerol, 0.0025% bromphenol) containing 5% β-mercaptoethanol. The supernatants were transferred into new microcentrifuge tubes and incubated with 2 µl of anti-G5 polyclonal antibody (1:200), and 20 µl of a 50% (v/v) suspension of protein A-agarose for 6 h at 4 °C. After extensive washing, the proteins were eluted from the beads with 1 × SDS loading buffer containing 5% β-mercaptoethanol. The proteins were then analyzed by SDS-PAGE and immunoblotting. The antibodies were stripped from the membrane by incubation for 40 min at 55 °C in stripping buffer (Pierce). Stripped membranes were confirmed to be free of signal by repeat autoradiography before incubating the filter with another antibody.

**Photolabeling of G5 and G8 with 8-Azido[α-32P]ATP and BeFx-induced Trapping of 8-Azido[α-32P]ATP by G5 and G8**—ATP binding was examined by photolabeling with 8-azido[α-32P]ATP using membrane proteins isolated from CRL-1601 cells expressing recombinant G5, G8, or G5 plus G8 as described previously (24). Briefly, membrane fractions (10–40 µg) were incubated in 30 µl of labeling buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.02% NaN₃) containing 10 µM 8-azido[α-32P]ATP on ice for 5 min and then subjected to UV irradiation for 3 min at 4 °C. The reactions were terminated by the addition of 400 µl of ice-cold stop buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, 5 mM MgCl₂), and the membranes were centrifuged at 16,000 × g for 30 min at 4 °C. The pellets were solubilized in 400 µl of lysis buffer, and G5 and G8 were immunoprecipitated as described above. The samples were size-fractionated on 8% gels by SDS-PAGE and then transferred to nitrocellulose membranes by electroblotting. The membranes were dried and exposed to F-BX810™ Blue X-Ray films overnight at −80 °C.

An identical protocol was used for the ATP competition experiment except that membrane fractions were incubated in labeling buffer containing 10 µM 8-azido[α-32P]ATP and various amounts of unlabeled ATP (0, 1, 10, 50 mM). To determine the binding affinity of G5 and G8 for 8-azido[α-32P]ATP, the experiment was performed in the presence of increasing concentrations of 8-azido[α-32P]ATP (ranging from 0.5 to 160 µM). The radioactivity incorporated into the G5 and G8 bands was competed by the addition of 8-azido[α-32P]ATP to bind to G8 when it was expressed in the absence of G5 (Fig. 2C).

Next we examined whether heterodimerization and transport of G5 and G8 out of the endoplasmic reticulum is required for the proteins to bind an analog of ATP, 8-azido[α-32P]ATP. In cells expressing both G5 and G8, the precursor and mature forms of G5 and G8 photolabeled with 8-azido[α-32P]ATP. The degree of labeling was proportional to the amount of protein present (Fig. 2A). Azido-ATP also bound to G5 and G8 when they were expressed individually in cells. Thus, heterodimerization of G5 and G8 was not required for ATP binding. Binding to both the precursor and mature forms of G5 and G8 was competed by the addition of excess cold nucleotide (Fig. 2B).

Binding of 8-azido[α-32P]ATP required Mg²⁺ (Fig. 2C) and was inhibited by the addition of EDTA (data not shown) in cells expressing both half-transporters. Magnesium ions were also required to photolabel G5 with 8-azido[α-32P]ATP when the half-transporter was expressed individually in cells (Fig. 2C). In contrast to these findings, magnesium was not required for 8-azido[α-32P]ATP to bind to G8 when it was expressed in the absence of G5 (Fig. 2C).

Next we examined the relative affinity of G5 and G8 for 8-azido[α-32P]ATP by measuring the amount of nucleotide bound to each half-transporter over a range of concentrations (Fig. 3). When G5 and G8 were expressed individually in cells, G5 bound ATP in a saturable manner with an apparent Kd of 66 µM, whereas ATP binding to G8 was not fully saturable up to a concentration of 160 µM (Fig. 3A). Co-expression of G5 and G8 did not affect the affinity of ATP binding to G5 (apparent Kd = 57 µM; Fig. 3B), whereas it increased the affinity of G8 for ATP. The binding of ATP to G8 became saturable with an apparent Kd of 174 µM. These data demonstrated that expressing G8 together with G5 resulted in a higher affinity, magnesium-dependent ATP binding to G8.

