Disruption of N-linked glycosylation promotes proteasomal degradation of the human ATP-binding cassette transporter ABCA3

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Disruption of N-linked glycosylation promotes proteasomal degradation of the human ATP-binding cassette transporter ABCA3. Am J Physiol Lung Cell Mol Physiol 305: L970–L980, 2013.—The lipid transport protein, ABCA3, expressed in alveolar type 2 (AT2) cells, is critical for surfactant homeostasis. The first luminal loop of ABCA3 contains three putative N-linked glycosylation sites at residues 53, 124, and 140. A common cotranslational modification, N-linked glycosylation, is critical for the proper expression of glycoproteins by enhancing folding, trafficking, and stability through augmentation of the endoplasmic reticulum (ER) folding cycle. To understand its role in ABCA3 biosynthesis, we utilized EGFP-tagged fusion constructs with either wild-type or mutant ABCA3 cDNAs that contained glutamine for asparagine substitutions at the putative glycosylation motifs. In A549 cells, inhibition of glycosylation by tunicamycin increased the electrophoretic mobility (Mr) and reduced the expression level of wild-type ABCA3 in a dose-dependent manner. Fluorescence imaging of transiently transfected A549 or primary human AT2 cells showed that although single motif mutants exhibited a vesicular distribution pattern similar to wild-type ABCA3, mutation of N124 and N140 residues resulted in a shift toward an ER-predominant distribution. By immunoblotting, the N53 mutation exhibited no effect on either the Mr or ABCA3 expression level. In contrast, substitutions at N124 or N140, as well as N124/N140 double mutation, resulted in increased electrophoretic mobility indicative of a glycosylation deficiency accompanied by reduced overall expression levels. Diminished steady-state levels of glycan-deficient ABCA3 isoforms were rescued by treatment with the proteasome inhibitor MG132. These results suggest that cotranslational N-linked glycosylation at N124 and N140 is critical for ABCA3 stability, and its disruption results in protein destabilization and proteasomal degradation.

ATP binding cassette, ABCA3; N-linked glycosylation; ER-associated degradation (ERAD); proteostasis; lung epithelium

THE ABCA3 (ATP binding cassette subfamily A, member 3) glycoprotein is a member of a highly conserved multispan transmembrane ABC superfamily of transporters that uses the energy of ATP hydrolysis to translocate a variety of substrates, ranging from ions to large molecules, across cell membranes. The human ABCA3 gene has been mapped to chromosome 16p13.3 and encodes a 1,704-amino acid protein (13, 30). Structure prediction algorithms suggest that ABCA3 is typical of most ABC transporters, consisting of four core domains forming a minimal functional unit (27) (Fig. 1). Two transmembrane domains (six α-helices per domain) form the conduit through which substrates cross the membrane. These domains also contain substrate-binding sites, which contribute to transport specificity. Two ATP binding cassettes (ABC1 and ABC2) (nucleotide binding domains) couple the energy of ATP hydrolysis for substrate translocation. Although the ABCA3 transporter is found in many tissues, it is highly expressed in the alveolar type 2 (AT2) cells of the distal lung epithelium (42, 59). Localized at the limiting membrane of the lamellar body (LB), an AT2-cell specific organelle (42, 59), ABCA3 functions as a transporter of phospholipids and cholesterol into the LB, and in recent years it has been recognized as one of the critical regulators of LB biogenesis and lung surfactant metabolism (2, 8, 9, 11).

Studies of ABCA3 biosynthesis have revealed several key components of its intracellular trafficking. After synthesis of the primary translation product and translocation to the endoplasmic reticulum (ER), ABCA3 is routed via the early endosome/multivesicular body network directly to the LB in AT2 lung epithelial cells or to lysosomes and lysosomal-related organelles (LROs) in A549 and HEK293 cell lines (2, 10, 11, 17, 38, 42, 44). Within these distal compartments ABCA3 also undergoes postranslational proteolysis of the proximal NH2-terminal region (17), which can be used as a biochemical marker of successful trafficking to distal vesicles. We have recently reported the identification of a novel xLxxKN targeting motif in the ABCA3 NH2-terminus that is also found in most other ABCA family members (4) and serves as a signal for their transit to post-Golgi compartments.

