Sterol Transfer by ABCG5 and ABCG8
IN VITRO ASSAY AND RECONSTITUTION*

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ATP-binding cassette transporters G5 and G8 are half-transporters expressed on the apical membranes of enterocytes and hepatocytes that limit intestinal uptake and promote secretion of neutral sterols. Genetic defects that inactivate either half-transporter cause accumulation of cholesterol and plant sterols, resulting in premature coronary atherosclerosis. These observations suggest that G5 and G8 promote the translocation of sterols across membranes, but the primary transport substrate of the G5G8 complex has not been directly determined. Here we report the development of a sterol transfer assay using “inside-out” membrane vesicles from Sf9 cells expressing recombinant mouse G5 and G8. Radiolabeled cholesterol or sitosterol was transferred from donor liposomes to G5- and G8-containing membrane vesicles in an ATP-dependent and vanadate-sensitive manner; net transfer of cholesterol was associated with an increase in vesicular cholesterol mass. CTP, GTP, and UTP, as well as ATP, supported transfer but with lesser efficiency (ATP > CTP > GTP > UTP). Transfer was specific for sterols and was stereoselective; minimal ATP-dependent and vanadate-sensitive transfer of cholesteryl oleate, phosphatidylcholine, or enantiomeric cholesterol was observed. These studies indicate that G5 and G8 are sufficient for reconstitution of sterol transfer activity in vitro and provide the first demonstration that sterols are direct transport substrates of the G5 and G8 heterodimer.

Members of the superfamily of ATP-binding cassette (ABC) transporters actively translocate a wide variety of substances, including anions, lipids, peptides, and other compounds across membranes (1). Two ABC half-transporters, ABCG5 (G5) and ABCG8 (G8), expressed in the absorptive cells of the intestine and in hepatocytes play critical roles in the trafficking of cholesterol and other neutral sterols (2). G5 and G8 form heterodimers in the endoplasmic reticulum and are transported to the apical membranes (3, 4). In enterocytes, the G5G8 complex limits the amount of dietary sterols that are incorporated into lipoproteins and delivered to the liver (3, 4). In hepatocytes, G5 and G8 are required for efficient cholesterol secretion into bile, the major pathway for cholesterol elimination in mammals (5). Mutations inactivating either G5 or G8 cause sitosterolemia, a recessive disorder characterized by hypercholesterolemia and phytosterolemia because of increased fractional absorption and reduced biliary secretion of sterols (2, 6). Detailed metabolic studies in genetically modified mice in which G5 and G8 are overexpressed or inactivated confirm the central role of G5 and G8 in sterol trafficking (5, 7). The fractional absorption of sterols is increased and biliary sterol secretion decreased in the G5G8−/− mice, whereas overexpression of G5 and G8 reduces the fractional absorption of dietary cholesterol and promotes biliary cholesterol secretion (5, 7).

All ABC transporters share a common molecular architecture, which includes a pair of nucleotide binding domains (NBD) and two transmembrane (TM) domains each containing multiple membrane-spanning α-helices (8–10). In eukaryotes, genes encoding members of the ABC transporter family are organized either as full transporters containing both TM domains and both NBDs, or as half-transporters, like G5 and G8, that contain a single TM domain and a single NBD. Half-transporters can either homodimerize (11) or heterodimerize (12) to form a functional complex. G5 and G8 are obligate heterodimers that are not transported out of the endoplasmic reticulum unless they are co-expressed (4). Expression of either recombinant G5 or G8 alone in livers of G5G8−/− mice does not rescue the ability to secrete sterols into bile (3).

Despite substantial progress in our understanding of the physiological roles of G5 and G8, little is known regarding the mechanisms by which these proteins promote movement of sterols into the intestinal lumen or into bile. Although heterodimerization is required for transport of G5 and G8 to the apical membrane of cells, it is not known if the dimer is the functional form of the protein or if higher order complexes are required. To understand the mechanism by which sterols are transported by the G5G8 complex, we have developed in vitro assays using recombinant G5 and G8 expressed in Spodoptera frugiperda (Sf9) ovary cells. Here we report the first reconstitution of sterol transfer by G5 and G8 in a cell-free system.

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2 The abbreviations used are: ABC, ATP-binding cassette; PC, phosphatidylcholine; MES, 2-(N-morpholino)ethanesulfonic acid; CBP, calmodulin-binding peptide; NBD, nucleotide binding domain; TM, transmembrane; IOV, inside-out membrane vesicles; RIVs, right side-in vesicles; BN, Blue Native; AMP-PNP, adenosine 5′-(β,γ-imino)triphasphate; GC-MS, gas chromatography-mass spectrometry; mut, mutant.

