Conserved Intramolecular Disulfide Bond Is Critical to Trafficking and Fate of ATP-binding Cassette (ABC) Transporters ABCB6 and Sulfonylurea Receptor 1 (SUR1)/ABCC8

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The ATP-binding cassette (ABC) transporter ABCB6 is a mitochondrial porphyrin transporter that activates porphyrin biosynthesis. ABCB6 lacks a canonical mitochondrial targeting sequence but reportedly traffics to other cellular compartments such as the plasma membrane. How ABCB6 reaches these destinations is unknown. In this study, we show that endogenous ABCB6 is glycosylated in multiple cell types, indicating trafficking through the endoplasmic reticulum (ER), and has only one atypical site for glycosylation (NX) in its amino terminus. ABCB6 remained glycosylated when the highly conserved cysteine (Cys-8) was substituted with serine to make a consensus sequence but reportedly traffics to other cellular compartments. This analysis led to the discovery of a disease-causing mutation (Cys-26) when Cys-26 was mutated alone or in combination with Cys-8, it also resulted in instability and ER retention. Further analysis revealed that these two cysteines form a disulfide bond. We discovered that other ABC transporters with an amino terminus in the ER had similarly configured conserved cysteines. ATP-binding cassette (ABC) transporters utilize ATP to facilitate the transmembrane movement of a variety of biologically important molecules (1). ABC transporters are required for many essential biological processes such as heme biosynthesis, [Fe-S] cluster formation, antigen presentation, and insulin secretion. Some point mutations in ABC genes produce only alterations in substrate specificity (e.g. P-glycoprotein (ABCB1) and ABCG2), whereas others cause profound conformational changes producing defects in trafficking (e.g. \( \Delta S508-CFTR \)) (2, 3). The endoplasmic reticulum (ER) has a protein quality control system that monitors conformational changes in proteins. Membrane proteins have multiple domains (cytoplasmic, ER lumen, and membrane-spanning) that are recognized by the ER and are currently being elucidated. Some of these domains may determine the fate of a protein within the ER (e.g. retention or degradation), and it is likely that ABC transporters contain characteristic domains determining their fate. Recent studies suggested that the ER contains multiple protein “quality control” checkpoints, each with defined criteria for recognizing protein folding (4–6). In membrane proteins, an initial ER checkpoint appears to require interrogation of the cytoplasmic domains to scan for lesions in folding that could activate protein degradation processes. A second checkpoint monitors domains located in the ER lumen. Some post-translational modifications occurring in the ER (e.g. disulfide bond formation) may be required by a domain to ensure proper folding, avoid activation of a checkpoint, and escape ER-associated protein degradation. Typically, disulfide bonds are maintained by the oxidizing environment of the ER and protein-disulfide isomerases. If the necessary disulfide bonds are not formed, the proteins might be retained in the ER and then degraded (7). Among the ABC transporters, it was discovered that ABCG2 contains an intramolecular disulfide bond, which is critical for protein stability (8). In the absence of one of the cysteines in the
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disulfide bond, ABCG2 was retained in the ER and then degraded. One explanation for ER retention and degradation of ABCG2 may be the presence of the “free” cysteine thiols. Unpaired cysteine thiols have been shown to produce ER retention of adiponectin (9) and IgM (10–12), and this process is referred to as “thiol retention.”