In addition to this novel targeting motif, treatment of membrane fractions from ABCA3 expressing HEK293 cells by glycosidases endoglycosidase H (Endo H) and glycosidase F (PNGase F), which cleaves N-linked glycans including complex carbohydrate chains, indicate that the transporter undergoes N-linked glycosylation (10, 37). The specific glycosylation site(s) and the role glycosylation plays in ABCA3 biosynthesis, however, remain unknown.

N-linked glycosylation is one of the most common co-/postranslational modifications that occurs during protein synthesis in the ER and has a pivotal role in the folding, stability, and cellular localization of proteins (24, 40). N-linked glycosylation is characterized by the addition of a carbohydrate moiety to the polypeptide via a β-glicosidic linkage between an N-acetylglucosamine residue and the δ-amide of an asparagine (N) residue in the consensus sequence NX(5,7)T, where X is any amino acid except proline (24, 25, 33, 40). Terminally misfolded glycoproteins undergo mannose cleavages and that
render them recognizable by lectin-like proteins such as EDEMs (ER degradation-enhancing α-mannosidase-like proteins), OS-9, and SEL-1 as adaptor molecules for ER-associated degradation (ERAD) (12, 24, 40).

In the present study we tested the hypothesis that N-linked glycosylation of ABCA3 is crucial to its biosynthetic metabolism. On the basis of an initial sequence/domain analysis, we identified several putative N-linked glycosylation motifs, with three of the four located within the first luminal loop of the transporter (Fig. 1). Using site-directed mutagenesis, we generated green fluorescent protein (GFP)-tagged fusion constructs of ABCA3 substituting glutamine for asparagine to evaluate trafficking patterns and expression levels. By multiple approaches, N-linked glycans were found to be present at only two of the four candidate sites, N124 and N140, and these were shown to be critical for the proper anterograde trafficking and stability of the nascent ABCA3 protein. In addition, the data also establish a critical role for quality control pathways in the degradation of mistrafficked ABCA3 resulting from defects in N-linked glycosylation.

MATERIALS AND METHODS

Materials. The pEGFP-N1 monomer plasmid was purchased from Clontech (Palo Alto, CA). Tagged constructs were necessary to follow trafficking of the ABCA3 transporter owing to lack of commercially available reliable antibodies. Tissue culture medium was produced by the Cell Center Facility, University of Pennsylvania. Except where noted, all other reagents were electrophoresis, tissue culture, or analytical grade and were purchased from Sigma Chemical (St. Louis, MO) or Bio-Rad (Melville, NY).

ABCA3/EGFP constructs. The full-length human ABCA3WT/EGFP was generated by amplifying three overlapping ~2-kb segments of cDNA by the RT-PCR method described previously (42). The plasmid was used as a template to generate EGFP fusion asparagine (N) mutant constructs (Fig. 2) via the one-step PCR amplification and subcloning method using the QuikChange kit (Santa Clara, CA). The primers [primer nucleotide sequence is obtained from the National Center for Biotechnology (NCBI) of human ABCA3, data accession number NM_001089] generated for these mutant constructs are as follows: for N53Q: forward, 5’-tgggaattgtgcccacgccacacctacccg-3’, reverse, 5’-cggttagatgtgcctgggcatgcctcga-3’; for N124Q: forward, 5’-ctacattagtgcaacagctgtgcctgcaggt-3’, reverse, 5’-gcctggaggagcagcgtgctg-3’; for N140Q, forward, 5’-tgaggaccacctcaggacagcaggaggcc-3’, reverse, 5’-ggtctgtggtggaaggggtcga-3’, and for N945Q: forward, 5’-cctcgcttgcacatctcctgcg-3’, reverse, 5’-gtgctgcaggtgctg-3’. All resultant plasmids were transformed into competent bacterial cells (Life Technologies, Grand Island, NY) for amplification.

DNA constructs. To ensure that no random alterations were introduced, the sequences of the coding regions of all wild-type (WT) and mutant constructs were verified by use of overlapping primers and by automated DNA sequencing performed by the Core Facility in the Department of Genetics at the University of Pennsylvania.

Cell lines, transfection, and reagent treatment. Human A549 and HEK293 epithelial cell lines were originally obtained through the American Type Culture Collection (ATTC, Manassas, VA). A549 cells grown to 85–90% confluence on glass coverslips in 35-mm plastic dishes were transiently transfected with fusion WT or mutant ABCA3 constructs (4 µg/dish) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), with consistently achievable transfection efficiencies of 40–60%. ABCA3WT/EGFP stable cell line in HEK293 cells were generated following a 48-h transient expression by the CaPO4 transfection method (41), with subsequent steps taken for clonal selection with 1 mg/ml G418. The cells were maintained in DMEM containing 500 µg/ml G418, 10% FBS, and 1% penicillin/streptomycin.