**Nucleotide Trapping by BeFx and AlFx**—Currently, no assays are available to detect the ATPase activity of G5 and G8. We therefore examined the ability of the G5/G8 heterodimer to bind and hydrolyze ATP by determining whether the protein complex can trap ADP after hydrolysis in the presence of various phosphate analogs (BeFx, AlFx, and V₅) (27, 28). Membranes isolated from cultured hepatocytes expressing recombinant G5 and G8 were incubated with 8-azido[α-32P] ATP (10 µM) for 15 min at 37 °C in the presence or absence of AlFx, BeFx, or V₅. The reaction was terminated, and the unbound nucleotide was com-
peted away by incubating the samples on ice for 5 min in the presence of excess cold ATP (30 mM). No labeling of G5 or G8 was observed in the absence of inhibitors (Fig. 4A). In cells expressing both proteins, the addition of BeFx or AlFx was associated with photolabeling of both the precursor and mature forms of G5 and with the mature form of G8. A trace amount of $^{32}$P signal was also apparent on longer exposures if a lower concentration of Vi was used (1 mM) and on a short exposure with higher concentration of Vi (5 mM) (data not shown), as has been observed for other members of the ABC transporter family (29, 30). The experiment was repeated under conditions in which ATP hydrolysis was prevented (4 °C); ATP bound G5 and G8 without the addition of inhibitor, and no increase in signal was apparent after adding AlFx or BeFx (data not shown).

To determine whether the BeFx-induced trapping of 8-azido[$^{32}$P]ADP in G5 and G8 resulted from ATP hydrolysis in the NBDs or was due to the hydrolysis of ATP by other ATPases present in the membrane preparations and subsequent trapping of the ADP in G5G8, we compared the amount of nucleotide trapped when membranes were incubated with 8-azido[$^{32}$P]ATP and processed as described in the legend to Fig. 2A. The amount of radioactivity in the G5 and G8 bands was quantified using ImageQuant software. The experiment was repeated twice, and similar results were obtained.

To determine whether the BeFx-induced trapping of 8-azido[$^{32}$P]ADP in G5 and G8 resulted from ATP hydrolysis in the NBDs or was due to the hydrolysis of ATP by other ATPases present in the membrane preparations and subsequent trapping of the ADP in G5G8, we compared the amount of nucleotide trapped when membranes were incubated with 8-azido[$^{32}$P]ATP (14.3 Ci/mmol) or 8-azido[$^{32}$P]ADP (15.4 Ci/mmol) (Fig. 4B). Despite the two nucleotides having a similar specific activity, more trapping of nucleotide was seen with 8-azido[$^{32}$P]ATP than with 8-azido[$^{32}$P]ADP. These data suggest that the
trapping of nucleotide in G5 and G8 induced by BeF₆ predominantly results as a consequence of ATP hydrolysis in the NBDs of the half-transporters. Taken together, these findings imply that both G5 and G8, when expressed in CRL-1601 cells, are capable of ATP hydrolysis.

BeF₆ also induced trapping of 8-azido[α-³²P]ADP when G5 was expressed alone in cells (Fig. 4C). In contrast to these results, only a trace amount of nucleotide was seen associated with G8 expressed in the absence of G5. Thus, both the precursor and mature forms of G5 were capable of trapping nucleotide; trapping was not dependent on the formation of G5G8 heterodimers, whereas efficient trapping of nucleotide in the NBD of G8 was dependent on the formation of G5G8 heterodimer.

Effects of Walker A Mutations in G5 and G8 on Protein Function—Next we examined the effects of mutating a conserved lysine residue in the Walker A motif of G5 (Lys⁹³) and the corresponding basic residue in G8 (Arg¹¹¹) on nucleotide binding and trapping in cultured hepatocytes and on sterol transport in mice. The lysine at amino acid 93 in the Walker A motif of G5 is completely conserved from humans to zebrafish. A basic residue at this position in the Walker A motif of other ABC transporters plays a critical role in the binding of ATP to the NBD (13, 15, 19, 31); substitution of this residue with a methionine in ABCB1 (MDR1), ABCC1 (multidrug resistance protein 1), and ABCC7 (cystic fibrosis transmembrane conductance regulator) is associated with reduced ATP binding and substrate transport (32–34). To determine whether Lys⁹³ in G5 was required for ATP binding, ATP hydrolysis, and sterol transport, we substituted methionine for lysine at this position.