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Experimental Procedures

Expression of Mouse G5 and G8 in Sf9 Cells and Preparation of Inside-out Membrane Vesicles (IOV)—Recombinant baculoviruses expressing wild type and mutant G5 and G8 were developed using the Bac-to-Bac baculovirus expression system (Invitrogen). cDNAs encoding mouse G5 and G8 were cloned into the pFastBac vector (Invitrogen) engineered to add 10 histidine residues to the C terminus of each protein. To purify the heterodimeric complex of G5 and G8, expression constructs were generated in which the sequence encoding the histidine tag was replaced with that of calmodulin-binding peptide (CBP). The coding sequence for CBP was obtained by PCR from a proteomics vector, pSGP18 (kindly provided by Dr. Ina Urbatsch, Texas Tech University). Mutations predicted to inactivate the ATPases were introduced into the Walker A motif of G5 (K93M) and into the signature motif of G8 (G216D) using the QuickChange site-directed mutagenesis kit (Stratagene) (13, 14). The recombinant pFastBac donor plasmids were transformed into DH10Bac competent Escherichia coli cells (Invitrogen) to transpose the cDNAs into recombinant bacmids. Recombinant bacmid DNAs were isolated and transfected into Sf9 ovary cells (9 × 10^5 cells/35-mm dish) with CellFECTION reagent (Invitrogen) to produce recombinant baculovirus. The recombinant baculovirus was amplified three times. The Sf9 cells were propagated at 27 °C in IPL-41 insect medium (IPL-41 powder (Atlanta Biologicals), Bacto TC yeastolate (BD Biosciences), tryptose phosphate broth (Invitrogen), 10% heat-inactivated fetal bovine serum (Gemini Bio-products), 0.1% pluronic F-68 (Invitrogen), gentamycin 30 μg/ml, and 0.1% antibiotic-antimycotic (Invitrogen)).

Four liters of Sf9 cells were transfected with recombinant virus. The cells were grown for 72 h and harvested by centrifugation at 500 × g for 5 min. The expression of recombinant proteins was analyzed by immunoblotting using antibodies against G5 or G8 (5). Inside-out membrane vesicles (IOV) were prepared as described (15) with some modifications. In brief, Sf9 cell pellets were resuspended in an ice-cold hypotonic buffer (0.5 mM sodium phosphate, pH 7.4, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml pepstatin) and incubated on ice for 30 min. After centrifugation at 100,000 × g for 40 min at 4 °C, the pellet was suspended in a buffer containing 50 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml pepstatin (TS buffer). The suspension was homogenized on ice with a tight pestle and centrifuged at 500 × g for 10 min. The supernatant was centrifuged at 4,000 × g for 10 min to precipitate the membranes. The membrane pellet was resuspended in TS buffer at a protein concentration of ~35–40 mg/ml, divided into aliquots of 50 μl, and stored at −80 °C. The IOVs were prepared fresh from frozen aliquots by adding TS buffer (final protein concentration of 10 mg/ml) and passage through a 27½-gauge needle 25 times. To obtain a plasma membrane-enriched vesicle preparation, the IOVs were loaded on top of TS buffer containing 35% sucrose in a centrifuge tube and centrifuged in an SW rotor at 100,000 × g for 1 h, and the membrane vesicles at the interface were collected.

Purification of Recombinant G5 and G8 Using Affinity Chromatography—Recombinant G8–his and G5–CBP were co-expressed and extracted from 3.5 g (wet weight) of membrane vesicles using 1% Nonidet P-40. Insoluble materials were removed by centrifugation, and the supernatant was diluted four times with buffer A (50 mM phosphate, pH 7.5, 100 mM NaCl, 5 μg/ml leupeptin, and 5 mM β-mercaptoethanol) to decrease the detergent concentration and incubated at 4 °C with 4 ml of Talon metal affinity resin (BD Biosciences) pre-equilibrated with buffer A. The protein/protein slurry was rotated for 50 min and poured into a column. The flow-through fraction was reloaded onto the column. The column was then washed with 100 column volumes of buffer B (buffer A plus 200 mM NaCl, 5 mM imidazole, 0.05% C12E9, and 7.5% glycerol). Bound proteins were eluted from the column with 20 ml of 250 mM imidazole in buffer B and collected in 1-ml fractions. The peak fractions were combined, and the imidazole concentration was lowered to 15 mM by dialysis of the peak fractions with a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 0.05% C12E9, 10 mM 2-mercaptoethanol, and 5% glycerol and 5 μg/ml leupeptin (CBP binding buffer) (53). The diluted peak fraction was then loaded onto a calmodulin-affinity resin (Sigma) column (4 ml). After washing the column with 20 ml of CBP binding buffer, the bound proteins were eluted in 4 bed volumes of buffer comprising CBP binding buffer in which CaCl2 and MgCl2 were replaced with 2 mM EGTA.