Naïve A549 and primary AT2 cells or cells transfected with the various WT or mutant ABCA3/EGFP fusion constructs were treated with 1 µM MG132 following overnight expression of the proteins and incubated for another 24 h before harvest. Similarly, nontransfected and transfected A549 cells were treated with the indicated concentrations of tunicamycin concurrently with transfection and incubated for
16 h (time of expression was reduced to minimize cell toxicity by tunicamycin).

Human fetal lung alveolar type 2 cell culture and transfection. Isolation and culture of human fetal AT2 cells was performed as previously described (22) with the knowledge of the Children's Hospital of Philadelphia Institutional Review Board. Briefly, enriched populations of epithelial cells were isolated from explants of second-trimester (13–20 wk gestation) human fetal lung tissue and were first transfected in suspension with fusion WT or mutant ABCA3/EGFP constructs by using electroporation with a proprietary transfection system protocol (AMAXA, Gaithersburg, MD) (15–25% transfection efficiency) prior to plating on glass coverslips in 30-ml plastic culture dishes in Waymouth’s medium containing 10% fetal calf serum. After overnight culture, attached cells were cultured for an additional 24–48 h in 1 ml of serum-free Waymouth’s medium containing 10 mM dexamethasone, 0.1 mM 8-bromo-cAMP, and 0.1 mM iso-butylmethylxanthine together (referred to as DCI), previously shown to maximally induce surfactant components while maintaining a morphological AT2 cell phenotype.

Immunocytochemistry. Colocalization studies were performed by immunostaining plated cells that were fixed by immersion of coverslips in 4% paraformaldehyde. Following permeabilization, cells were immunolabeled with primary antibodies for 1 h at room temperature at the following dilutions: anti-CD63 (Immunotech, Marseilles, France), 1:500; anti-calnexin (Stressgen, Victoria, Canada), 1:200; and anti-DC-Lamp (Beckman Coulter, Brea, CA), 1:100. Texas red-conjugated secondary goat anti-mouse IgG monoclonal or secondary goat anti-rabbit IgG polyclonal antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:200 dilutions were used for visualization.

Immunofluorescence with confocal microscopy. Confocal fluorescence images of permeabilized cells fixed in 4% paraformaldehyde and immunostained with various Texas red-conjugated antibodies were examined by a Bio-Rad Radiance 2000 confocal scanning system (Carl Zeiss, Thornwood, NY). The system was equipped with an argon laser line at 488 nm, a helium-neon laser line at 543 nm, and a red diode line at 637 nm coupled to a Nikon Eclipse TE300 inverted-based microscope with a 60× oil-immersion objective. Single-focal red and green fluorescent images of the same cells were saved and processed with Bio-Rad Zeiss LaserSharp 2000 software (Carl Zeiss).

SDS/PAGE. Cells were washed with ice-cold PBS (pH 7.4), scraped, and pelleted by centrifugation at 450 g for 5 min at 4°C. The pellet was washed three times with balanced salt solution and resuspended for cell count. Samples were normalized for equal number of cells by dilution with PBS as described (3), pelleted and resuspended in sample loading buffer (93.8 mM Tris·HCl (pH 6.8), 3% (wt/vol) SDS, 15% glycerol, 0.15% (wt/vol) bromophenol blue, and 20 mM DTT). Following sonication and centrifugation at 6,000 g for 30 s to remove nuclei, proteins were separated by electrophoresis on a 3–8% polyacrylamide gel (Life Technologies) and transferred to nitrocellulose membrane.