Expression of G5-K93M with wild-type G8 in CRL-1061 cells resulted in a moderate reduction in the level of immunodetectable G5-K93M and a more significant reduction in the level of G8 (Fig. 5A). The reduction in the amount of immunodetectable G8 coincided with the appearance of a lower molecular mass band (~55 kDa). This band was smaller than the precursor form of G8 (~70 kDa), and the relative amount of the band decreased with the addition of the calpain inhibitor N-acetyl-Leu-Leu-norleucinal (data not shown). Therefore, we concluded that the lower molecular mass band was likely a degradation product of G8. The K93M substitution was also associated with a marked reduction in the binding and trapping of azido-ADP to both G5 and G8. These results are consistent with the Walker A motif of G5 playing a crucial role in ATP binding and stabilization of the G5G8 complex with substitution of methionine for lysine interfering with ATP binding to the NBD formed by the Walker A and Walker B motifs of G5 and the signature motif of G8 (NBD1). Without a stable complex forming at NBD1, the protein complex is unstable, resulting in a failure of nucleotide to bind in the NBD formed by the Walker A and B sequences of G8 and signature motif of G5 (NBD2) (Fig. 1).

To determine whether another basic residue at position 93 in the Walker A motif of G5 could substitute for lysine, we replaced the residue with arginine (K93R), which is the corresponding amino acid in G8 (Fig. 1). Expression of G5-K93R with wild-type G8 only modestly affected photolabeling and 8-azido-[α-³²P]ADP trapping in G5 and had no effect on G8. These results are consistent with a basic residue at this position in the Walker A motif of G5 permitting ATP binding, increasing the stability of the G5G8 heterodimer.

In contrast to these results, replacement of the corresponding basic residue in the Walker A motif of G8 with methionine (R111M) or with lysine (R111K) had no effect on either the amount of immunodetectable G5 or G8 or on the ability of G5 or G8 to bind ATP or trap ADP (Fig. 5A). Thus, the Arg¹¹¹ in the Walker A motif of G8 appears not to be required for binding and trapping of nucleotide in the complex.

The effects of the Walker A mutations in G5 and G8 on cholesterol and plant sterol transport were examined using an in vivo, adenovirus-based expression system. Previously, we showed that biliary cholesterol levels in mice expressing no G5 and G8 are extremely low (8). Adenovirus-mediated expression of either wild-type G5 alone or wild-type G8
alone in the livers of G5G8−/− mice failed to increase biliary sterol levels, whereas co-expression of G5 and G8 resulted in a marked increase in biliary cholesterol and plant sterols (Fig. 5B). Substitution of Arg111 in G8 to methionine or lysine had no adverse effects on biliary cholesterol or plant sterol levels, even when steady state levels of mature G5 and G8 were significantly reduced, as occurred in the mice expressing G8-R111M with wild-type G5 (Fig. 5B). Taken together, these results are consistent with a positively charged residue at position 93 in the Walker A of G5 being essential for both G5 and G8 to bind and hydrolyze ATP, whereas arginine at position III in the Walker A motif of G8 appeared not to be required for binding or hydrolysis of nucleotide to the complex.

**Effects of the Mutations in the Signature Motif on G5 and G8 Functions**—Next we examined the relative importance of a highly conserved residue in the signature motifs of G5 and G8 on the ability of the G5G8 complex to bind and trap nucleotide and to promote sterol transport. In other ABC transporters, the fourth residue of the signature motif is almost invariably glycine, which interacts with the oxygen of the γ-phosphate of the ATP bound at the Walker motifs of the opposite subunit (Fig. 1) (11, 14). Substitution of this residue in the signature motif of NBD1 in ABCC7 (cystic fibrosis transmembrane conductance regulator) (Gly551) causes severe cystic fibrosis (35). We therefore substituted aspartate for glycine in the signature motif of G5 and G8 (G5-G197D and G8-G216D) to investigate the possible role of this motif in protein function. Recombinant adenoviruses expressing these mutant forms of G5 and G8 were used to co-infect CRL-1601 cells with the wild-type partner. Photolabeling and trapping experiment was performed. In general, the intensity of 32P signal in G5 and G8 was proportional to the relative expression levels of the mutant and wild-type proteins (Fig. 6A). Thus, G5-G197D and G8-G216D did not affect significantly the ATP labeling and ADP trapping in G5 and G8. Although the cells expressing G5-G197D and wild-type G8 had less immunodetectable protein and reduced binding of 8-azido[α-32P]ATP and trapping of 8-azido-[α-32P]ADP, co-expression of G5-G197D with wild-type G8 in vivo fully reconstituted sterol transport (Fig. 6B). In contrast to these results, co-expression of G8-G216D with wild-type G5 did not result in any increase in biliary cholesterol level and produced only a modest increase in the level of biliary plant sterols (Fig. 6B). Thus, the highly conserved glycine residue in G8, but not in G5, was required for efficient sterol transport.