Preparation of Liposomes—Commercially purified lipids (Avanti) dissolved in chloroform were mixed at designated compositions and dried under N2. Residual solvent was completely removed by placing the dried lipid mixtures under vacuum for 1 h. For reconstitution of proteoliposomes, phosphatidylincholine (PC), phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, and cholesterol were mixed at a weight ratio of 46:22:6:6:6:16:4 (liposomes R). For the donor liposomes (liposomes D), PC, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, and cholesterol were mixed at a weight ratio of 35:20:7:8:14:16. A lipid suspension was made by the adding buffer (10 mM Tris/ MES, pH 7.0, 1 mM dithiothreitol plus detergent (1%)) to a dried lipid mixture so that the final lipid concentration was 100 mg/ml. The lipid preparation for proteoliposome reconstitution contained octyl glucoside and that for donor liposomes contained cholate. The lipid suspension was sonicated in a bath-type sonicator (model G112SPIT, Laboratory Supplies Co., Inc.) three times for 15 min until the milky suspension became clear. Both lipid preparations were maintained at 4 °C as stock solutions (100 mg/ml) and were used within a week.

For lipid transfer assays, the stock solution of donor liposomes was diluted in 20 mM Tris, pH 7.4, and 0.5 mM EGTA to a final lipid concentration of 1 mg/ml. A total of 10 μl of [3H]-labeled sterols (1 mg/ml in ethanol; PerkinElmer Life Sciences) was added to the liposome solution, which was sonicated for 2 min to facilitate incorporation of tritiated sterol into the liposomes. Donor liposomes labeled with [3H]cholesterol olate (PerkinElmer Life Sciences), [3H]PC (PerkinElmer Life Sciences), or with both [3H]cholesterol and [3C]cholesterol olate were prepared after the tolueno in which the labeled lip-
ids were provided was removed. Toluene interferes with liposome preparation and limits the functionality of the liposomes as a donor, even at concentrations below 1%. Donor liposomes were prepared on the day of the experiment.

**Reconstitution of Recombinant G5 and G8 in Proteoliposomes**—Reconstitution was performed using either detergent-solubilized crude membrane proteins (10–12 mg of protein/ml in 1% detergent) or the affinity-purified G5G8 complex as the starting material. A total of 3 μl of the stock liposomes (liposomes R) and 3.5 μl of a reconstitution buffer were added sequentially to 10 μl of either the crude detergent extract or the purified proteins (final concentration 0.3 μM sucrose, 0.15 M KCl, 2 mM MgCl₂, and 1 mM dithiothreitol). After vortexing, the mixture was incubated at room temperature for 1 h and then placed on ice. The proteoliposomes were used within 12–16 h of preparation.

**Measurement of ATP-dependent [3H]Sterol Transfer by IOVs and by Reconstituted Proteoliposomes**—Inside-out membrane vesicles containing 35 μg of protein were added to 300 μl of assay buffer (20 mM Tris-Cl, pH 7.4, 60 mM KC1, 30 mM NaCl, and 2 mM MgCl₂). A total of 30 μl of donor liposomes (30 μg of lipids) containing either radiolabeled free sterol ([3H]cholesterol) or [3H]sitosterol (~100,000 cpm/assay), [3H]cholesterol oleate, or [3H]PC was added to the reaction mixture. After a 60-min incubation at 37 °C in the absence or presence of 2.75 mM ATP, the reaction mixture was applied to the top of a centrifuge tube containing 2.5 ml of separation buffer (20 mM Tris-Cl, pH 7.4, 0.5 mM EGTA, and 150 mM sucrose) and centrifuged using a Beckman TL100.4 rotor at 100,000 rpm for 30 min. The supernatant was transferred to a scintillation vial, and the sides of the tube were wiped clean. The pellet was suspended in 300 μl of assay buffer and transferred to a second scintillation vial. Both fractions were mixed with scintillation mixture, and the radioactivity was measured in a Beckman scintillation counter. The same procedure was used to measure sterol transfer from donor liposomes to reconstituted proteoliposomes except that the proteoliposomes (4–5 μl) were first diluted at least 20-fold in 100 μl of buffer containing 20 mM Tris-Cl, pH 7.4, 2 mM MgCl₂, 100 mM NaCl, and 300 mM sucrose to seal the proteoliposomes with the high density buffer. The difference in the densities of the proteoliposomes and the donor liposomes was sufficient to separate the two by centrifugation.

**Measurement of Mass Cholesterol Transfer**—Transfer assays were performed using IOVs in the presence or absence of 2.75 mM ATP as described above except that the cholesterol in the donor liposomes was not labeled. IOVs were incubated with donor liposomes in reaction mixture, isolated by centrifugation, and then suspended in 0.1 ml of distilled water. Ent-cholesterol was labeled by incorporation of six deuterium atoms during the initial synthesis. The transfer of cholesterol and of cholesterol-oleate, or [3H]PC was added to the reaction mixture. After a 60-min incubation at 37 °C in the absence or presence of 2.75 mM ATP, the reaction was terminated by addition of cold ATP to a final concentration of 10 mM after 15 min. For IOVs, the unbound 8-azido-[α-32P]ATP was removed by washing the vesicles with a buffer containing 1 mM cold ATP followed by centrifugation. The vesicles were resuspended in 10 μl of reaction buffer and UV-irradiated on ice for 1 min to cross-link the trapped 8-azido-[α-32P]ADP to proteins. The resulting membrane vesicles were then solubilized using 1% Nonidet P-40, centrifuged to remove insoluble materials, and loaded onto BN-PAGE gels. The gel was fixed, extensively washed, and dried prior to autoradiography to detect the binding of radioactive-labeled nucleotide. For detergent-solubilized G5 and G8, the reaction mix was size-fractionated on a BN-PAGE (5–13%) using a running buffer containing 1 mM cold ATP to compete out the unbound azido-ATP. After the native gel electrophoresis, the samples in the gel were UV-irradiated on ice for 1 min to cross-link the trapped 8-azido-[α-32P]ADP to proteins, before the gel was fixed, washed, dried, and autoradiographed.