Our previous studies demonstrated that the porphyrin transporter ABCB6 is a homodimer that localizes to the mitochondrial outer membrane where it facilitates heme biosynthesis (13). Although others have also shown mitochondrial localization of ABCB6 (14, 15), it has also been reported to localize to the plasma membrane (15). One explanation for this could be cell context. It is not known whether ABCB6 traffics the same in all cells or if its pattern of trafficking depends upon cell type. Previous studies in overexpression systems have shown that ABCB6 contains N-glycans, indicating that ABCB6 traffics to the ER (15–17). In this study, we extended this to show that endogenous ABCB6 is a glycoprotein. Notably, we demonstrate that ABCB6 uses a single atypical but evolutionarily conserved glycosylation site (NXC) site. Although these NXC glycosylation sites are rare in the glycoproteome (18), this ER luminal cysteine (Cys-8) in ABCB6 is not required for glycosylation; however, it is required for ABCB6 stability because it forms a disulfide bond with another conserved luminal cysteine (Cys-26), which is also required for ABCB6 stability. Upon close examination of ABCB subfamily members with an amino terminus predicted to localize in the ER lumen, we discovered similar conserved cysteines that were separated by non-conserved amino acids. The biological significance of these cysteines was then demonstrated for sulfonylurea receptor 1 (SUR1)/ABCC8 (19, 20) because we discovered a single mutation in one of these cysteines in SUR1/ABCC8 from a patient with a defective SUR1-regulated K+ channel. In addition, we show that MSD0 of MRP1, which has been shown to traffic to the plasma membrane (21), requires the amino-terminal cysteines for proper trafficking. In total, our studies reveal that, in ABC transporters, conserved ER-localized cysteines form an important ER checkpoint.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH3T3, HEK293, Mel, and K562 cells were maintained in DMEM containing 4500 mg/liter glucose, 10% FBS (HyClone, Logan, UT), 2 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin in humidified 5% CO2 at 37 °C. NIH3T3 or HEK293 cells were transiently transfected with expression plasmids by using Lipofectamine Plus (Invitrogen) according to the manufacturer’s protocols. Where specified, NIH3T3 cells were incubated with 50 µg/ml cycloheximide, 10 µM MG132, or 25 mM N-ethylmaleimide. The samples were then fractionated by SDS-PAGE on a 7.5, 10, or 12.5% gel. Proteins were transferred to a nitrocellulose membrane (GE Healthcare) and immunoblotted as described (13). A point mutation was introduced into pcDNA3.1-hABCB6-V5-His by using a QuiChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and primers listed in the supplemental table. All genes were sequenced after mutagenesis. The pcDNA3.1-hABCB6-C8S/C26A-V5-His was generated by introducing a point mutation into pcDNA3.1-hABCB6-C8S-V5-His at Cys-26. To generate truncated mutant constructs to determine disulfide bond formation, point mutations were introduced into construct pcDNA3.1-hABCB6-V5 or pcDNA3.1-hABCB6-C8S-V5 at Cys-50 and then at Cys-120 using primers listed in the supplemental table. The region encoding the amino-terminal 210 amino acids was then amplified with a FLAG tag at the carboxyl terminus from pcDNA3.1-hABCB6-C50A/C120A-V5 or pcDNA3.1-hABCB6-C8S/C50A/C120A-V5 by using primers (sense, 5’-GCCATGGTACTGGCGCAAATCTAGTCTGGACGCCG-3’; antisense, 5’-CTCTATATAGTCTGTACATCCTGTTAATATCAGAAGTCCAGGGGCCCAGAG-3’) under the following conditions: heating at 94 °C for 1 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and reaction at 68 °C for 1 min. The PCR product was purified from an agarose gel and TA-cloned into pcCR2.1-TOPO vector (Invitrogen). The inserts were subcloned into pcDNA3 using HindIII/Xbal sites to obtain pcDNA3.1-hABCB6-C50A/C120A-V5-FLAG or pcDNA3.1-hABCB6-C8S/C50A/C120A-V5-FLAG constructs.

The amino-terminal 203-amino acid segment of hMRP1 (hMRP1-MSD0) (21) was cloned from a pool of cDNA generated from K562 cells by using PCR with the following primers: sense, 5’-GCCACCGGCATGGCGCTCCGGGGCTTC-3’; antisense, 5’-CGTGGATGGTTTCCGAGAACAGGGGTGAG-3’. The purified product was TA-cloned into pcDNA3.1/GT-FLAG-TOPO (Invitrogen) to generate the pcDNA3.1-hMRP1-MSD0-FLAG construct. Using this wild-type construct as a template, we introduced a point mutation at Cys-32 to generate hMRP1-MSD0-C32A-FLAG by using the QuiChange mutagenesis kit and the primers listed in the supplemental table. Oligonucleotide synthesis and DNA sequencing were performed by Hartwell Center for Bioinformatics and Biotechnology (St. Jude Children’s Research Hospital).

Immunoblotting—Twenty-four hours after transfection, cells were washed with PBS, scraped into 1 ml of cold PBS containing 1× protease inhibitor mixture (Complete EDTA-free, Roche Applied Science) and 10 mM N-ethylmaleimide where indicated, pelleted by centrifugation at 1,000 × g for 4 min at 4 °C, and solubilized in buffer A (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 1× commercial protease inhibitor mixture) or Nonidet P-40 lysis buffer containing 10 mM N-ethylmaleimide. Cell lysates were centrifuged at 17,000 × g for 15 min at 4 °C to remove cell debris. For immunoblotting, Laemmli sample buffer containing β-mercaptoethanol or DTT (where indicated) was added to the supernatant followed by addition of N-ethylmaleimide. The samples were fractionated by SDS-PAGE on a 7.5, 10, or 12.5% gel. Proteins were transferred to a nitrocellulose membrane (GE Healthcare) and immunoblotted as described previously (13) using anti-GFP-HRP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).
Cruz, CA), anti-FLAG (M2 or polyclonal, Sigma), anti-V5 polyclonal (Medical Biological Laboratories Co., Ltd., Nagoya, Japan), anti-ABCB6 monoclonal (developed in our laboratory), anti-ABCB6 polyclonal (Rockland Inc., Gilbertsville, PA), or anti-apoptosis inducing factor (AIF) polyclonal (Chemicon International, Billerica, MA) antibody. The intensity of the bands was quantified using ImageJ. A non-linear regression curve was generated using GraphPad Prism 5 (GraphPad Software, La Jolla, CA) to estimate half-life in the experiments using cycloheximide.

