Mutations in ABCG5 (G5) or ABCG8 (G8) cause sitosterolemia, an autosomal recessive disease characterized by sterol accumulation and premature atherosclerosis. G5 and G8 are ATP-binding cassette (ABC) half-transporters that must heterodimerize to move to the apical surface of cells. We examined the role of N-linked glycans in the formation of the G5/G8 heterodimer to gain insight into the determinants of folding and trafficking of these proteins. Site-directed mutagenesis revealed that two asparagine residues (Asn585 and Asn592) are glycosylated in G5 and that G8 has a single N-linked glycan attached to Asn619. N-Linked glycosylation of G8 was required for efficient trafficking of the G5/G8 heterodimer, but mutations that abolished glycosylation of G5 did not prevent trafficking of the heterodimer. Both G5 and G8 are bound by the lectin chaperone, calnexin, suggesting that the calnexin cycle may facilitate folding of the G5/G8 heterodimer. To determine the effects of 13 disease-causing missense mutations in G5 and G8 on formation and trafficking of the G5/G8 heterodimer, mutant forms of the half-transporters were expressed in CHO-K1 cells. All 13 mutations reduced trafficking of the G5/G8 heterodimer from the endoplasmic reticulum to the Golgi complex, and most prevented the formation of stable heterodimers between G5 and G8. We conclude that the majority of the molecular defects in G5 and G8 that cause sitosterolemia impair transport of the sterol transporter to the cell surface.

Mutations in either ABCG5 (G5) or ABCG8 (G8) cause sitosterolemia, an autosomal recessive disorder characterized by the accumulation of both plant-derived (primarily sitosterol) and animal-derived sterols (cholesterol) in plasma and tissues (1–3). In mice, G5 and G8 are located on the apical surfaces of enterocytes and hepatocytes, where they limit the absorption of dietary sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8). Individuals with sitosterolemia have a generalized increase in the absorption of dietary neutral sterols and promote the excretion of cholesterol into bile, respectively (4–8).
acids in G5 and G8 to which the N-linked glycans are attached were mapped, and the roles of N-linked glycans in chaperone interaction, dimer formation, and ER to Golgi translocation of G5/G8 were examined. The processing of the N-linked glycans on G5 and G8 was then monitored to determine the effect of disease-causing, missense mutations in ABCC5 and ABCC8 on the trafficking of heterodimers containing mutant G5 or G8 in cultured cells. The results indicate that the majority of disease-causing mutations in G5 and G8 impair transport of the G5/G8 heterodimer beyond the ER.

MATERIALS AND METHODS
Cell Culture—CHO-K1 cells (ATCC) were cultured in Ham’s F-12 and Dulbecco’s modified Eagle’s medium 50% (v/v) containing 5% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) in a humidified incubator (8% CO₂). CRL-1601 cells stably expressing G5, G8, or both proteins were cultured in Dulbecco’s modified Eagle’s medium (glucose, 1 g/liter) containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 500 µg/ml G418 (Invitrogen), and 500 µg/ml Zeocin in a humidified incubator (5% CO₂).

Site-directed Mutagenesis—Single bp changes in mouse ABCC5 and ABCC8 cDNA expression constructs (11, 20) were introduced using sense and antisense oligonucleotides (25-mers) containing the mutant base(s) in the central codon. Each mutation was generated with Pfu Turbo (Stratagene, La Jolla, CA), dNTPs, and the appropriate oligonucleotide pair (18 cycles of 94°C, 45 s; 65°C, 45 s; 68°C, 18 min). After PCR, template DNA was removed by digestion with DpnI endonuclease, and the reaction components were removed with a QiaQuick Spin PCR, template DNA was removed by digestion with DpnI endonuclease,