Protein deglycosylation assay. Pellets from HEK293 cells (3 × 10⁸) stably expressing EGFP-tagged WT ABCA3 protein were treated with PNGase F or Endo H and analyzed by immunoblotting for EGFP (Fig. 3A). Endo H cleaves N-linked mannosederive oligosaccharides, but not highly processed complex carbohydrates, whereas PNGase F cleaves both forms. Treatment with PNGase F increased the electrophoretic mobility of the 220-kDa isoform, yielding a deglycosylated ~210-kDa ABCA3 product, whereas Endo H treatment resulted in partial deglycosylation of the protein, indicating that ABCA3 is modified with Endo H-insensitive complex sugar chains. In addition, we consistently detected a second GFP immunoreactive band with Mr 180,000, previously demonstrated to represent a proteolytically cleaved ABCA3 isoform (17), that was insensitive to enzymatic treatment. The differential sensitivity of ABCA3 to PNGase F and Endo H profile of these isoforms is consistent with a model suggesting that this represents a processed product of ABCA3 generated by removal of the proximal NH₂-terminal region (including N-glycosylation sites) in post-Golgi, LAMP-3-positive compartments (17).

Inhibition of cellular glycosylation by tunicamycin alters trafficking and reduces steady-state levels of ABCA3 protein expression. To test the hypothesis that N-linked glycosylation is required for ABCA3 biosynthesis, A549 lung epithelial cells transiently transfected with WT ABCA3 were concurrently treated with tunicamycin. Fluorescence immunocytochemistry revealed a dose-dependent retention of ABCA3 within a calnexin-positive (ER) (Fig. 3B, top) and CD63-negative (Fig. 3B, bottom) compartment. SDS/PAGE of cell lysates confirmed the identity of the 220-kDa band as an N-linked glycosylated isoform (34) (Fig. 3C). Importantly, when compared with the fully mature isoform, steady-state expression levels of the ABCA3 primary translation product (Mr ~210,000) were markedly reduced by tunicamycin treatment in a dose-dependent manner. Moreover, the processed ABCA3 product at 180 kDa was observed only in control (untreated) cells, indicating.
that N-linked glycosylation is required for delivery to peripheral processing compartments (Fig. 3C, middle).

Glycosylation at N124 and N140 is required for normal ABCA3 protein trafficking. Using online sequence analyses (ExPASy Molecular Biology/NetGlyc 1.0 prediction server), we identified three highly conserved consensus N-linked glycosylation motifs within the first luminal loop of the ABCA3 protein (Table 1). To assign functional roles to these putative motifs, we employed site-directed mutagenesis. In A549 cells transiently transfected with EGFP-tagged WT ABCA3 or single asparagine (N) mutants, electrophoretic mobility was significantly increased by glutamine (Q) substitution at two of the three potential sites for N-linked glycosylation (N124, N140) compared with the WT ABCA3 isoform (Fig. 3D). Similar findings were seen with transient expression of the same constructs in HEK293 cells (data not shown). As with tunicamycin treatment, a loss of glycosylation was accompanied by considerably reduced expression of both the primary ABCA3 isoforms and processed products (Mr 180,000) (Fig. 3D). The decrease in ABCA3 expression was not influenced by the transfection efficiency of cDNA plasmids since comparative mRNA levels were detected in transfected cells (Fig. 5D).

Given that ABCA3 is normally routed to LROs in lung epithelial cells (4, 10, 37, 59), the effect of consensus N-linked glycosylation on intracellular trafficking of ABCA3 was assessed. Since data obtained thus far suggested that glycosylation takes place at N124 and N140, but not at N53, we next generated a double mutant construct containing both N124Q and N140Q mutations. Representative confocal fluorescence colocalization images acquired for each mutant ABCA3 isoform by either CD63 or calnexin staining in transiently expressing EGFP-tagged WT or Q for N mutant isoforms of ABCA3 at indicated asparagine residues. Nontransfected (NT) cells were used for control of antibody specificity.
Comparison of the sequences coding for the 3 potential N-glycosylation sites, N53, N124, and N140, in the first luminal/extracellular loop of ABCA3