**Glycosidase Digestion**—Lysates prepared as described above from NIH3T3, K562, or Mel cells were denatured in 1× denaturing buffer (0.5% SDS and 1% β-mercaptoethanol) and incubated at 37 °C for 1 h in reaction buffer (50 mM Na2PO4 (pH 7.5) and 1% Nonidet P-40) with or without PNGase F (New England Biolabs, Beverly, MA) according to the manufacturer’s recommendations. For Endo H digestion, the denatured samples were incubated with or without the enzyme in 50 mM sodium citrate buffer. Crude mitochondria prepared from liver from C57Bl/6/129 female mice (2–6 months of age) using MITOISO1 (Sigma) were treated with glycosidases as described as above. The Abcb6 knock-out mouse line was established in our laboratory and will be described in details elsewhere. Where indicated, Mel cells were treated with DMSO (vehicle) or 1 µg/ml brefeldin A for 24 h prior to mitochondrial preparations.

**Pulse-Chase**—K562 vector or ABCB6-FLAG cells (~2 × 10⁶ cells) were washed with warm 1× Hank’s buffer and incubated in labeling medium comprising DMEM without l-methionine or l-cysteine (Cellgro, Manassas, VA), 10% dialyzed FBS (HyClone), and 2 mM l-glutamine containing 0.1 mCi/ml ³⁵S (Tran²⁵⁵S-label; l-[³⁵S]methionine and l-[³⁵S]cysteine, 1175 Ci/mmol; MP Biomedicals, Inc., Irvine, CA) for 0 or 5 min at 37 °C. Cells were then washed once with chase medium (complete DMEM supplemented with 2 mM l-methionine and l-cysteine) and chased for the times indicated in the figures. At the end of each chase time interval, cells were washed with cold PBS, pelleted, flash frozen in liquid N₂, and stored at ~80 °C until use. Cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholate, and 0.5% Nonidet P-40) for 10 min on ice, and cell debris were removed by centrifugation at 15,000 × g for 10 min at 4 °C. The lysate was then washed once with chase medium (complete DMEM supplemented with 2 mM l-methionine and l-cysteine) and chased for the times indicated in the figures. At the end of each chase time interval, cells were washed with cold PBS, pelleted, flash frozen in liquid N₂, and stored at ~80 °C until use. Cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholate, and 0.5% Nonidet P-40) for 10 min on ice, and cell debris were removed by centrifugation at 15,000 × g for 10 min at 4 °C. The lysate was then washed once with chase medium (complete DMEM supplemented with 2 mM l-methionine and l-cysteine) and chased for the times indicated in the figures.

**RESULTS AND DISCUSSION**

**ABCB6 Is Glycosylated at Atypical NXC Site**—We have previously demonstrated that ABCB6 expression increases during erythroid differentiation and that overexpression of ABCB6 increases porphyrin synthesis (13). In one of our models (the erythroid progenitor Mel cells), we extended this to show that erythroid differentiation by hexamethylene bisacetamide produced the expected increase in endogenous ABCB6 expression, which is highly correlated with increased intracellular PPIX concentrations (supplemental Fig. 1A). An analysis of ABCB6 expression suggested glycosylation (in erythroid and non-erythroid cell types) based upon the formation of a broad band on immunoblots as well as previous results with exogenously expressed ABCB6 (15–17); therefore, we tested whether endogenous ABCB6 is also modified by N-linked glycans. To analyze endogenous ABCB6, mitochondria were purified from Mel cells and mouse liver and treated with either Endo H, which only reacts with the high mannose-containing proteins that remain in the ER, or PNGase F to remove high mannose and complex glycans from asparagine residues (22–26). Immunoblot analysis revealed faster migration of ABCB6 only after PNGase F but not Endo H treatment (Fig. 1, A and B), indicating that ABCB6 is modified by N-glycans and traffics through the ER to the Golgi. The glycan sensitivity of a previously established functional tagged ABCB6 (13) after either stable expression in Mel or K562 cells or transient expression in NIH3T3 cells demonstrated a similar sensitivity to PNGase F (supplemental Fig. 1B). Notably, inherent differences in glycosylation between human and mouse cells were demonstrated when the same ABCB6 expression vector was introduced into either murine (Mel) or human (K562) cells (Fig. 1C). Using tunicamycin to disrupt the first step in N-glycosylation provided additional confirmation that ABCB6 is a glycoprotein (Fig. 1D). We further tested the requirement for ER to Golgi trafficking in the maturation of ABCB6 by using brefeldin A to