Transfections and Cell Lysates—CHO-K1 cells were seeded (1 × 10⁶ cells/dish) in 35-mm dishes. Expression plasmids (1 µg/dish) were transiently transfected into CHO-K1 cells using FuGENE (6 µl of FuGENE/µg of DNA) according to the manufacturer’s protocol. The appearance of the fully processed form of G5 was enhanced by coexpressing an excess of normal G5. Similarly, maturation of G8 was enhanced by coexpression of excess G5. To provide optimum conditions for trafficking, each mutant construct was coexpressed with a 2-fold excess of its normal partner. Cell lysates were prepared 48 h after transfection. Cells were washed twice in phosphate-buffered saline, pH 7.4, incubated in 0.3 ml of Triton lysis buffer (50 mM Tris, 80 mM NaCl, 2 mM CaCl₂, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 5 µg/ml pepstatin A, pH 8) for 30 min at 4°C, and harvested by scraping with a rubber policeman. Cell membranes were transferred to 1.5 ml tubes and centrifuged for 15 min (15,000 × g, 4°C). Supernatants were transferred to new 1.5-ml tubes and subjected to SDS-PAGE and immunoblot analysis.

Analysis of Glycosylation—CHO-K1 cells were transiently transfected with epitope-tagged murine G5, G8, and the expression constructs containing mutations. G5 contained three copies of the Myc epitope and G8 contained three copies of a HA epitope, each at the C terminus (11). Proteins were denatured by heating in 0.5% SDS, 1 mM β-mercaptoethanol (95°C, 5 min) and incubated in the presence or absence of 10 units of N-glycosidase F (PNGase F, Calbiochem) in 60 mM Tris, pH 8.6, and 1% (v/v) Triton X-100. Samples were diluted with protein sample buffer, heated to 95°C for 5 min, and subjected to SDS-PAGE and immunoblot analysis.

SDS-PAGE and Immunoblot Analysis of G5 and G8—Protein concentrations of cell lysates and fractions were determined using the Bio-Rad DC assay according to the manufacturer’s protocol. Protein sample buffer was added to a final concentration of 1×, and samples were heated to 95°C for 5 min. Proteins were size fractionated on 10% SDS-PAGE polyacrylamide gels at 50 mA and subsequently transferred to nitrocellulose membranes at 50 V for 2 h. Membranes were incubated in buffer A (20 mM Tris, pH 7.6, 137 mM NaCl, 0.5% Tween 20, 5% milk) for 60 min at 22°C prior to the addition of primary antibodies. Primary antibodies were diluted in buffer A and incubated with membranes for 60 min at 22°C. Membranes were washed three times for 5 min in buffer B (20 mM Tris, pH 7.6, 137 mM NaCl, 0.5% Tween 20, 0.2% nonfat milk). Horseradish peroxidase-conjugated donkey anti-rabbit IgG or goat anti-mouse IgG (Pierce) was diluted (1:20,000) and incubated with membranes for 60 min at 22°C. Membranes were washed three times for 5 min in buffer B and visualized using SuperSignal enhanced chemiluminescence (Pierce). Protein loading was assessed by visual inspection of Ponceau S-stained membranes.

Immunosolation—Comunoprecipitation of G5 and G8 was conducted essentially as described previously (20). Transfected CHO-K1 cells or CRL-1601 cells harboring empty plasmids or plasmids containing cDNAs encoding G5, G8, or both G5 and G8 (11) were grown to 90% confluence in 100-mm plates. Cells were washed twice with prechilled detergent-free IP buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂) and scraped on ice in 1 ml of freshly prepared IP buffer containing 0.5% (w/v) digitonin (Calbiochem). Cells were passed through a 25-gauge needle 20 times, transferred to a 1.5-ml microcentrifuge tube and incubated at 4°C on a rotator for 30 min. Samples were centrifuged for 15 min (15,000 × g, 4°C) and supernatants were transferred to a new microcentrifuge tube. Antibodies (10 µg) directed against the epitope tag at the C terminus of G5 (Mye, 9E10) or G8 (HA, 12CA5) and protein A-agarose (20 µl) were added, and samples were incubated for 16 h (4°C, with rotation). Protein A-bound antibodies and proteins were separated from supernatants by centrifugation (15 s, 800 × g) and washed three times for 15 min in IP buffer (4°C). For precipitations with pAbs directed against calcineurin and calreticulin, antisera were prebound to protein A-agarose and washed with IP buffer to remove serum proteins. Immunoprecipitation experiments also were conducted in magnesium free IP buffer supplemented with 5 mM EDTA. Results were identical in the presence or absence of the divalent cation.