We also swapped the sequences of the signature motifs of G5 and G8. We made the signature motif of G5 identical to that of G8 and visa versa (Fig. 1). Ile194 was changed to valine, and Ser196 was changed to glycine in G5 (G5-I194V+S196G), and complementary changes were made in the signature motif of G8 (G8-V213I+G216D). These substitutions had
no apparent effects on the levels of G5 or G8 expression either in cultured cells (Fig. 6A) or on sterol transport in vivo (Fig. 6B). Taken together, these results demonstrated that both signature motifs are competent to support ATP binding and hydrolysis and that the glycine in the signature motif in G8 but not in G5 is critical for sterol transport. Thus, the integrity of the Walker A motif of G5 and the highly conserved glycine residue in the signature motif of G8 are required for G5G8-mediated sterol transport.

Effects of Mutations of the Conserved Glutamic Acid Residue Adjacent to Walker B Motif on G5 and G8 Function—A highly conserved glutamate residue immediately adjacent to the Walker B motif plays a critical role in ATP catalysis in other ABC transporters (16, 36). We examined the effect of substituting this highly conserved residue with either an aspartate or a glutamine. Expression of G5-E219D with wild-type G8 in cultured cells was associated with reduced expression of both half-transporters and a proportional reduction in nucleotide binding and trapping (Fig. 7A). Replacement of the conserved glutamate with glutamine (G5-E219Q) resulted in a dramatic reduction in the stability of G5 and G8; therefore, the effect of this mutation on ATP binding and hydrolysis could not be assessed. Both of these substitutions in G5 essentially abolished cholesterol and plant sterol transport when co-expressed with wild-type G8. In contrast to these results, substitution of the conserved glutamate in G8 with aspartate had no effects on cellular levels of G5 or G8 and also did not alter the binding of nucleotide to either protein. Substitution of the same residue to glutamine (G8-E238Q) resulted in relative reduction in binding of 8-azido-[\alpha-^32P]ATP and the trapping of 8-azido-[\alpha-^32P]ADP to G8 without having a significant effect on nucleotide binding and trapping in G5. Neither of these substitutions in G8 affected sterol transport when co-expressed with wild-type G5 in vivo (Fig. 7B). Therefore, the conserved glutamate in G5, but not in G8, is essential for biliary sterol transport by the G5G8 heterodimer.

DISCUSSION

The major finding of this paper is that the two NBDs formed by heterodimerization of G5 and G8 are not functionally equivalent. Both NBDs can bind and trap ATP in vitro, but mutations in highly conserved residues in the Walker A and B motifs of G8 had no effect on sterol transport in vivo, whereas the corresponding substitutions in G5 abolished sterol transport. Conversely, substitution of a conserved residue in the signature motif of G5 fully supported sterol transport, whereas the identical substitution in G8 failed to reconstitute biliary sterol transport in the G5G8 heterodimer mouse. These data are consistent with the model of Jones and George (37) and suggest that the Walker A and B motifs in G5 and the signature motif of G8 form one functional NBD (NBD1) and that the integrity of NBD1 is essential for ATP binding, stabilization of the G5G8 complex, and sterol transport. The second NBD (NBD2), which includes the Walker A and B motifs of G8 and the signature motif of G5, does not appear to play an essential role in powering sterol transport.

The finding that the two NBDs in the G5G8 heterodimer are functionally distinct is similar to other ABC transporter family members, including ABCG1 (multidrug resistance protein 1) and the peptide transporter formed by heterodimerization of ABCB2 and ABCB3 (TAP1/TAP2). Mutations in the conserved residues of either NBD of ABCG1 or of ABCB2/ABC3 alter transport function, although the severity of the functional impairment is dependent on which NBD is compromised (34, 38 – 40). The significance of the functional asymmetry in the two NBDs in these transporters and in G5 and G8 is not known. One possibility is that the two NBDs have different roles, with
one performing a catalytic function while the other serves a structural or regulatory function. However, some ABC transporters with identical NBDs, such as HisP, substrate transport is maintained when one NBD is inactivated (41), suggesting that ATP hydrolysis at only a single NBD is required for transport.