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3 W. S. Rasband, National Institutes of Health, Bethesda, MD, 1997–2009.
disrupt ER to Golgi transit (27). Brefeldin A prevented the formation of Endo H-resistant mature ABCB6 in Mel cells, further demonstrating the requirement for Golgi-mediated processing and maturation of the glycan status of ABCB6 (Fig. 1E). Pulse-chase analysis revealed that the conversion of ABCB6 to the 94-kDa mature form occurred over 1–2 h (Fig. 1F). A PROSITE motif search (28) identified four potential consensus N\(\times\)(S/T)\(\times\)-glycosylation sites in the ABCB6 sequence (Fig. 2A).

However, the available topological predictions (17, 30, 31) (supplemental methods) place these sites in the cytosol or in the transmembrane helices but not in ER lumen where \(N\)-glycosylation occurs, suggesting that these sites are unlikely to be used (supplemental Fig. 2A). To rule out these consensus \(N\times(S/T)\times\) sites as the potential \(N\)-glycosylation sites, we performed site-directed mutagenesis yielding glutamine substitution at the asparagines in each of the four consensus glycosylation motifs and at both residues 447 and 498 (supplemental Fig. 2) or at all four consensus residues ("Q4" mutant) (Fig. 2B). The wild-type and mutant ABCB6 expression plasmids were transiently expressed in NIH3T3 cells. The Q4 mutant had a migration pattern identical to that of wild-type ABCB6 in the absence of glycanase treatment. The sensitivity of the Q4 mutant to PNGase F (Fig. 2B) indicates that other non-consensus glycosylation site(s) exist, which is supported by the topological predictions. Moreover, the acquisition of Endo H resistance indicates that the Q4 mutant leaves the ER.

An atypical \(N\)-glycosylation motif containing a cysteine residue (\(N\times C\) rather than the conventional \(N\times(S/T)\)) has been reported in a few proteins (18, 32–36). We identified one such atypical motif (NYC) starting at position 6 in the ABCB6 amino terminus. Glutamine was substituted for Asn-6 in the wild-type and Q4 ABCB6 expression plasmids to generate N6Q and Q5 ABCB6, both of which showed faster electrophoretic mobility than wild-type ABCB6. Moreover, their mobility was identical to that of the PNGase F-treated wild-type protein and was unchanged by either PNGase F or Endo H treatment (Fig. 2C), indicating that this atypical site is the sole glycosylation site in ABCB6. This finding also supports that the ABCB6 amino terminus is in the ER lumen (17). Although the cysteine residue is highly conserved among human, chimpanzee, mouse, rat, and
zebrafish ABCB6, the asparagine residue for glycosylation (NXC) is not conserved in zebrafish (Fig. 2D).

**Cys-8 Is Dispensable for Glycosylation but Critical to Avoid ER Retention and Proteasomally Mediated Degradation—** Other proteins reported to have an atypical N-glycosylation motif have additional typical glycosylation sites; thus, ABCB6 with only one such atypical site presented an opportunity to test whether the cysteine in NXC was required for glycosylation. We developed the ABCB6 substitution mutants described in Fig. 3A. The cysteine residue was substituted with a serine residue to form the consensus glycosylation site NXS (labeled C8S). The C8S mutant was transiently expressed in NIH3T3 cells, and glycosylation status was evaluated by PNGase F treatment. The ABCB6-C8S was PNGase F-sensitive, indicating that glycosylation does not require the cysteine (Fig. 3A, right panel). As a control, we showed that glycine substitution to form a non-consensus site caused a loss of glycosylation, which demonstrates that ABCB6 glycosylation requires either the consensus NXS(T) or NXC motif. Unexpectedly, substitution of this cysteine resulted in decreased protein levels despite glycosylation (Fig. 3, A and B) as well as ER retention as shown by Endo H sensitivity (Fig. 3C). Notably, the point mutation disrupting the Walker A (13) did not result in reduced ABCB6 expression. Our multiple sequence alignment of ABCB6 homologs (Fig. 2D) revealed a conserved downstream cysteine residue at position 26, and a transmembrane (TM) helix prediction algorithm, TMHMM, predicted that Cys-26 is in the ER lumen (30). Proteins containing free cysteine thiol residues in the ER lumen are one cause of ER retention (11) and may explain the ER retention of ABCG2 after mutation of one cysteine in an intramolecular disulfide bond created a free thiol (8). Therefore, we next determined whether ABCB6 engineered to contain free thiols in the ER was ER-retained by generating the ABCB6 mutants C26A and C26S as well the mutants lacking both cysteine residues, C8S/C26S. Both the single cysteine mutants and the double mutants, which now lack a thiol in the ER lumen, were Endo H-sensitive, indicating ER retention. This finding excludes free thiols in ABCB6 as a mechanism of ER retention. Nonetheless, the expression of these cysteine mutants was reduced (Fig. 3, B and C), indicating that both Cys-8 and Cys-26 are required for maximal ABCB6 expression. We next hypothesized that we could rescue the trafficking defect and restore protein expression by adding a cysteine residue downstream from the serine now at position 8; therefore, a cysteine residue was inserted between Glu-9 and Ala-10 to create C8S/C10insertion mutant. However, this construct failed to restore maximal ABCB6 protein expression and was also ER-retained as shown by Endo H sensitivity. This finding indicates that either the spacing