Blue Native PAGE—Total cellular membranes were prepared from transiently transfected CHO-K1 cells and subjected to native-PAGE (28). Cells were equilibrated and harvested on ice in membrane buffer (10 mM Tris, pH 7.8, 150 mM NaCl, 0.4 mM EDTA, 4°C), lysed by passing 20 times through a 25-gauge needle, and centrifuged (1,000 × g, 4°C, 5 min) to remove cellular debris and nuclei. Supernatants were transferred to TLA 100.2 tubes and centrifuged (100,000 × g, 4°C, 60 min) to pellet membranes. Supernatants were aspirated, and membrane pellets were solubilized in EGTA-free membrane buffer containing 1% (w/v) CHES by passing through a 25-gauge needle (10 times).
proic acid, 15% (v/v) glycerol) and electrophoresed (cathode, 50 m M
precursor form of G5 has an apparent molecular mass of 70
N
sites are utilized in G5 and G8, site-directed mutagenesis was
to assess trafficking of normal and mutant forms of G8. B, two additional
mutants of G8 (NA, and NQ) were examined for their ability to support
trafficking of G5. Samples were processed and analyzed as in A.

and incubating for 30 min at 4 °C on a rotator. The insoluble fraction
was removed by centrifugation (15,000 × g, 4 °C, 15 min). Supernatants
were collected, and blue native loading buffer (50 mM BisTris, pH 7.0,
750 mM 6-aminocaproic acid, 7 mM phenylmethylsulfonyl fluoride, 10%
v/v glycerol, 5% v/v Coomassie G-250) was added to a final concentra-
tion of 1×. Samples were loaded onto a 5–15% (v/v) acrylamide gradi-
et gel prepared in native gel (100 mM BisTris, pH 7.0, 1 mM 6-aminocaproic
acid, 15% (v/v) glycerol) and electrophoresed (cathode, 50 mM
Tricine, 15 mM BisTris, 0.02% w/v Coomassie G250, pH 7.0; anode, 50
mM BisTris, pH 7.0; 150 V, 16–18 h, 4 °C). Proteins were transferred
to polyvinylidene difluoride membranes. Membranes were washed in
methanol to remove Coomassie Blue stain and subjected to immunoblot
analysis as described above.

RESULTS

Mapping N-Linked Glycosylation Sites—Mouse G5 contains
canonical N-linked glycosylation sequences (NXS/T) at aspar-
agine positions 437, 585, and 592 (Fig. 1A). Two of these sites
(Asn585 and Asn592) are conserved between mice and humans.
The third (Asn437) nonconserved site is predicted to reside
within a cytoplasmic loop and is not expected to be modified
(Fig. 1A). Mouse G8 contains five canonical N-linked sites.
Three of these (Asn at 90, 160, and 619) are conserved between
mice and humans, but only Asn619 is expected to be utilized
because Asn80 and Asn160 are predicted to be in cytoplasmic
regions of the protein.