The Walker A motif in the NBD of G5 (GXXGKTT) conforms to the consensus Walker A sequence (GXXGK[T/S]T) and is completely conserved in human, chimp, monkey, lemur, dog, cow, rabbit, mouse, rat, opossum, chicken, frog, and fish (fugu, tetraodon, stickleback, and zebrafish). The crystal structures of several bacterial ABC transporters reveal that the main chain nitrogens and side chains of the lysine and threonine/serine in the Walker A sequences form hydrogen bonds with the oxygen atoms from the β- and γ-phosphate of ATP to stabilize binding of nucleotide to the NBD (11, 13, 15, 31). Substitution of methionine for lysine in the Walker A motif of G5 destabilized the G5G8 complex, causing a reduction in protein mass and azido-ATP binding to G8 as well as to G5 (Fig. 5A). Thus, the Walker A motif of G5 is required to form a stable association with ATP and with the signature motif of G8 to form a NBD (NBD1). Formation of this sandwich-like complex is required for the second NBD to form (NBD2) and to support transport of sterols (Fig. 5).

Co-expression of the two half-transporters influenced the binding and trapping of ATP by G8. G8 bound 8-azido-ATP with a lower affinity and in a magnesium-independent manner when the half-transporter was expressed alone in cells (Figs. 2 and 3). The affinity of 8-azido-ATP binding to G8 increased and became magnesium-dependent when G5 and G8 were co-expressed. This finding is consistent with the notion that binding of ATP to NBD 1 promotes conformational changes in NBD2. Similar differences in ATP binding affinity and specificity are seen for TAP1 and TAP2. Co-expression of TAP1 and TAP2 increases the binding affinity of TAP2 for ATP but does not affect the binding of ATP to TAP1 (42).

The Walker A motif of human G8 (GSSGCGRAS) differs from that of G5 (and of most other ABC transporters) by having an arginine rather than a lysine at residue 7 and an alanine rather than a threonine or a serine at position 8. These deviations from the canonical sequence appear to be a relatively recent evolutionary development; in all species prior to opossum in the evolutionary chain in which G5 has been sequenced, position 7 is a lysine, and position 8 is either a serine or threonine. In opossum and all higher species, the residue at position 7 is an arginine, and at position 8 is an alanine. The substitution of these two critical residues in the Walker A motif of G8 may have conferred new functional capabilities to the transporter, or alternatively, the substitutions may have been tolerated because they appear not to affect protein stability, ATP binding, ATP trapping, or sterol transport (Fig. 5).

Further differences between the NBDs of G5G8 were revealed by mutating the glutamate residue immediately downstream of the Walker B motif. Heterodimers in which the catalytic glutamate of G5 (Glu219) was substituted with either aspartate or glutamine failed to support sterol transport in vivo (Fig. 7). The introduction of glutamine at residue 219 destabilized the protein complex. When G5-E219D was expressed with G8, both G5 and G8 were reduced slightly in amount and a proportional decrease in ATP binding was observed in vitro. Despite the modest effects of the substitution on protein stability and ATP binding, this mutation completely abolished sterol transport in vivo. These data provide further support for the importance of formation of NBD1.

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finding that mutation of the catalytic glutamate of G8 (Glu\textsuperscript{238}) to either aspartate or glutamine did not affect sterol transport suggests that ATP hydrolysis at NBD2 is not required for substrate transport.

Substitution of the catalytic glutamate residue in G5 with aspartate (G5-E219D) abolished sterol transport \textit{in vivo}, confirming a specific requirement for the carboxyl side chain of glutamate. Perhaps surprisingly, this substitution did not significantly decrease the trapping of azido-ATP in cultured cells (Fig. 7A). Similar results have been obtained when the corresponding glutamate residue of other ABC transporters was mutated; for example, substitution of the catalytic glutamate with aspartate or glutamine in either NBD1 or NBD2 of mouse ABCB4 (MDR3) markedly reduced ATPase activity but did not interfere with V\textsubscript{i}-induced-trapping of labeled nucleotide (18). Elution of the trapped nucleotides from the mutant proteins revealed that a significant fraction (26 – 47\%) was ATP, whereas essentially all of the nucleotide eluted from the wild-type protein was ADP. This is most consistent with the mutation greatly reducing but not abolishing hydrolysis. This finding indicates that mutation of the catalytic glutamate residue promotes an NBD conformation in which nucleotide is tightly bound but poorly hydrolyzed. Thus, low residual ATPase activity may allow accumulation of ADP by the NBD1 of G5-E219D during the 15-min incubation period of the trapping assay.