ATPase activity was measured in 200 μl of assay buffer containing 50 mM Tris/MES, pH 7.0, 30 mM KCl, 2.5 mM MgCl₂, 2 mM [γ-32P]ATP (400 cpm/nmol) and in the absence or presence of NaVO₄ as an inhibitor. The ATP hydrolysis reaction was carried out at 37 °C for 1 h and terminated by addition of 1 ml of 1.25 N perchloric acid. Liberated ³²P, was extracted and measured as described previously (20).

**RESULTS**

**Expression of Recombinant Mouse G5 and G8 in Sf9 Cells**—Recombinant wild type and mutant (mut) forms of mouse G5 and G8 were expressed in Sf9 cells, either individually or together, using a baculoviral expression system. The mutant forms of G5 (mutG5) and G8 (mutG8) used in these experiments contained point mutations predicted to inactivate the ATPase. Lysine was substituted for methionine at residue 93 in the Walker A motif of G5; mutation of the corresponding residue in other ABC transporters inactivates transport activity (13, 21, 22). A missense mutation in a highly conserved residue in the signature motif was introduced into G8 (G216D); substitution of the corresponding residue in two other ABC transporters impairs the ATPase and transport activity of both transporters (14, 23).

The apparent molecular mass of the histidine-tagged G5 and G8, as determined by migration on an SDS-polyacrylamide gel, was between 65 and 68 kDa (Fig. 1). Both proteins were glycosylated. Treatment with peptide N-glycanase F was associated...
ABCG5 and ABCG8 Form Heterodimers When Co-expressed—To confirm that co-expression of G5 and G8 in Sf9 cells was associated with the formation of heterodimers, partially purified membrane proteins from Sf9 cells expressing G5 alone, G8 alone, or both G5 and G8 were subjected to immunoprecipitation using antibodies against either G5 or G8. The immunoprecipitated proteins were then analyzed by immunoblotting (Fig. 3). Proteins were immunoprecipitated using a monoclonal antibody to G8 (3) and then immunoblotted for G5. G5 was detected only in cells co-expressing G5 and G8. Similarly, when G5 was immunoprecipitated, G8 was detected only in the immunoblot of cells also expressing G5. Because no monoclonal antibody was available for G5, we used a polyclonal antibody against G8 or against the His tag to blot the recombinant proteins. The results of these experiments demonstrated that G5 and G8 formed heterodimers when the two proteins were co-expressed in Sf9 cells.

**Dimers of G5 and G8 Catalyze ADP Trapping**—After ATP binds to an ATPase, the high energy phosphate bond is hydrolyzed, and ADP and phosphate are released. In some ATPases, ADP can be “trapped” in the nucleotide binding domain in the presence of ATPase inhibitors such as sodium vanadate (NaVO₄) (24, 25), beryllium fluoride (BeF₂) (19, 26), or aluminum fluoride (AlF₃) (19). To determine which form of the par-...
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When the experiments were repeated using 8-azido-[α-32P]ATP and BeF₆⁻, no labeling was seen (data not shown). ADP trapping was also seen with samples prepared from cells expressing G5 or G8 individually. Thus, homodimers as well as heterodimers catalyzed ATP hydrolysis.

ATP-dependent Cholesterol Transfer by IOVs from Sf9 Cells Expressing Recombinant G5 and G8—Inside-out membrane vesicles were prepared from Sf9 cells expressing recombinant G5 and G8 using a method modified from Zelcer et al. (15). ADP trapping was observed, indicating that the NBD domains were exposed to the medium in these vesicles (Fig. 5A). To confirm that the majority of the vesicles prepared were IOVs, the vesicles were subjected to trypsin digestion and compared with right side-in vesicles (RIVs) prepared from mouse liver apical membranes. The cytoplasmic loop, which has an estimated molecular mass of 40 kDa, was used to generate the anti-G5 antibody. Therefore, the epitope for the antibody should be protected in the RIVs but not the IOVs. No immunoreactive bands were observed in trypsin-treated IOVs, whereas a 37-kDa band was apparent in the RIVs (Fig. 5B). This finding indicates that most of the NBDs in the IOVs were exposed to the medium and were thus in the inside-out orientation.