To determine which of the canonical N-linked glycosylation
sites are utilized in G5 and G8, site-directed mutagenesis was
used to replace the candidate asparagine (N) codons with glut-
amine (Q) codons in the mG5-Myc and mG8-HA cDNAs. The
N-glycosylation status of the normal and mutant proteins was
analyzed in transiently transfected CHOK1 cells (Fig. 1B).
Cell lysates were prepared and incubated in the presence or
absence of PNGase F, which removes all N-linked glycans, and
then subjected to SDS-PAGE and immunoblot analysis. The
precursor form of G5 has an apparent molecular mass of 70–72
kDa, whereas the apparent mass of the mature form ranges
from 85 to 100 kDa. When the normal forms of the two half-
transporters were coexpressed, the mature forms of the protein
predominated. Mutation of Asn437 to glutamine (construct
QNN) in G5 had no effect on the apparent molecular mass,
whereas mutation of either Asn585 (construct NQN) or Asn592
(construct NNQ) resulted in a decrease in the apparent
molecular mass of the mature protein. No mature forms of the
protein were present when both Asn585 and Asn592
were changed to glutamine (QQQ) or when all three asparagines
were changed to glutamine (QQQ). The reductions in apparent
molecular mass of normal (NNN), Asn585 (NQN), or Asn 592
were examined for their ability to support
molecular mass was used to determine whether the complex trafficked
beyond the ER. Normal G8 (NN) was used to assess trafficking of
normal and mutant forms of G8. B, two additional
mutants of G8 (NA, and NQ) were examined for their ability to support
afferent G5 after PNGase F treatment indicated that each of
these proteins contained at least one N-linked glycan. In con-
tраст to these results, no change in size of the proteins was seen
with PNGase F treatment when both Asn585 and Asn 592
mutated to glutamine (QQQ) or when all three positions (QQQ)
were changed to glutamine. Collectively, these data indicate that amino acids 585 and 592 are the sites to which the N-linked glycans are added in mouse G5.

An identical strategy was used to identify sites of N-linked
glycosylation in G8. Mouse G8 contains a potential N-linked
glycosylation site at residue 369, which resides N-terminal to
the first predicted membrane spanning segment (Fig 1A). Mu-
tation of Asn437 (QN) had no effect on the size of the protein or
its sensitivity to PNGase F (Fig. 1B). Substitution of glutamine
for asparagine at position 619 (NQ), alone or together with
substitution of a glutamine for asparagine at amino acid 369
(QQ), reduced the apparent molecular mass of the untreated
proteins and rendered them resistant to PNGase F, indicating
that neither protein contained N-linked sugars. Collectively,
Methods.

G5 and G8 were immunoprecipitated ("A G8 in CRL-1601 cell lines. were washed and lysates prepared as described under /H11001 and G8 (8) or plasmids encoding cDNAs for G5 only, G8 only, or G5 C24884 these data indicate that mouse G8 contains a single N or G8 (Y11). GRP-78 (Bip), cells were cultured and processed as in A. Antisera to a glutamine, greatly diminished or abolished trafficking of N-linked glycosylation was required for trafficking of 

G5/G8 trafficking independently of N-linked glycosylation. Consequently, two additional mutations were made in G8; codon 619 was changed from asparagine to alanine (NA), and a new canonical N-linked site was introduced seven amino acids upstream of residue 619 in glycan-deficient G8 (N2NQ). Loss of the N-linked site in G8, either by substitution to an alanine or to a glutamine, greatly diminished or abolished trafficking of the G5/G8 heterodimer out of the ER (Fig. 2B). Introduction of an N-linked site at position 619 of G5 (N6N1Q) partially restored trafficking of the G5/G8 heterodimer. These data are consistent with N-linked glycosylation of G8 being required for the efficient trafficking of the G5/G8 heterodimer.

Effect of G8 Glycosylation on G5/G8 Dimer Formation—To determine whether the reduction in trafficking of the glycan-deficient G8 was the result of a failure to dimerize with G5, two different experiments were performed (Fig. 3). First, we determined whether glycan-deficient forms of G5 and G8 coimmunoprecipitated with their normal partners when expressed together in cells (Fig. 3A). When either normal or glycan-deficient G8 was coexpressed with G5, more than half of the G8 coprecipitated with G5. To ensure that the coimmunoprecipitation of glycan-deficient G8 with G5 represented G5/G8 dimers rather than coaggregated proteins, microsomal proteins from cells expressing either G5 or G8 alone or G5 in the presence of normal or glycan-deficient forms of G8 were subjected to native gel electrophoresis (Fig. 3B). G5 migrated as a broad, very high molecular mass band (~670 kDa) when expressed alone in cells; this band is presumed to represent aggregated G5. When G5 was coexpressed with glycosylated forms of G8 (NN or QN) almost all of the protein migrated as a single band with a molecular mass of ~150 kDa, the predicted size of a G5/G8 heterodimer. Glycan-deficient forms of G8 enabled some G5 to migrate in the lower molecular mass form, although a significant fraction of the G5 remained in high molecular mass aggregates.