FIGURE 2. **ABCB6 is modified at single conserved atypical glycosylation site.** A, ABCB6 contains four consensus N-glycosylation (NX(S/T)) motifs and a single atypical N-glycosylation motif. B, ABCB6 with amino acid substitution of all four asparagines in the consensus motif (Q4) was analyzed for PNGase F and Endo H sensitivity by immunoblotting using anti-V5 antibody. The presence of glycans was detected by protein mobility shifts. The migration patterns of all mutants were similar to those of the wild-type protein. C, NIH3T3 cells were transiently transfected with single mutant N6Q, Q4, or Q5 in which glutamine was substituted for all five asparagine residues. Cell lysates were treated with the indicated glycosidases and immunoblotted with anti-V5 antibody (lower arrow, non-glycosylated ABCB6; upper arrow, glycosylated ABCB6). D, multiple sequence alignment of ABCB6 shows two conserved cysteines (red boxes) and one N-glycosylation site (green box) among the species. Each experimental result was independently performed in all iterations shown at least twice with a single representative blot from one experiment used. vec, vector; H.sapiens, Homo sapiens; P.troglodytes, Pan troglodytes; B.taurus, Bos taurus; M.musculus, Mus musculus; R.norvegicus, Rattus norvegicus; D.reio, Danio rerio. Pro, protein (amino acid) sequence.
between the two cysteines and/or their context is important for expression (Fig. 3C).

To determine whether ABCB6 with cysteine substitutions at either position 8 or 26 was unstable, we performed pulse-chase experiments using K562 cells expressing either ABCB6, C8S, or C26A. The half-life for wild-type ABCB6 could not be estimated but appeared to be greater than 24 h. The initial rise in labeling of wild-type ABCB6 was unexpected but may be due to its long half-life. In contrast, both C8S-ABCB6 and C26A-ABCB6 had almost identical decay curves with an estimated half-life of 9.4 and 8.0 h, respectively (Fig. 3D). Collectively, these results demonstrate that loss of either Cys-8 or Cys-26 renders ABCB6 unstable; both mutants exhibit kinetically similar degradation curves, suggesting a similar mechanism.

We next determined whether N-glycosylation altered the stability of ABCB6. We compared wild-type ABCB6 and ABCB6 mutants lacking either Cys-8 (C8S), N-glycosylation (N6Q), or both (C8G). Cells were transiently transfected with either wild-type ABCB6 or ABCB6 harboring either the C8S or C8G substitution. At 17 h post-transfection, cycloheximide was added to inhibit protein synthesis, and cells were harvested at the indicated times (Fig. 4A). ABCB6 protein expression was evaluated by immunoblotting. Notably, the degradation of wild type assessed by this method confirms the pulse-chase, which showed an extremely long half-life for ABCB6 (＞24 h), and C8S appears to be just as unstable in this assay as in the pulse-chase. Non-glycosylated C8G exhibited an even shorter half-life (2.9 h) compared with C8S (7.1 h). The absence of glycosylation of ABCB6 (N6Q) did not alter the wild-type ABCB6 degradation pattern as it had a degradation pattern similar to that of the wild-type protein (Fig. 4A). Therefore, lack of glycosylation affects the stability of ABCB6 only in the absence of the conserved disulfide bond.
conserved cysteine. This suggests that the amino-terminal cysteines are a dominant factor stabilizing ABCB6 and that glycosylation modifies this but only in the context of the ABCB6 cysteine mutants.