Because of the low solubility of sterols in aqueous solutions, donor vesicles were used to deliver substrate to the membrane vesicles. Freshly prepared unilamellar liposomes with a lipid composition similar to that of mammalian plasma membranes were labeled with [3H]cholesterol (or other lipids) and incubated with the IOVs for 1 h in the presence or absence of ATP and inhibitors. A two-step density gradient ultracentrifugation was used to effectively separate donor and acceptor vesicles after the transfer reaction. When centrifuged separately, 98.6% of the donor liposomes remained in the supernatant, whereas over 95% of IOVs precipitated to the bottom of the centrifuge tube with minimal release of radiolabeled lipid to the supernatant (data not shown).

When G5G8-containing IOVs were incubated with radiolabeled donor liposomes, ATP-stimulated sterol transfer from the donor liposomes to the IOVs was observed (Fig. 6A). To ensure that the ATP-dependent sterol transfer was not because of the contamination of donor liposomes with the IOVs during the process, the donor liposomes were co-labeled with [3H]cholesterol and the transport-inert sterol ester [14C]cholesteryl oleate. ATP-dependent transfer of [14C]cholesteryl oleate was less than one-fifth that observed for cholesterol, indicating that contamination from donor liposomes was minimal.

Addition of ATP to the membrane vesicles containing wild type G5 and G8 resulted in a 3.5-fold increase in [3H]cholesterol uptake (Fig. 6B). The transfer activity was sensitive to vanadate at concentrations ranging from 15 μM to 0.15 mM. Beryllium fluoride (BeF₆⁻) also inhibited cholesterol uptake activity. Thus, the cholesterol transfer activity of G5G8-expressing IOVs was ATP-dependent and vanadate-sensitive. Very little ATP-dependent cholesterol transfer was observed when membrane vesicles containing mutant G5 and G8 were used (Fig. 6B) or when bovine serum albumin was used as a donor (data not shown).

The time course of the ATP-dependent [3H]cholesterol uptake is shown in Fig. 7. Cholesterol uptake continued...
throughout the 2-h observation period. However, the rate of sterol uptake decreased progressively over the course of the assay. Accordingly, we were not able to determine the rate of G5G8-mediated sterol transfer in this assay. Whether this decrease reflects changes in the composition of the donor and acceptor vesicles because of bulk transfer of sterol (see below) or instability of the G5G8 complex under these conditions is not known.

To determine whether mass transfer of cholesterol by G5 and G8 occurred under these conditions, the cholesterol content of the IOVs was measured by GC-MS. Addition of ATP was associated with an almost 2-fold increase in the cholesterol content of the IOVs containing wild type G5G8. The increase in sterol mass was abolished by vanadate (Fig. 8A). No detectable increase in ATP-dependent cholesterol mass was seen in vesicles containing mutG5 and mutG8. Uptake of [3H]sitosterol was comparable with that of cholesterol (Fig. 8B). To determine the specificity of lipid transfer, we measured ATP-stimulated uptake of [3H]PC, cholesteryl oleate, and the deuterated enantiomer of cholesterol (ent-cholesterol). The uptake of PC and cholesteryl oleate was much lower than that of free cholesterol and was not inhibited by vanadate (Fig. 8B). The uptake of ent-cholesterol was also much lower than that of natural cholesterol but was inhibited by vanadate.

**Triphosphate Nucleotide Specificity and Divalent Cation Requirement for G5G8-mediated Cholesterol Uptake**—Other members of the ABC transporter family bind and hydrolyze triphosphate nucleotides other than ATP (27–29). To examine the triphosphate nucleotide specificity of the G5G8 heterodimer, we monitored transfer of radioactive cholesterol from donor liposomes to membrane vesicles in the presence of ATP, GTP, UTP, dCTP, and AMP-PNP (Fig. 9A). ATP was the most effective substrate, but other triphosphate nucleotides also supported cholesterol uptake, albeit at lower levels (ATP > GTP > UTP). Thus, ATP was the preferred nucleotide substrate for G5G8. No activity was detected when divalent cation was omitted from the reaction condition (Fig. 9B).

**Homodimers of G5 and G8 Do Not Catalyze ATP-dependent and Vanadate-sensitive Cholesterol Transfer**—Because G5 or G8 homodimers catalyze ADP trapping (Fig. 4), we next examined if homodimers of G5 or G8 can transfer sterols. Although
IOVs containing homodimers of G5 or G8 showed a small increase in nucleotide-stimulated cholesterol uptake, the increase was not inhibited by vanadate (Fig. 10) or BeF$_x$ (data not shown). Expression of increasing amounts of G8 in cells expressing G5 was associated with a concomitant increase in cholesterol transfer (Fig. 10).