In contrast to the Walker A and Walker B motifs, where G5 is under greater sequence constraints than G8, the opposite is true for the signature motifs. The two most highly conserved residues in the signature motifs of ABC transporters are the serine and the second glycine residue (LSGGQ). Both of these residues are completely conserved through evolution in G5 and G8. In other transporters, these two residues bind the γ-phosphate of ATP (12,14). Substituting glycine at position 197 in the signature motif of G5 had no effect on sterol transport \textit{in vivo}, whereas the corresponding amino acid substitution at the conserved glycine in G8 completely abolished G5G8-mediated sterol transport (Fig. 6B). The different functional consequences of the glycine substitutions do not reflect intrinsic differences in the signature motifs, because switching the sequence of the entire motif was compatible with sterol transport (Fig. 6B). These results support the notion that the Walker A and Walker B motifs of G5 form a functional NBD with the signature sequence of G8 and that the two NBDs in the G5G8 heterodimer are not functionally equivalent.

The finding that the signature motifs of G5 and G8 were both competent to form a functional NBD and support biliary transport \textit{in vivo} (Fig. 6) raises the possibility that homodimers of G5 may support ATP binding and hydrolysis. Both G5 and G8 bound the ATP analog, 8-azido[α-\textsuperscript{32}P]ATP, whether the transporters were expressed individually or together in cells. In cells expressing only one of the half-transporters, we did not determine whether ATP is binding the monomer, the homodimer, or larger multimers of G5 or G8. We have previously shown that G5 and G8 can multimerize in cells expressing large amounts of the recombinant protein (2), but there is no evidence that G5 or G8 form functional homodimers \textit{in vivo}.

Although substitution of the second glycine in the signature motif of G8 inactivated sterol transport \textit{in vivo} (Fig. 6B), the mutation did not reduce 8-azido-ATP binding or BeF\textsubscript{2}-induced ADP trapping in either G5 or in G8. Similar findings have been reported for ABCB1 (P-glycoprotein). Substitution of the conserved glycine in the signature motif of human P-glycoprotein to aspartate resulted in reduced ATPase activity despite no detectable differences in ATP binding and V\textsubscript{i} or BeF\textsubscript{2}-induced trapping of 8-azido-ADP (43). Substitution of the conserved serine (to alanine) in either signature motif of mouse MDR3 also had no adverse effects on either ATP binding or ADP trapping (44). The conserved glycine in the signature sequence may not be required for the initial ATP binding event or participate directly in ATP hydrolysis. Senior and co-workers (44) have suggested that the signature motif stabilizes the transition state by interacting directly with the bound transition state complex.

Currently, we cannot detect any ATPase activity associated with G5 and G8 expression, either in the presence or in the absence of added sterols (data not shown). Therefore, we cannot formally exclude the possibility that both NBDs of the G5G8 heterodimer are required to bind and hydrolyze ATP. It remains possible, although unlikely, that the mutations we introduced into highly conserved sequences in the Walker A motif and in the catalytic glutamate of G8 simply did not affect ATPase activity in the second NBD. The maintenance of normal ATPase activity in the absence of these residues would imply a novel catalytic mechanism that has not been observed in other ABC transporters. Accordingly, our data are most compatible with a model in which the two NBDs of G5 and G8 fulfill different functions.

An interesting and unexpected observation was that substitution of arginine for the highly conserved lysine in the Walker A motif of G5 changed the substrate specificity of the transporter. Although this substitution abolished cholesterol transport, biliary plant sterol transport was preserved when G5-K93R and wild-type G8 were expressed together in the livers of G5G8\textsuperscript{−/−} mice. Molecular cross-talk between the NBDs and the transmembrane domains in other ABC transporters has been documented (45–47), and substitution of this residue in the Walker A of G5 may influence the conformation of the membrane-spanning domain, affecting substrate specificity. Alternatively it is possible that G5G8 can sustain the transport of noncholesterol sterols even when the efficiency of ATP hydrolysis is reduced but that the transport of cholesterol requires an intact NBD. Further studies will be required to determine the mechanism by which an amino acid substitution in the NBD domain affects the substrate specificity of the transporter.

Acknowledgments—We thank Christina Zhao, Julie Pourot, Yuannan Liao, Linda Donnelly, and Jill Fairless for excellent technical assistance and Fang Xu for the measurement of biliary sterols.

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