When expressed alone, G8 was poorly resolved on native gels. Immunoblotting revealed that a portion of the protein migrated as a diffuse band of ~140 kDa. Coexpression of G5
increased the amount of G8 detected on the blot and increased the apparent mass to \(150\) kDa. These shifts appeared to be somewhat greater for the glycosylated forms (NN, NQ) of G8 than for the nonglycosylated forms (NQ and QQ). Levels of normal and mutant G8 were similar when analyzed by SDS-PAGE, indicating that differences in the signal intensities of the various constructs observed on native gels did not reflect differences in protein expression. Taken together, these findings suggest that glycosylation of G8 is not required for dimerization with G5 but is required for efficient transport of the two proteins out of the ER.

Lectins Coimmunoprecipitate with G5 and G8—To determine whether the ER lectins calnexin and calreticulin associate with G5 and G8, we performed coimmunoprecipitation studies in cells that stably expressed empty plasmids, G5-Myc (5), G8-HA (8), or G5-Myc and G8-HA (5/8, Fig. 4). Calnexin coprecipitated with G5 in the presence or in the absence of G8. Calnexin coimmunoprecipitated with G8 in the absence of G5, but only trace amounts of calnexin coprecipitated with G8 in the presence of G5 (as judged by long exposures of the gel; data not shown). Under these conditions, associations between G5 or G8 and GRP-94 or Bip were not detected (Fig. 4). Nor were ERp57, ERp53, or protein disulfide isomerase found in association with G5 or G8 (data not shown). Calreticulin precipitated nonspecifically with agarose beads in these experiments, therefore we used antibodies to calreticulin to determine whether G5 and G8 associated with this chaperone (Fig. 4B). pAbs to calreticulin and calnexin were prebound to protein A-agarose and washed extensively prior to incubation with cell lysates. In cells expressing G5 alone anti-calnexin antibodies coimmunoprecipitated a form of G5 with slightly reduced mobility relative to that which remained in the supernatant. In cells expressing G5 and G8, calnexin immunoprecipitated a form of G5 of the same molecular mass as that observed in cells expressing G5 alone, but the mature form of G5 remained in the supernatant. A similar pattern was observed for G8; calnexin immunoprecipitated the lower molecular mass form of G8, but the higher molecular mass, mature form remained in the supernatant. Thus, both immature G5 and G8 are bound by calnexin, suggesting that calnexin participates in the folding of these proteins. No G5 and only a trace amount of G8 coimmunoprecipitated with calreticulin, suggesting that calreticulin does not participate in the folding of the G5/G8 heterodimer.

Glycan Dependence of G5 and G8 Binding to Calnexin—To determine whether the N-linked glycans on G5 and G8 were required for calnexin binding, CHO-K1 cells were transiently transfected with normal or glycan-deficient forms of G5 and G8 (Fig. 5). The ability of calnexin to associate with each form of G5 and G8 was assessed by immunoprecipitation. Calnexin coimmunoprecipitated with G5 when it contained either two (NNN, QNN) or one (NQN, NNQ) N-linked glycans but failed to associate with glycan-deficient G5 (NQQ, QQQ) (Fig. 5A). For G8, calnexin associated with both the glycosylated G8 (NN, QN) and the nonglycosylated forms of G8 (NQ, QQ), although the association was stronger for the glycosylated forms of the half-transporter.

