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N-glycosylation is essential for ileal ASBT function and protection against proteases

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Muthusamy S, Malhotra P, Hosameddin M, Dudeja AK, Borthakur S, Saksena S, Gill RK, Dudeja PK, Alrefai WA. *N*-glycosylation is essential for ileal ASBT function and protection against proteases. *Am J Physiol Cell Physiol* 308: C964–C971, 2015. First published April 8, 2015; doi:10.1152/ajpcell.00023.2015.—The bile acid transporter ASBT is a glycoprotein responsible for active absorption of bile acids. Inhibiting ASBT function and bile acid absorption is an attractive approach to lower plasma cholesterol and improve glucose imbalance in diabetic patients. Deglycosylation of ASBT was shown to decrease its function. However, the exact roles of *N*-glycosylation of ASBT, and how it affects its function, is not known. Current studies investigated the roles of *N*-glycosylation in ASBT protein stability and protection against proteases utilizing HEK-293 cells stably transfected with ASBT-V5 fusion protein. ASBT-V5 protein was detected as two bands with molecular mass of ~41 and ~35 kDa. Inhibition of glycosylation by tunicamycin significantly decreased ASBT activity and shifted ASBT bands to ~30 kDa, representing a deglycosylated protein. Treatment of total cellular lysates with PNGase F or Endo H glycosidases showed that the upper 41-kDa band represents a fully mature *N*-acetylglucosamine-rich glycoprotein and the lower 35-kDa band represents a mannose-rich core glycoprotein. Studies with the glycosylation deficient ASBT mutant (N10Q) showed that the *N*-glycosylation is not essential for ASBT targeting to plasma membrane. However, mature glycosylation significantly increased the half-life and protected ASBT protein from digestion with trypsin. Incubating the cells with high glucose (25 mM) for 48 h increased mature glycosylated ASBT along with an increase in its function. These results unravel novel roles for *N*-glycosylation of ASBT and suggest that high levels of glucose alter the composition of the glycan and may contribute to the increase in ASBT function in diabetes mellitus.

bile acid absorption; *N*-glycans; bile acids; intestinal transport proteins

APICAL sodium-dependent bile acid transporter (ASBT) is a plasma membrane protein expressed in the distal ileum and mediates the Na^+-dependent transport of bile acids across the apical membrane of intestinal epithelial cells (2). ASBT function represents the first and rate-limiting step of reabsorption of bile acids that are then transported back to the liver via the portal blood where they influence cholesterol metabolism and insulin signaling, as well as lipid and glucose metabolism (24). The essential role of ASBT in controlling enterohepatic circulation of bile acids is supported by the fact that surgical removal of the distal ileum and genetic deletion of ASBT in mice leads to bile acid malabsorption and the depletion of their circulating pool between the liver and intestine (1, 14). On the other hand, a decrease in ASBT expression was shown in disorders with lipid abnormalities such as familial type IV hypertriglyceridemia (16). Further, ASBT function and expression are increased in diseases that are associated with bile acid overload in the liver like necrotizing enterocolitis (NEC) and progressive familial intrahepatic cholestasis (PFIC) (10, 19). Therefore, much attention has been focused on investigating ASBT regulation and defining molecular targets for modulating its function and expression.

The transcriptional regulation of ASBT has been extensively investigated. ASBT mRNA expression and promoter activity is decreased by proinflammatory cytokines and by cholesterol (3, 11). Also, bile acids through FXR-SHP axis suppress ASBT expression by reducing the binding of RAR/RXR activating complex to ASBT promoter (25). On the other hand, the activation of glucocorticoid receptor increases ASBT expression and promoter activity (22). Besides the transcriptional regulation, recent studies also demonstrated posttranscriptional modulations of ASBT. For example, the activation of PKC ζ-dependent pathway decreases ASBT level on the plasma membrane (27). Also, ASBT function is dependent on lipid raft domains of the plasma membrane and the depletion of plasma membrane cholesterol decreases ASBT function (7).

N-glycosylation represents one of the most important post-translational modifications of plasma membrane proteins. The attachment of glycans to membrane proteins is known to influence many functional aspects including targeting to plasma membrane (18, 21, 26, 32, 33). Further, changes in the pattern of glycosylation of plasma membrane proteins occur in diseases with hyperglycemia affecting their function and contributing to the associated pathophysiology as shown for the urea transporter UT-A1 in the kidney (12). ASBT has been previously shown to be *N*-glycosylated on the asparagine residue N10 in the first extracellular loop of the protein (34). Deglycosylation led to a decrease in ASBT function without affecting its targeting to plasma membrane (34); however, the exact mechanism by which the deglycosylation of ASBT alters its function remains elusive. Also, bile acid absorption was increased in diabetic patients and animal models of diabetes mellitus, and whether hyperglycemia affects ASBT function and *N*-glycosylation is not known (5, 8, 29).

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The current studies were focused at investigating the mechanisms by which glycosylation affects ASBT function and surface expression in HEK293 cells stably transfected with ASBT-V5 fusion protein. Our studies provide novel molecular insights into the roles of *N*-glycosylation in increasing the half-life of ASBT protein on the plasma membrane and protecting ASBT against digestion by proteases. Further, these data suggest that hyperglycemia-induced changes in the glycosylation may underlie the increase in ASBT function and bile acid absorption associated with diabetes mellitus.