**The Cholesterol Uptake Activity of G5G8 Is Not Mediated by Membrane Binding or Fusion**—To examine the possibility that the G5G8 complex serves as site of attachment between donor liposomes and IOVs, we added trypsin to the assay solution at the beginning or at the end of the assay (Fig. 11). Addition of trypsin at the beginning abolished transfer, whereas addition of trypsin at the end of the assay did not reduce ATP-dependent $[^{3}H]$cholesterol transfer to the IOVs. This result is not consistent with a G5G8-mediated increase in IOV/liposome binding. Taken together with the low uptake of $[^{3}H]$cholesterol oleate and $[^{3}H]$PC (Fig. 8B), these data suggest that the observed sterol uptake of the G5G8 containing IOVs is unlikely to be because of liposome/vesicle fusion.

**Functional Reconstitution of Purified G5G8 Complex into Proteoliposomes**—As a first step in the functional reconstitution of purified G5G8 heterodimers, we reconstituted the detergent-solubilized proteins. We tested a series of detergents (Nonidet P-40, C$_{12}$E$_{9}$, Triton X-100, digitonin, zwittergent 3-16, and octyl glucoside) to determine their ability to support functional reconstitution of the solubilized G5G8 complex into proteoliposomes. Sterol transfer was observed with octyl glucoside or cholate but not with Nonidet P-40 or C$_{12}$E$_{9}$ (data not shown). However, transfer activity could be restored if Nonidet P-40 or C$_{12}$E$_{9}$ was used for the initial extraction and then exchanged with octyl glucoside using chromatography. Because Nonidet P-40 solubilized G5G8 most efficiently, it was used for initial solubilization of G5G8 from Sf9 membranes. G5(His-tagged)G8(CBP-tagged) complex was then purified by sequential affinity chromatography using a metal-affinity column (Talon resin) followed by a calmodulin-affinity column (Fig. 12). Proteoliposomes reconstituted with the purified G5G8 complex demonstrated an ATP-dependent $[^{3}H]$cholesterol transfer activity that was almost completely inhibited (90%) by vanadate (150 $\mu$M) (Table 1). The specific ATPase activity of the purified G5G8 complex ranged from 0.12 to 0.14 $\mu$mol of P$_i$/mg/min and was moderately inhibited by vanadate (Table 1). Addition of cholesterol, sitosterol, or phospholipids did not increase ATPase activity. The detergent-solubilized and purified G5G8 complex was functionally very unstable; unlike
the transfer activity in the G5G8-containing IOVs, which was stable at −80 °C for several months, the purified G5G8 complex was freeze-labile even in the presence of lipids and 0.25 M sucrose. When reconstituted into proteoliposomes and main-
tained on ice, transfer activity gradually decayed after 12–16 h, with a t_1/2 of 6–8 h (data not shown).

**DISCUSSION**

The major finding of this study is that recombinant G5 and G8 promote transfer of cholesterol and sitosterol in vitro. ATP-dependent transfer was documented using membrane vesicles isolated from Sf9 cells expressing high levels of recombinant G5 and G8. The transfer activity is sensitive to both vanadate and BeF_4^−, two commonly used inhibitors of ABC transporters. When co-expressed in Sf9 cells, G5 and G8 formed heterodimers, as demonstrated by BN-gel electro-
phoresis and co-immunoprecipitation. The G5G8 heterodimer trapped ADP in the presence of BeF_4^− and AlF_3, indicating that the recombinant proteins hydrolyzed ATP. IOVs prepared from Sf9 cells expressing G5 and G8 catalyzed ATP-dependent transfer of cholesterol and sitosterol from donor liposomes. Sterol transfer was confirmed by demonstrating mass transfer of cholesterol from the donor liposomes to the membrane vesicles. ATP was the preferred nucleotide substrate of G5G8, although other triphosphate nucleotides supported transfer. These data provide the first biochemical evidence that cholesterol and sitosterol are direct substrates of G5 and G8.

G5- and G8-mediated transfer of cholesterol is stereoselective; ATP-dependent transfer of natural cholesterol was much more efficient than that of the enantiomer of cholesterol. Therefore, cholesterol transfer by G5G8 must involve direct interaction between the sterol and the transporter and is not a secondary consequence of phospholipid flipping or other alteration of the lipid environment of the membranes. However, the sterol transfer assay reported here does not reveal the mecha-
nism by which G5 and G8 transfer sterols. G5G8 may act as a sterol flippase, promoting the movement of sterols from the cytoplasmic to luminal leaflet of the membrane. Energetic con-
siderations and evidence from several model systems suggest that trans-bilayer movement of cholesterol is very rapid in most biological membranes. Thus protein-mediated cholesterol flip-
ning would only be required if the canalicular membrane is in the gel-ordered phase (30). Alternatively, G5 and G8 may increase the accessibility of membrane cholesterol to acceptors such as bile acid-PC micelles. Steck and co-workers (31) have shown that at the equimolar cholesterol-phospholipid ratios observed in red cell membranes, cholesterol is largely inaccessible to cyclodextrin and cholesterol oxidase. G5 and G8 may
use the energy of ATP hydrolysis to push cholesterol out of the plane of the membrane, increasing its accessibility to acceptors, as was originally proposed by Small (30). Further studies will be required to define the mechanism by which G5 and G8 promote sterol efflux.