<table>
<thead>
<tr>
<th>Species</th>
<th>NCBI Accession No.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig</td>
<td>NM_0010889</td>
<td>NVP ATIYPGQSIQELPLFFTFPPPGDTWELAYIPSHSDAAKTVTETVRRALVINMRVRGFPSEKDFEDYIRYD HSKEPL CSSSVLAAVVFEHPF</td>
</tr>
<tr>
<td>Monkey</td>
<td>EAF36503</td>
<td>NVP ATIYPGQSIQELPLFFTFPPPGDTWELAYIPSHSDAAKTVTETVRRALVINMRVRGFPSEKDFEDYIRYDHSTNVLAAVVFEHAPRSSSVLAAVVFEHPF</td>
</tr>
<tr>
<td>Pig</td>
<td>JAA53723</td>
<td>NVP ATTYPDQSIRELPLFFSFPPPGDAWELVYIPSQSEAVRTVVETARRTALVINMRARGFRSEKDFEDYVRYD</td>
</tr>
<tr>
<td>Bovine</td>
<td>DAA15550</td>
<td>NVP ATLYPSQSIQELPLFFSFPPPGATWELAYIPSQSEAVRTVVENVQRALVINLRAHGFASEKDFEDYVRYD RSTNVLAALVFEHAPRSSNVLAALVFEHPF HSRDPL</td>
</tr>
<tr>
<td>Sheep</td>
<td>XP_004021172</td>
<td>NVP ATLYPSQSIQELPLFFSFPPPGATWELAYIPSQSEAVRTVVENVQRALVINLRAHGFASEKDFEDYVRYD RSSNVLAALVFEHAPRSSNVLAALVFEHPF HSRDPL</td>
</tr>
<tr>
<td>Cat</td>
<td>XP_004001633</td>
<td>NVP ATIYPGQSIRELPLFFSFPPPGDAWELAYIPSHSDAVKTTITETARRTALVINMRARGFRSEKDFEDYVRYD HSREPL RSSNVLAAIVFEHAPRSSNVLAAIVFEHPF</td>
</tr>
<tr>
<td>Rat</td>
<td>XP_003750835</td>
<td>NVP ATVYPDQHIQELPLFFSFPPPGGSWELAYVPSHSDAARTITEAVRREFMIKMRVHGFSSEKDFEDYVRYD HSXDPL</td>
</tr>
<tr>
<td>Mouse</td>
<td>NM_001039581</td>
<td>NVP HSQDPL HSSSVLAAVVFEHSPH</td>
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Sequences of mammalian ABCA3 were aligned using NCBI/BLAST (blast2seq). Amino acids fitting the consensus N-X-S/T for asparagine-linked glycosylation are boldfaced.

Glycosylation-deficient ABCA3 is cleared by the proteasome. Quantitative immunoblot analysis of cell lysates (Fig. 5, A and C) revealed decreases in levels of ABCA3 protein expression by ~50% in single mutants of N124 and N140 and by as much as 85% when both N residues were mutated. Real-time quantitative PCR analysis using primers specific for EGF FP demonstrated that ABCA3 message levels were comparable for WT and single N mutants whereas the double N mutant showed even higher message levels (Fig. 5D), suggesting that the reduced protein expression is most likely due to posttranslational degradation and is not accounted for by either transfection efficiency or transcriptional mechanisms.

To examine the role of the ubiquitin-proteasome proteolytic pathway in cellular quality control of aberrant ABCA3 protein expression, A549 cells transfected with EGF P-tagged WT and N mutant isoforms of ABCA3 were treated for 24 h in the presence of 1 μM MG132, an inhibitor of proteasomal proteolysis. As shown in Fig. 5, B and C, MG132 significantly restored expression of both the single and double N mutant forms of ABCA3.

Glycan-deficient ABCA3 is misrouted and degraded by primary human AT2 cells. Primary human AT2 cells were transfected with EGF P-ABC A3 isoforms. Confocal immunofluorescence imaging of cells 72 h after introduction of plasmid DNA revealed two distinct ABCA3 trafficking patterns that were dependent on complete posttranslational glycosylation (Fig. 6, A and B). Both WT and single N mutants colocalized in DC-Lamp-positive compartments (a marker antigen for human AT2 cell LBs and LROs) and did not colocalize with calnexin. In contrast, double N mutation showed markedly increased expression in calnexin (+) compartments with some residual staining in DC-Lamp vesicles, suggesting partial ER retention.

Further analysis of protein subcellular distribution pattern, in both A549 and AT2 cells, of the double ABCA3 isoform revealed the relative significance of N-linked glycosylation in the trafficking of the transporter. As demonstrated in Fig. 6C, expression of WT ABCA3 in both cell types showed little or no effect on the transporter’s cellular distribution. In contrast, double mutation at N124 and N140 resulted in more ER-retained protein.