Trafficking of G5 and G8 Mutants—The appearance of high molecular mass forms of G5 and G8 indicates that they have escaped the ER quality control system and reached the Golgi complex. We monitored the appearance of these forms of G5

![Diagram](https://example.com/diagram.png)

**Fig. 6. Disease-causing missense mutations in G5 and G8.** The location of each missense mutation in human G5 and G8 is shown in the context of the major structural components of each half-transporter. Each mutation and its codon number are depicted in **bold** text (normal amino acid-amino acid number-mutant amino acid). Below each mutation, the normal residue (bold) and its 6 flanking residues are shown for human (h), mouse (m), and puffer fish (pf). Walker A, B, and C motifs are indicated, and the putative transmembrane spanning segments are shown as gray cylinders. The putative locations for attachment of N-linked glycans are indicated by gray circles with branched lines.
and G8 to determine the effects of disease-causing missense mutations on the trafficking of the G5/G8 dimer. The 13 reported missense mutations are shown in Fig. 6 (1, 29). 10 of the 13 mutations occur in residues that are conserved among human, mice, and puffer fish. Three exceptions, L596R, G574E, and G574R in G8, result in the substitution of charged for neutral amino acids. Normal and mutant G5 and G8 were expressed alone or in the presence of their normal partners in CHO-K1 cells (Fig. 7).

First, we determined whether any of the mutations in G5 or G8 resulted in trafficking of the protein independently of its dimerization partner (Fig. 7, A and B, top panels). No mature forms of either protein were seen in cells transfected with single mutant forms of G5 or G8. Next, the trafficking of the mutant proteins in cells cotransfected with normal partners was examined. No mature G5 was present in cells expressing G5 with the R389H, R419H, or N437K mutations. Trafficking was less efficient in cells expressing the E146Q mutation, and only trace amounts of mature G5 were seen in cells expressing G5 with the R419P mutation. Six of the eight missense mutations in G8 markedly decreased the proportion of G8 protein present in the mature form (Fig. 7B). The remaining two mutations (R543S and G574R) decreased, but did not eliminate, the proportion of G8 present in the mature form. In addition, qualitative variations in the pattern of the mature form were observed, perhaps because of differential glycosylation within the Golgi complex. Although none of the mutations in G5 were associated with reduced protein expression, coexpression of G5 with the mutant forms of G8 resulted in a reproducible decrease in the amount of immunodetectable G8 (Fig. 7B). The reasons for the reduced levels of detection of G8 are not known.

Dimer Formation among Mutant G5 and G8 Proteins—To determine whether the failure of the mutant proteins to traffic beyond the ER was the result of an inability to form heterodimers, the proteins were examined on native gels (Fig. 8A). As observed previously, normal G5 migrated as a high molecular mass aggregate when expressed alone and as an apparent dimer when coexpressed with normal G8. When the mutant forms of G5 were coexpressed with normal G8, most of the G5 remained in the high molecular mass aggregates. The appearance of the 150 kDa band was only apparent in cells expressing the G5 mutants E146Q and R419P and correlated with the appearance of fully processed G5 as determined by SDS-PAGE.

Similar analyses were performed with G8. When expressed alone, G8 was poorly resolved on native gels, and immunoblotting revealed a disperse population extending from −140 kDa to the top of the gel. When normal G5 was coexpressed with normal G8, the population condensed into a band of −160 kDa. G8 mutants that failed to traffic beyond the ER were not detectable on nondenaturing gels, in part because of reduced expression (Figs. 7B and 8A, lower panel). Only those mutant forms of G8 that were traffick-competent (R543S and G574R) and to a lesser extent L596R were detected in the position of the dimer.

Because the mutant forms of G5 and G8 were poorly detected on the native gels, we used glycerol density gradient ultracentrifugation to confirm that these mutants formed high molecular mass aggregates (Fig. 8B). These analyses were performed using a trafficking-defective mutant of G5, N437K. In cells expressing G5 alone, three forms of the protein were detected. The form with the greatest apparent molecular mass appeared exclusively in fraction 3. The middle, and most abundant form, was present in fraction 4 and extended to the cushion of the gradient, where the bulk of this form was found. The lowest molecular mass form was only present in the cushion. In contrast to cells expressing G5 alone, the mature form of G5 was present in the cells coexpressing G8 and was concentrated in