To understand the pathway of degradation of cysteine mutant ABCB6, we used either MG132 or NH4Cl to inhibit the proteasomal or the lysosomal degradation pathway, respectively. Cells transiently expressing either wild-type ABCB6 or C8S mutant were incubated with MG132 for 12 h and analyzed for ABCB6 proteins. Although untreated cells express C8S protein at a lower level (51.6/11006 5.4% of wild-type protein), the protein expression of C8S mutant was comparable with that of untreated wild-type ABCB6 after MG132 incubation (112.6/11006 5.2%; Fig. 4B). However, incubation with NH4Cl failed to restore the C8S protein expression (Fig. 4B), indicating that the mutant ABCB6 is not degraded by the lysosomal pathway.

These studies demonstrate that the cysteines in the amino terminus of ABCB6 are important for stability by escaping proteasomal degradation.

Cys-8 Is Not Required for Substrate Binding of ABCB6 but Forms a Disulfide Bond with Cys-26—The reduced stability of ABCB6 cysteine mutants and Endo H sensitivity indicate ER retention and degradation. The signal for this degradation process is protein misfolding. However, the determinants of the stability of a misfolded protein can be location-specific (e.g. cytosol or ER lumen) (6). Because substrate binding requires a native conformation, we tested whether cysteine substitution (Cys-8) altered substrate binding. We interrogated ABCB6 substrate binding using hemin coupled to hemin-agarose in a pull-down assay. The C8S-ABCB6 protein produced in NIH3T3 cells was competent at substrate (hemin) binding. Moreover, the kinetics of hemin-agarose association between ABCB6 and ABCB6-C8S showed an identical rate of hemin association with ABCB6-C8S, which suggests that the substrate binding domain is properly folded despite being unstable (Fig. 5A). We extended this to show that the non-glycosylated form of ABCB6, N6Q, also bound hemin-agarose, suggesting that a lack of N-glycosylation does not affect substrate binding (Fig. 5B).

The ER retention and instability of ABCB6-C8S, -C26S, and -C8S/C26S suggests that these residues have a crucial role in the folding of the ABCB6 amino terminus, a domain that appears to be important in determining the stability of ABCB6. Because both cysteine residues (Cys-8 and Cys-26) are predicted to reside in the ER lumen and the ER has a unique oxidizing potential, we postulated that they form an intramolecular disulfide bond. Depending upon the distance between the two cysteine residues such bonds cause proteins to migrate faster in SDS-PAGE under non-reducing conditions. We reasoned that the small number of amino acids separating the two conserved cysteines (17 amino acids) would not produce a readily detectable band shift in a 94-kDa ABCB6. Therefore, we generated chimeric expression plasmids that contained segments of varying length of ABCB6 protein fused with GFP. Each of these constructs was compared with the full-length ABCB6-GFP with respect to Endo H and PNGase F sensitivity, and the constructs with sensitivity comparable with full length were used to evaluate the disulfide bond for mutation (supplemental Fig. 2). Two of the constructs were poorly expressed (1–50 and

FIGURE 4. Glycosylation is not critical for ABCB6 instability, and MG132 partially rescues C8S instability. A, NIH3T3 cells were transfected with ABCB6-V5, C8S-V5, C8G-V5, or N6Q-V5, and cycloheximide (final concentration, 50 μg/ml) was added 17 h post-transfection. Cells were harvested at the indicated times and analyzed for ABCB6 proteins using anti-V5 antibody. Intensity of the bands was analyzed using densitometry and expressed as percentage of ABCB6 protein at 0 h for each construct. Curve fitting was performed by non-linear regression analysis using GraphPad Prism. Values shown are the mean from two independent experiments with the range indicated by the error bars. B, NIH3T3 cells were transiently transfected with ABCB6-V5 or C8S-V5, incubated with 10 μM MG132 or 25 mM NH4Cl for 12 h, and analyzed for ABCB6 proteins using an anti-V5 antibody. CHX, cycloheximide. A representative image from two separate experiments is shown.

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1–90), whereas a third did not achieve the required Endo H resistance. Only the construct containing the amino-terminal 210 amino acids (N1–210) mimicked the wild-type protein by showing PNGase F sensitivity and Endo H resistance (supplemental Fig. 3). To rule out the contribution of the two additional cysteines (Cys-120 and Cys-150) present in this segment, we then developed a chimeric expression plasmid encoding the amino-terminal 210 amino acids of ABCB6 fused to a carboxyl-terminal FLAG epitope where both Cys-120 and Cys-150 were substituted with Ala (ABCB6-C50A/C120A-N1–210-FLAG). A schematic drawing shows the predicted location of the amino-terminal cysteines in the ER lumen (Fig. 6A).