As was observed in epithelial cell lines, immunoblot analysis of primary human AT2 cells revealed increased electrophoretic mobility of the primary translation products of single and double mutants of N124Q and N140Q but not the single mutant of N53Q (Fig. 7A). Similarly, a reduced level of expression of the primary translation products of ABCA3 isoforms was observed in AT2 cells expressing single and double mutants at N124 and N140 (Fig. 7, A and C). Moreover, treatment with MG132 increased levels of ABCA3 expression of single and double mutants of N124 and N140, suggesting proteasomal proteolytic involvement during the expression of the two N mutant isoforms (Fig. 7, B and C). Noticeably, despite an increase in total ABCA3 protein levels with MG132, levels of processed ABCA3 products (M, 180) remain unchanged in both transiently transfected AT2 cells (Fig. 7B) as well as A549 cell lines (Fig. 5) and HEK cells (data not shown).
suggests that MG132 exerts its effects by inhibiting ubiquitin/proteasome proteolysis but that enhanced expression does not necessarily promote anterograde transport of glycosylation mutants of ABCA3.

A potential glycosylation site also exists at N945 (Fig. 1), although this site appears to be located within or very close to the putative seventh transmembrane domain. As shown in Fig. 8, glutamine for asparagine mutation at residues 945 did not alter either the subcellular distribution (Fig. 8A) or the size and expression level of ABCA3 (Fig. 8B).

DISCUSSION

In the present study we investigated the functional role N-linked glycosylation plays in the biosynthesis and trafficking of the ABCA3 lipid transporter. With both cell lines and primary human AT2 cells, inhibition of glycosylation by tunicamycin altered the Mr of ABCA3 and resulted in a dose-dependent diminution of steady-state expression level. Among the four putative N-linked glycosylation consensus motifs, our studies have identified that two sites, corresponding to N124 and N140, both located within the first luminal loop of the ABCA3 transporter, are glycosylated. As shown in Table 1, the primary amino acid sequence of this region is highly conserved among the orthologs in different species. Double missense substitution at N124 and N140 to glutamine resulted in retention of ABCA3 within the ER, alterations in electrophoretic mobility, and reduced protein expression levels. Moreover, the dual mutations of N124 and N140 produced an additional visibly ER-retained ABCA3 expression pattern, suggesting that, although cells are able to clear the ER of single ABCA3 mutations (either by ERAD and anterograde trafficking or both), they are less efficient doing so with the double mutation. Furthermore, the N124Q, N140Q, and N124Q+N140Q substitutions all resulted in reduced total ABCA3 protein expression. Importantly, the decrease of ABCA3 expression was not related to transfection efficiency (Fig. 5D), whereas treatment with the proteasome inhibitor MG132 partially or totally restored normal levels of all three of these mutant isoforms, indicating their rapid intracellular degradation via ubiquitin-
proteasome-mediated quality control, suggesting that the initial glycosylation event is critical for ABCA3 protein stability. Decreased stability and rapid degradation in the absence of N-linked glycosylation have also been observed with other polytopic membrane proteins. Mutation of the N-linked glycosylation sites of the human μ-opioid receptor resulted in a threefold increase in turnover of the protein (33). Similarly, other ABC transporters including ABCG2, P-glycoprotein (ABCB1), and the cystic fibrosis transmembrane conductance regulator (CFTR) have been shown to turn over at a significantly faster rate when N-linked glycosylation is disrupted (21, 45, 62). This degradation apparently occurs as a consequence of increased ubiquitin–proteasome activity, and it appears that a similar mechanism might contribute to the reduced expression of nonglycosylated ABCA3.

Although it can be argued that the increased degradation observed in the mutations of N124 and N140 may not be the result of lack of glycosylation but solely due to an acquired protein misfolding phenotype, it would be unlikely for various reasons. First, studies using tunicamycin treatment demonstrated that ABCA3 is indeed glycosylated (Fig. 3, A and C) (10, 36, 37) and that trafficking of native ABCA3 isoforms beyond the ER can be disrupted with drug treatment (Fig. 3B). Second, the immunoblot bands of single and double mutants of N124Q and N140Q supports the notion that the prominent changes in molecular weights (compared with WT ABCA3) are likely due to the absence of large sugar moiety and not due to the replacement of a single amino acid. Third, because of asparagine’s close resemblance to glutamine in both size and structure, the reliability of glutamine for asparagine substitution mutation in the study of N-linked glycosylation is frequently demonstrated in previous studies of glycoproteins (1, 39, 53, 57, 63) including other ABC transporters such as the multidrug resistance protein (MRP) (28), ABCC7 (CFTR) (21), and ABCG2 (16, 45).