![Fig. 7. Trafficking of G5 and G8 mutants in CHO-K1 cells. A, normal G5 was expressed alone (5, V) or with normal G8 (5, 8). Mutants of G5 were expressed alone (top) or coexpressed with normal G8 (bottom). 48 h after transfection, cell lysates prepared and subjected to SDS-PAGE and immunoblot analysis for G5 using the anti-Myc antibody. B, normal G8 was expressed alone (8, V) or with G5 (5, 8). Mutants of G8 were expressed alone (top) or coexpressed with G5 (bottom). Cells were processed as A for analyzed by immunoblot for G8 using a mAb. V, empty vector.](image)

![Fig. 8. Dimerization and molecular mass of normal and mutant G5 and G8 in CHO-K1 cells. A, CHO-K1 cells were transfected with normal (n) G5, G8, or both constructs (controls). The indicated mutants of G5 (left) and G8 (right) were coexpressed with their normal partners. 48 h after transfection, membrane proteins were isolated, solubilized in digitonin, and subjected to Blue Native PAGE (top) and SDS-PAGE (bottom). Proteins were transferred to polyvinylidene difluoride (Blue Native) or nitrocellulose (SDS) membranes and analyzed by immunoblotting for G5 (left, anti-Myc) or G8 (right, IB10). B, cells were transfected and membranes prepared and solubilized as in A. Membranes were loaded onto preformed glycerol gradients (12–24%, 80% cushion) and centrifuged for 4 h (30,000 × g, 4 °C). Fractions were collected by pipetting, and 10% of each fraction was analyzed by immunoblotting after SDS-PAGE as in A. Calibration of the gradients was performed in a parallel, and molecular masses are indicated above the peak fraction for each standard.](image)
fractions 4 and 5 of the gradient. Trace amounts of an immature form of G5 were present in fractions 4 and 5 but were also present in the cushion. The lowest molecular mass form was confined to the cushion of the gradient. The gradient from cells expressing normal G5 and G8 was also analyzed for G8. Similar to G5, both precursor and mature forms of G8 peak in fractions 4 and 5, with the majority of the precursor present in the cushion. When the trafficking-defective mutant of G5 (N437K) was coexpressed with G8, the pattern of G5 was virtually identical to that obtained when G5 was expressed alone, indicating that the presence of G8 had no effect on the size or on the processing of the mutant G5. These data suggest that mutant G5 failed to associate with the normal G8.

**DISCUSSION**

In this study, we examined the role of glycosylation in the heterodimerization and trafficking of G5 and G8 from the ER to the Golgi complex and the effects of disease-causing missense mutations on these processes. The data indicate that G5 is glycosylated at two sites (Asn585 and Asn592) and that G8 is glycosylated at one site (Asn619). Glycosylation of G5 was required for binding of the lectin chaperone calnexin, suggesting a role for the calnexin cycle in the folding of this protein. Glycosylation was not required for heterodimerization of G5 and G8, but the complex failed to traffic efficiently out of the ER unless an N-linked glycan was present G8. G8 behaved like a molecular chaperone for G5, as demonstrated by the ability of G8 to prevent G5 from forming high molecular mass aggregates. These data, together with the observation that 10 of 13 disease-causing missense mutations in G5 and G8 interfere with formation of the G5/G8 heterodimer and/or its movement to the cell surface, provide further evidence that interactions between these G5 and G8 are essential for the intracellular trafficking as well as functioning of these two proteins.

The folding of many ABC transporter family members appears to be an inefficient process so that a significant fraction of the newly synthesized protein fails to reach its final destination in the cell. For example, 45–80% of CFTR made in cells is degraded in the ER with a half-life of ~30 min (26). The trafficking of G5 and G8 also appears to be inefficient. In cultured cells, the half-lives of the immature forms of G5 and G8 (2.5 h) are much shorter than those of the fully processed forms of the proteins (2–3 days) (11), but the two forms of the proteins are present in similar amounts. A similar distribution of the mature and immature forms of G5 and G8 is observed in mouse liver (5). Thus, under steady-state conditions the major fraction of G5 and G8 never reaches the Golgi complex.