Identification of the physiological substrates of ABC transporters remains a formidable challenge. Several ABC transporters, such as ABCB1 and ABCG2, were isolated based on their ability to confer resistance to chemotherapeutic agents (32, 33), but the natural substrates of these transporters have not been identified. For some transporters, the natural substrates have been inferred from the substances that accumulate when the transporter is inactivated. The accumulation of cholesterol and plant sterols in humans and mice lacking G5 and G8 suggested that the G5G8 complex is a neutral sterol transporter (2). The reconstitution of G5G8-mediated sterol transfer in vitro strongly supports this notion and provides a system for dissection of the transport mechanism.

The transport activities of ABC transporters have been studied using diverse experimental systems, including cell-based assays, membrane vesicles, and reconstituted proteoliposomes. Cell-based assays most closely resemble in vivo conditions and have been successfully employed to examine the transport characteristics of several ABC transporters, including some that transport sterols (ABCA1 and ABCG1) (34, 35). Our efforts to develop a cell-based transport assay for G5G8 in cultured mammalian cells (CRL-1601 or Madin-Darby canine kidney cells) and in insect cells (Sf9 cells) expressing recombinant G5 and G8 were unsuccessful (data not shown). Prior to the studies described here, the only available in vivo assay system demonstrated that G5 and G8 are obligate heterodimers that promote the efflux of neutral sterols into bile but could not be used to determine whether sterols are direct substrates of the transporter.

Membrane vesicles can be used to study transport in isolation from other cellular components. When vesicles are prepared in an “inside-out” orientation (i.e. so the ATP binding domains of the transporter are on the outside), the ATP and the transport substrate can be added to the medium. Transport to the membrane vesicles can then be monitored. Such assays have been established for some mammalian ABC transporters, including ABCB11, the bile salt export pump (36), several members of the ABCB subfamily (15, 37), and ABCG2 (38). Developing such assays for a sterol transporter is particularly challenging because of the very low solubility of cholesterol in aqueous solution and the high concentration of cholesterol in the plasma membrane. Tam et al. (39) circumvented this limitation by using 25-hydroxycholesterol as the substrate in an in vitro assay of ABCA1. Others have assessed sterol transport by using sterols as a competing substrate for the transport of a soluble substrate (40) or by testing for stimulation of ATPase activity by sterols (38). In this study, crude membrane vesicles expressing G5 and G8 took up cholesterol and sitosterol, the presumptive substrates for the transporter, but not cholesteryl esters or phosphatidylcholine. Affinity-purified G5 and G8 also promoted sterol uptake when reconstituted in proteoliposomes. The development of a cell-free cholesterol transfer assay for G5G8 represents the first direct measure of neutral sterol transfer by an ABC transporter.

When G5 and G8 were co-expressed in Sf9 cells, the transporters formed heterodimers. This finding is consistent with a series of prior observations suggesting that G5 and G8 function as a heterodimer in vivo (2). Thus, expression of recombinant G5 and G8 in Sf9 cells recapitulates the oligomeric structure of the native transporter.

We previously demonstrated that both the precursor and mature forms of G5 and G8 bind ATP (41). When G5 and G8 were expressed individually in Sf9 cells, they formed homodimers that catalyzed ADP trapping. Could homodimers of G5 and G8 form and promote sterol transport in vivo? When recombinant G5 or G8 is expressed at high levels in cultured cells, a trace amount of homodimer can be detected (4). However, when recombinant G5 or G8 are expressed individually in

**TABLE 1**

Substrate transfer and ATPase activity by recombinant G5G8

Recombinant G5G8 was expressed in Sf9 cells, and assays were performed using IOVs or affinity-purified G5G8 reconstituted into proteoliposomes as described under “Experimental Procedures.” A silver-stained SDS-polyacrylamide gel of the starting material from the solubilized membranes and the purified complex is shown in Fig. 12. The content of G5 and G8 in the crude IOVs was estimated using immunoblotting. ATP hydrolysis was assayed using soluble G5G8 eluted from the calmodulinaffinity column. ATP hydrolysis activity was not measured in the crude membrane vesicles due to high background activity.

| Preparation | Sf9 membrane vesicles | Affinity-purified G5G8 complex |
|-------------|-----------------------|------------------------------|
| **Purification** | | |
| Fold purification | 1 | >1000 |
| Estimated purity (% of total protein) | <0.1% | >95% |
| **[1H]cholesterol uptake** | | |
| Activity (pmol/µg/h) | 37 | 3330 |
| Specific activity (nmol/mg/min) | 0.6 | 50 |
| VO₄ inhibition (¹⁵µM) (%) | 65% | 70% |
| VO₄ inhibition (0.15 mM) (%) | 70% | 90% |
| ATPase activity (µmol/mg/min) | | |
| - cholesterol | Not measured | 130 |
| + cholesterol | Not measured | 105 |
| VO₄ inhibition (0.15 mM) | Not measured | 35% |
the livers of G5G8−/− mice, biliary sterol levels are not increased (3, 41). Furthermore, when co-expressed in cells, G8 is almost completely co-immunoprecipitated together with G5, indicating that the proteins preferentially heterodimerize (4). These data suggest that under normal conditions in vivo, G5 and G8 homodimers do not participate in sterol transport to any appreciable extent.