The subcellular distribution patterns observed for either the WT or double mutant ABCA3 expressed proteins are independent of the reporter cell line utilized. Comparison of each isoform in ABCA3-deficient A549 cells vs. AT2 cells suggest that endogenous ABCA3, which is highly expressed in AT2 cells (30, 42), is unlikely to influence the trafficking of the glycan-deficient ABCA3 through heteromeric association. When an ER-retained ABCA3 mutant lacking the xLxxKN targeting motif (4) is coexpressed with WT ABCA3, neither did this ER-retained mutant inhibit trafficking of the WT to distal vesicles nor does WT ABCA3 correct proper trafficking of the mutant (S. Mulugeta, unpublished data). Moreover, coimmunoprecipitation and cross-linking studies have also confirmed the non-self-associative properties of the transporter (S. Mulugeta, unpublished data). This is in contrast to surfactant protein C (SP-C), which we had previously shown to exhibit a high degree of homomeric oligomerization (5, 55) and to be capable of interacting with mutant SP-C isoforms (heteromeric association) resulting in a profound influence on each other’s trafficking either by promoting the trafficking of ER retained proteins to distal vesicles or by inhibiting ER exit of the WT proteins (a dominant negative effect), in a stoichiometric fashion (43, 54). Also, with few exceptions, the ABC family of full transporters is not known to utilize posttranslational dimeric or oligomeric association for their biosynthesis. The few ABC transporters reported that are likely to form dimers or oligomers include ABCA1 (51), ABCCl (MRP1) (48, 60), ABCC7 (CFTR) (47, 61), ABCG2 (15), and the yeast Pdr5p (18). Among these, ABCA1 oligomerization and CFTR dimerization appear to take place during the ATPase cycle (51) or assembled at the plasma membrane (32), respectively.
The presence of glycosylation sites at N124 and N140 in the first luminal loop of ABCA3 is the first direct evidence that supports the current topological model as depicted in Fig. 1 (29, 52) that this long-spanning domain of ABCA3 faces either the lumen of cytosolic organelles or the extracellular space when expressed on the surface of the plasma membrane. In other model transmembrane proteins, it has been demonstrated that the minimal distance from the transmembrane domain to a luminal glycosylation site must be between 12 to 15 residues in order for N-linked glycosylation to take place (46). Therefore, it is not surprising that the potential glycosylation site at N53 is not modified since it puts this amino acid within 11 residues from the predicted first transmembrane domain (4, 7, 52). Similarly, the potential glycosylation site at N945 (Fig. 1) would be an unlikely site for glycosylation, as confirmed in Fig. 8, because N945 is also located within or very close to the predicted seventh transmembrane domain of ABCA3. By using NCBI/BLAST (bl2seq) sequence alignment (similar to that shown in Table 1), comparisons of the entire first luminal loop of ABCA3 between human and rodents showed over 82% sequence consensus, whereas comparison between human and sheep, bovine, cat, and pig revealed over 85% sequence consensus. The high extent of amino acid sequence conservation of this luminal loop suggests a critical function for this segment, apparently including mediation of proper protein stability via N-linked glycosylation.

The importance of this highly conserved luminal domain is further underscored by the growing number of ABCA3 mutations associated with fatal surfactant deficiency in newborns and with interstitial lung disease (ILD) in older children and adults. Since ABCA3 is critical for LB biogenesis and surfactant function (2, 11), it is not surprising that ABCA3 mutations have been linked to a range of lung disorders. To date, over 100 distinct ABCA3 mutations have been identified (58) in associ-
with various pulmonary abnormalities, of which a great
many have been mapped to regions encoding the first luminal
segment of the protein (14, 19, 20, 50). Although very little is
known about the cellular and molecular mechanisms underly-
ing lung disease linked to ABCA3 mutations, including those
within the first luminal loop, the limited available reports
appear to suggest that mutations in this region may affect
ABCA3 biosynthesis by one of several mechanisms.