To evaluate the presence of disulfide bonds, we eliminated spontaneous disulfide bond formation by N-ethylmaleimide treatment. We next compared the electrophoretic migration of ABCB6-C50A/C120A-N1–210-FLAG and the cysteine mutant ABCB6-C8S/C50A/C120A-N1–210-FLAG in the presence versus absence of the reducing agent DTT (Fig. 6B). Glycosidase treatment with either PNGase F or Endo H distinguished the ABCB6 location in either the ER or Golgi. In addition, we noted that the N1–210 ABCB6 was partially Endo H-sensitive, which is probably due to the amount of plasmid transfected. ABCB6-C50A/C120A-N1–210-FLAG exhibited three faster migrating bands in the absence of DTT (Fig. 6B). However, consistent with disulfide bond formation, addition of DTT reduced migration of each form of ABCB6. In contrast, the migration of ABCB6-FLAG with the C8S mutation was not affected by DTT (Fig. 6B, right panel, band II, lane 3 and 4). The ABCB6 with a C8S mutation exhibited no Endo H resistance, demonstrating ER retention like the full-length ABCB6 with amino-terminal cysteine substitution. We previously found that ABCB6 overexpression enhances de novo porphyrin biosynthesis monitored by an increase in fluorescent heme precursor PPIX (13). We hypothesized that this effect would be absent if ABCB6 was retained in the ER. As ABCB6 is expressed almost exclusively in the mitochondria of K562 cells (13, 37), these cells were transduced with retroviruses expressing ABCB6-FLAG, ABCB6-K629G-FLAG (a non-functional Walker A lysine mutant (13)), or ABCB6-C26A/S-FLAG by using plasmids containing IRES-GFP. PPIX concentration was measured in cells with comparable GFP expression by flow cytometry. The mean PPIX fluorescence in cells expressing the ABCB6-C26A mutant or nonfunctional ABCB6 was reduced in cells expressing ABCB6 (Fig. 6C and supplemental Fig. 4), indicating a loss of ability to stimulate porphyrin synthesis. Because these mutants still bind porphyrins (Fig. 5), the loss of function can be attributed to ER retention.

Only ER Luminal Cysteines Affect Stability and ER Retention — We next evaluated whether the localization of the amino-terminal cysteines is an important factor regulating either protein stability or ER exit. ABCA1 is essential for the transport of lipids across membranes and for the formation of high density lipoprotein. Our sequence analysis showed that ABCA1 contained the conserved amino-terminal cysteines (Cys-3 and Cys-23; supplemental Fig. 5); however, the predicted topology indicates that these residues reside in the cytoplasm and not the ER (38). We therefore used ABCA1 to test, regardless of predicted subcellular localization, whether amino-terminal cysteines affect protein stability. We combined confocal microscopy and differential glycosidase sensitivity to assess ER exit (supplemental Fig. 5). However, the predicted topology indicates that these residues reside in the cytoplasm and not the ER (38). We therefore used ABCA1 to test, regardless of predicted subcellular localization, whether amino-terminal cysteines affect protein stability. We combined confocal microscopy and differential glycosidase sensitivity to assess ER exit (supplemental Fig. 5). When the cysteines at ABCA1 residues 3 and 23 were substituted with alanine, confocal microscopic analysis showed that the mutant C3A/C23A-ABCA1 localized to both the plasma membrane and intracellular compartments (supplemental Fig. 5A). Immunoblot analysis showed resistance to Endo H digestion, confirming that both mutant and wild-type
ABCA1 had exited the ER (supplemental Fig. 5B). Moreover, the expression level of the mutant protein was comparable with that of the wild-type protein. Therefore, this finding supports the concept that location of the amino-terminal cysteines is an important determinant in ABC protein trafficking.

Two Amino-terminal Cysteines Are Conserved among Multiple ABC Transporters—Because of the conservation of this cysteine motif among ABCB6 homologs (Fig. 2D), we investigated whether cysteine motifs were found in other ABC transporters. We used the predicted topology of 43 additional human ABC transporters (31) to identify candidates with amino-terminal cysteines predicted to be in the ER. To determine whether two amino-terminal cysteines with the appropriate spacing were conserved, we visually examined the amino acid sequences of ABC transporters and other transporters (see supplemental Fig. 6) aligned by using ClustalW. Because the carboxyl-terminal nucleotide binding domain in most ABC transporters resides in the cytoplasm, an odd number of TM helices suggests projection of the amino terminus into the ER lumen, and therefore, we included transporters whose amino termini do and do not project into the ER. We found the two cysteines to be conserved but separated by 17–25 non-conserved amino acids in the so-called "long multidrug-related proteins (MRPs)" that are members of the ABCC subfamily, including ABCC1, ABCC2, ABCC3, ABCC6, ABCC8 (SUR1), and ABCC9 (SUR2) (Fig. 7C). These transporters contain an additional membrane-spanning domain0 (MSD0) at the amino terminus with five TMs, causing the amino terminus to reside in the ER lumen during maturation. Disease-causing mutations have been identified in these ABC transporters, and therefore, it is of interest to determine...
whether these cysteines affect the folding and ultimately trafficking of the proteins.