The folding of glycoproteins is facilitated by two molecular chaperones in the ER, calnexin and calreticulin, which recognize monoglucosylated N-linked glycans in the growing polypeptide chain. Calnexin, but not calreticulin, binds to G5 and to G8 when these proteins were expressed independently in cells. The preference of G5 and G8 for calnexin may reflect the membrane localization of these two glycoproteins and the membrane-bound chaperone, calnexin. Alternatively, the association of these proteins with calreticulin may be transient and not detectable in steady-state conditions used in these experiments. Calnexin failed to coimmunoprecipitate with G8 when G5 was coexpressed in the same cells (Fig. 4). This finding suggests that calnexin dissociates from G5 after heterodimerization with G5 (Fig. 4). In contrast, calnexin continued to bind to G5 even in the presence of G8, which may reflect differences in the expression levels of G5 relative to G8 resulting in an excess of G5 in cells. Alternatively, intrinsic differences in the stability or folding of G5 and G8 may account for these findings. In cell lines expressing both G5 and G8, a greater proportion of G8 was in the mature form than was G5 (see Fig. 4). In mouse liver, a higher percentage of the immunodetectable G5 is in the unprocessed form (5). Thus, G5 may not fold as efficiently as G8.

The interaction of G5 with calnexin required at least one N-linked glycan (Fig. 5). However, the loss of N-linked glycans in G5 did not reduce the ratio of immature to mature G8, suggesting that ER to Golgi transport of dimers containing glycans-deficient G5 is not substantially diminished. The loss of glycans in G5 reduced the amount of immunodetectable G8 and had modest, but reproducible effects on the dispersion and electrophoretic mobility of the mature form. Therefore, the loss of N-linked glycans in G5 was not without consequence, but the significance of this observation is not known and will require additional studies. In contrast, the loss of the glycan in G8 reduced, but did not abolish, its association with calnexin. G8 behaves like a molecular chaperone for G5 to other proteins has been documented (30–32). Mutants of calnexin that lack a functional lectin domain associate with proteins via a polypeptide binding site and retain chaperone activity; however, the lectin site within calnexin enhances both the number and amount of substrates bound by calnexin (32–35). The incomplete dissociation of calnexin with glycans-deficient G8 is consistent with the dual binding model of calnexin and suggests that the reduction in trafficking of G5/G8 dimers containing glycans-deficient G8 may reflect the reduced ability of calnexin to mediate folding of this dimer.

Calnexin presents monoglucosylated proteins to ERp57, a thiol oxidoreductase that facilitates intramolecular disulfide bond formation and proper folding (36, 37). In the conditions used in the present study, neither G5 nor G8 associated with ERp57, suggesting that other thioredoxins mediate the folding of G5 and G8 when bound by calnexin. Similarly, no evidence for association of either protein with other ER luminal chaperones was observed (Bip, GRP-94, or protein disulfide isomerase). Because most of the G5 and G8 protein is not exposed to the ER lumen, cytosolic chaperones, such as Hsp90 or Hdj-2/Hsc70, may play are greater role in facilitating folding as has been suggested for other ABC transporters including CFTR (38–40) and P-glycoprotein (41). Further studies will be required to determine the role of cytoplasmic chaperones in the folding of G5/G8.

Several missense mutations in G5 and G8 cause sitosterolemia; however, the majority of these are not located in known functional domains of the protein, and the mechanism by which they disrupt transporter function has not been determined previously. The current results indicate that a majority of disease-causing missense mutations in G5 and G8 interfere with the formation of the G5/G8 heterodimer and prevent efficient trafficking of the transporter out of the ER. These defects are common to many disease-causing mutations in ABC transporters (42, 43), including the most common CFTR mutation (AF508) (26). The added requirement of G5 and G8 having to heterodimerize further complicates the trafficking of the protein complex out of the ER to the apical surfaces of cells where it functions to enhance sterol transport.

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Missense Mutations in ABCG5 and ABCG8 Disrupt Heterodimerization and Trafficking

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