Firstly, expression of the leucine-to-proline mutation at
residue 101 (L101P) results in ER-retained, unprocessed
ABCA3 product that induces the unfolded proteins response
and ER stress (10, 37, 56), suggesting that this mutation causes
gross misfolding of ABCA3. In contrast, a second group of
lung disease-associated mutations that are proximal to N-gly-
can sites and/or that could profoundly alter the structural
makeup of the loop such as P147L (23) and R43L (6, 20) may
adversely influence glycosylation and subsequent ABCA3 bio-
synthesis. Thirdly, missense substitutions adjacent to the puta-
tive asparagines may interfere with posttranslational addition
of N-linked glycan side chains. Specifically, mutation of as-
dpartate to asparagine at residue 123 (D123N) has also been
associated with familial ILD with fibrotic lung remodeling.
Although this mutation has only been reported in conjunction
with a SP-C gene (SFTPC) mutation (14), this ABCA3 mis-
sense substitution is adjacent to the site of the N-linked glycan
chain, N124, and potentially capable of altering ABCA3 bio-
synthesis by this mechanism.

Finally, ABCA3 sequence variants carrying a N124S
(rs142977595) or N140H (rs45447801) substitution naturally
occur in the human population [Human Amino Acid Missense
Variant Server Database (humsavar) (http://decrypthon.igbmc.
fr/msv3d/cgi-bin/humsavar?gene=ABCA3)]. On the basis
of our present data, whereas single mutations in either glycosyl-
ation site fail to disrupt the trafficking pattern of ABCA3 to
cytosolic LROs or alter their function as lipid transporters, the
role of glycosylation of ABCA3 as a “proteostatic modifier”
cannot be understated. As Fig. 5 shows, the level of expression
of single glycosylation mutants (N124Q; N140Q) are highly
susceptible to MG132 treatment, indicating that as many as
half of the glycosylation-deficient isoforms are susceptible to
proteasomal degradation. Similar issues have been observed
for another ABC protein, the ABCG2 transporter. That is,
whereas N-linked glycosylation at residue N596 stabilizes
ABCG2 and averts its proteasomal degradation (45), it is not
essential for protein targeting to the plasma membrane or its
function (16). This raises the possibility that naturally occur-
ing defects in glycosylation are capable of producing a disease
susceptible phenotype of “functional ABCA3 haploinsuffi-
ciency” whereby total lung levels of ABCA3 protein expres-

Fig. 7. Proteasome inhibition restores glycan-deficient ABCA3 expression in
primary human AT2 cells. Representative anti-GFP immunoblots of whole cell
lysates of primary AT2 cells transfected with WT or various Q for N mutant
isoforms of EGFP-tagged ABCA3 plasmids including single and double
mutants of putative N-linked glycosylated sites. At 16 h following introduction
of plasmid DNA, cells were incubated for another 24 h in the presence (B)
or absence (A) of MG132 and immunoblot bands of the primary translation
product from at least 3 separate experiments were quantified (C). β-Actin was
used to normalize for equal loading. *P < 0.05; **P < 0.01; ***P < 0.005.

Fig. 8. N 945 of ABCA3 is not glycosylated. A: repre-
sentative confocal images of A549 cells 24 h following
plasmid introduction of EGFP-tagged to either WT or a
mutant (N945Q) ABCA3 isoform showing predominant
localization of both isoforms within CD63-positive
LROs. Bar, 5 μm. B: representative anti-GFP immuno-
blots (from 2 separate experiments) of whole cell lysates
of A549 cells NT or transfected with either WT or
N945Q mutant EGFP-tagged ABCA3 cDNAs showing
similar band electrophoretic mobility and protein expres-
sion levels. β-Actin was used to normalize for equal
loading.
sion are significantly reduced and could increase the propensity for lung injury. Indeed, ABCA3 haploinsufficient mice (ABCA3<sup>+/–</sup>) have been shown to have increased susceptibility to acute lung injury from bleomycin, hyperoxia, and ventilator-induced stretch (26, 49).

In conclusion, the present study provides evidence that N-linked glycosylation is functionally essential for regulating the stability of human ABCA3 transporter and that the ER has intrinsic checkpoint for the state of N-linked glycosylation of ABCA3. Removal of N-linked glycans at N124 and N140 increases the susceptibility of ABCA3 to proteasomal proteolysis. The evaluation of the cellular quality control pathways activated in response to glycan-deficient ABCA3 expression is crucial not only to understand the overall ER quality control mechanisms for the clearance of other disease-associated misfolded ABCA3 proteins but also to provide insights for new therapeutic strategies for treatment of ABCA3 mutation-induced lung disease.

**REFERENCES**