The ATPase activity of purified reconstituted G5G8 was comparable with that of ABCG2 (42), but the ATPase activity was slightly decreased, rather than stimulated by sterols. Interestingly, the ATPase activity of ABCA1, another ABC transporter that is involved in cholesterol transport, was slightly reduced by addition of cholesterol (43). It is not known if the lack of substrate-stimulated ATPase activity is an artifact of the systems used to analyze these transporters or an intrinsic property of ABCA1 and G5G8.

ADP could be trapped in a complex with G5 and G8 in the presence of BeF− and AlF3−, which mimic the structure of phosphate. No labeling was seen with either inhibitor when ATP was radioactively labeled at the γ-position (data not shown), confirming that the trapped nucleotide was ADP. This finding indicates that the NBDs of G5 and G8 are both capable of binding and hydrolyzing ATP. These findings are consistent with those obtained previously with recombinant G5 and G8 expressed in mammalian cells (41). Interestingly, in vivo reconstitution studies in G5G8−/− mice indicated that the NBD formed by the Walker A and B domains of G5 and the signature sequence of G8 is essential for sterol efflux, whereas the integrity of the NBD formed by the Walker A and B domains of G8 and the signature sequence of G5 is not (41). The relative contributions of the two NBDs to sterol transfer can now be directly determined using the in vitro assay developed in this study.

Although the sterol transfer activity of the G5G8 complex was very sensitive to vanadate, a potent inhibitor of many phosphofructokinase enzymes, vanadate inhibited the ATPase activity only modestly and did not induce detectable ADP trapping. Previous studies have revealed diverse responses to inhibitors of phosphofructokinase, such as vanadate, BeF−, and AlF3−. Some transporters, such as P-glycoprotein (ABC1), trap ADP in the presence of all three inhibitors (26, 44). Others, such as TAP1 and TAP2 (ABC2 and ABC3), trap ADP with both BeF− and AlF3− but only weakly with vanadate (19). MRP6 (ABCC6) traps ADP in the presence of BeF− and Mg2+ but requires Ni2+ for the reaction to occur with vanadate (45). The differences in inhibitor sensitivities between the various ABC transporters are presumed to reflect structural differences in the ATP-binding pocket, but the basis of these differences is not known. X-ray crystallographic studies of the myosin-Mg-ADP-vanadate complex revealed that ADP trapping resulted in significant conformational changes, which are considered to represent the transition state of the catalysis cycle (46). The complex of myosin-Mg-ADP with AlF3− was similar, although not identical to that seen with vanadate (46). In contrast to these findings, BeF− formed a complex with myosin-ADP that adopted the observed configuration of the protein prior to ATP hydrolysis (47). Structural studies of the NBDs of G5 and G8 will be required to provide further insights into the catalytic mechanism of this transporter.

ATP was the preferred nucleotide substrate for G5 and G8. Triphosphate nucleotide specificity varies between different ATPases. For some ATPases, such as mammalian V-ATPase (48), ATP is the only nucleotide that can be used as an energy source. Other ATPases, such as ATPase II (ATP8A1) (49), are quite promiscuous and hydrolyze a broad spectrum of triphosphate nucleotides. Most ABC transporters have been studied using ATP as the energy source, although some can use CTP, GTP, and UTP (27–29). These data are consistent with our findings that although ATP was the preferred substrate of G5G8, CTP, GTP, and UTP also supported sterol transfer, albeit not as efficiently.

Many questions regarding G5G8 function remain to be answered. Among the most important are whether the sterol transfer activity observed in this study reflects G5G8 function in vivo. Although the observed sterol transfer activity was G5G8-specific, ATP-dependent, and inhibitor-sensitive, we have not been able to experimentally demonstrate the destination of the transferred sterols, whether it is the outer or inner leaflet of the membrane or the interior of the vesicles. Does G5G8 acquire cholesterol from a cytosolic donor in vivo and transfer it across the membrane to release it into bile or does it act as a flippase? Is a phospholipid co-transported with cholesterol by G5G8? Are other proteins required for maximum transfer activity? The reconstitution system described here provides a tool with which these questions can potentially be addressed. In addition, the purified and reconstituted transport system can be used to identify and characterize the structural determinants of sterol transfer activity by G5 and G8, as well as regulatory effects of other factors, including proteins and lipids. Characterization of sterol transfer in this system may also provide insights into the mechanisms by which other ABC proteins promote sterol transport.

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