This analysis enabled us to identify a role for the previously unrecognized defective SUR1/ABCC8 allele in a patient with hyperinsulinemic hypoglycemia, a recessive genetic disease where a point mutation results in a Cys-26 to serine substitution (Fig. 7A). The patient was a compound heterozygote: one mutant ABCC8/SUR1 allele was a previously reported truncation.
Conclusions—We have determined that ABCB6 undergoes glican modification in many cell types at a conserved single atypical amino-terminal NXC site. This cysteine forms an intramolecular disulfide bond in the ER that appears to be a key event in the trafficking of ABCB6 as mutation of either cysteine led to ER retention. Quality control checkpoints in the ER are required to ensure that properly folded proteins reach their final destinations in the appropriate conformation to guarantee normal biological activity. The topological ER checkpoints for membrane proteins comprise both ER luminal and cytoplasmic signals, but the formation and type of integral membrane domains might also be a signal (6). Our studies reveal a conserved amino-terminal disulfide bond as an important conserved ER luminal checkpoint for many ABC transporters. Our analysis facilitated the identification of a new ABCC8/SUR1 mutant allele that produces ER retention as the cause of hyperinsulinemic hypoglycemia.

Intriguingly, an intramolecular disulfide bond has been previously identified in ABCG2 that when disrupted (by mutation) produces ER retention, although mechanistically it is unclear whether ER retention was due to an unpaired thiol causing thiol retention or the lack of disulfide bond formation (8). In the case of ABCB6 and ABCC8/SUR1, we showed that it was not thiol retention but instead loss of the disulfide bond. To further extend the idea that the disulfide bond might be conserved among ABC transporters, an amino acid sequence alignment of ABCG subfamily members revealed that ABCG5 also contains two cysteines in the region similar to ABCG2 (Fig. 7E). Collectively, these findings suggest that the formation of an intramolecular disulfide bond in an ER luminal loop may be a general conformational requirement for ER exit of many ABC transporters. Moreover, we showed that a subtle change in the topology of a short ER luminal segment is an important checkpoint that does not appear to appreciably alter transporter-ligand interactions. Finally, although disruption of this disulfide bond led to ER retention and, in the case of ABCB6 and ABCG2, proteasomal degradation (8), it is not clear whether this is the case for all ABC transporters with disruption of ER luminal domains. For example, SUR1 appears to be relatively stable but does not leave the ER. This finding suggests that some ABC transporters may only be retained in the ER when their ER luminal disulfide bond is disrupted, and other factors may determine whether they are degraded. If this is true, it raises the possibility that changes in ER oxidation state could impact the trafficking of many ABC transporters, and this might be the basis for some cases of loss of function.

FIGURE 7. Two amino-terminal cysteine residues are conserved and functional in ABCB transporters. A, a point mutation resulting in Cys-6 to Ser substitution in SUR1/ABCC8 was identified in one allele of a hyperinsulinemic patient as shown in the electropherogram. B, SUR1 and mutant SUR1 constructs containing various cysteine substitutions were co-expressed with K_\text{ATP} in COS7 cells. SUR1 proteins were labeled with \textsuperscript{125}I-azidoglibenclamide and detected by autoradiography after SDS-PAGE. Mature SUR1 that traffics to the cell surface was identified by its slower electrophoretic migration. Two independent experiments in green show the wild-type protein (amino acid) sequence. Conclusions—We have determined that ABCB6 undergoes glican modification in many cell types at a conserved single atypical amino-terminal NXC site. This cysteine forms an intramolecular disulfide bond in the ER that appears to be a key event in the trafficking of ABCB6 as mutation of either cysteine led to ER retention. Quality control checkpoints in the ER are required to ensure that properly folded proteins reach their final destinations in the appropriate conformation to guarantee normal biological activity. The topological ER checkpoints for membrane proteins comprise both ER luminal and cytoplasmic signals, but the formation and type of integral membrane domains might also be a signal (6). Our studies reveal a conserved amino-terminal disulfide bond as an important conserved ER luminal checkpoint for many ABC transporters. Our analysis facilitated the identification of a new ABCC8/SUR1 mutant allele that produces ER retention as the cause of hyperinsulinemic hypoglycemia.

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Conserved Disulfide Bond in ABC Transporters

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