MATERIALS AND METHODS

Materials. Taurocholic acid and cycloheximide were purchased from Sigma (St. Louis, MO); [^{3}H]taurocholic acid (TCA) was purchased from Perkin-Elmer (Boston, MA). Anti-V5-HRP was purchased from Invitrogen (Carlsbad, CA). Anti-GAPDH-HRP antibody was purchased from Santa Cruz Biotechnology (Paso Robles, CA). Cell culture medium was purchased from ATTC (Manassas, VA), and the transfection reagents were purchased from Invitrogen. Agarose-bound lectins were purchased from Vector Laboratories (Burlingame, CA).

Cell culture and stable cell lines. We have previously established human embryonic kidney (HEK)-293 cells that are stably transfected with human ASBT-V5 fusion protein (designated as 2BT cells) (4, 7) as a suitable model to investigate posttranslational regulation of ASBT. Cells were cultured in MEM supplemented with 10% FBS and were plated at a density of 2×10^5/well in 24-well Falcon plates. 2BT cells reached 90–100% confluence after 2–3 days in culture and were utilized for experiments. The medium was supplemented with 300 μg/ml of geneticin in case of the stably transfected cells. Transient transfections in HEK-293 cells were carried out using Lipofectamine 2000 (Invitrogen) as previously described (7).

Western blotting. Cells were lysed in RIPA buffer (Sigma) supplemented with freshly added protease inhibitor cocktail (Roche, Branford, CT). Protein concentration was measured by the method of Bradford using protein assay kit (Bio-Rad, Hercules, CA). Proteins (25 μg) were then solubilized in Laemmli sample buffer (2% SDS, 10% glycerol, 100 μM DTT, 60 mM Tris, pH 6.8, 0.01% bromophenol blue) and separated on 4–15% gradient SDS-PAGE gels (Bio-Rad). Separated proteins were then transferred to nitrocellulose membrane, and the blots were incubated with the anti-V5 HRP-conjugated antibody diluted in the blocking solution for 3 h at room temperature. Blots were washed extensively with PBS containing 0.1% Tween-20, and then the bands were visualized by enhanced chemiluminescence ECL kit according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

Cell surface biotinylation. HEK-293 cells stably or transiently transfected were washed with ice-cold PBS and incubated with 1 mg/ml of EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL) dissolved in borate buffer pH 9.0 for 1 h. Incubating with 100 mM glycine in ice-cold PBS for 20 min quenched the reaction; cells were then lysed in RIPA buffer and insoluble debris was cleared by centrifugation (6). Biotinylated proteins were separated by NeutrAvidin agarose beads (Pierce), washed with RIPA buffer, and eluted by boiling in 2X SDS buffer (Bio-Rad). The eluted proteins were analyzed by Western blotting. To validate biotinylation of surface proteins, the same blot was stripped and probed with anti-GAPDH-HRP antibodies.

Lectin pull-down assay. Lectin-pull downs were performed in total cell lysates prepared with RIPA buffer. Four hundred micrograms of total cell lysates was added to 75 μl of agarose bound lectins (Vector Laboratories) in a total volume of 500 μl RIPA buffer and mixed for overnight at 4°C. The beads were washed three times in RIPA buffer; boiled in 2X SDS buffer for 5 min to elute bound proteins, and the resulting eluent was analyzed by Western blotting with anti-V5-HRP antibodies.

Treatment with glycosylation inhibitors and glycosidases. Tunicamycin (Sigma, St. Louis, MO) at a concentration of 0.5 μg/ml was used to inhibit *N*-glycosylation in stably transfected HEK-293 cells for 24 h. Cell lysates were digested with endoglycosidase H (Endo H) for 1 h at 37°C, (New England BioLabs, Ipswich, MA) to remove high mannose glycans (core-glycosylated). Peptide-*N*-glycosidase F (PNGase F) (New England BioLabs) was used to remove *N*-linked glycans from both complex and high mannose glycoproteins for 1 h at 37°C. Digested proteins were analyzed by Western blotting with anti-V5-HRP antibodies.

Taurocholic uptake assay. HEK-293 cells stably transfected with hASBT were used for uptake experiments. Cells were seeded in 24-well plates, and uptake was carried out 3–4 days postplating. Uptake was carried out as described previously (6). To determine the sodium dependency of the uptake, Na^+ in the buffer was isosmotically replaced with choline chloride. Uptake rates were measured as picomoles per milligram of protein per 5 min.

Statistical analysis. Results were expressed as means ± SE of 3–4 experiments performed on separate occasions. Student's *t*-test was utilized for statistical analysis. $P \leq 0.05$ was considered statistically significant.

RESULTS

Deglycosylation decreases ASBT protein levels on plasma membrane. Since ASBT has previously been shown to be a glycoprotein, we aimed at investigating how *N*-glycosylation affected ASBT function and membrane expression. We first examined the pattern of ASBT glycosylation in HEK-293 cells stably transfected with ASBT-V5 fusion protein (designated as 2BT cells). As shown in Fig. 1*A*, ASBT-V5 fusion protein was detected as two bands with molecular masses of about 41 kDa and 35 kDa as previously reported in Cos-7 cells (34). Cell surface biotinylation presented in Fig. 1*A* demonstrated that the two ASBT bands were detected on the plasma membrane fractions. Also, Fig. 1*A* shows that the higher molecular weight band was more enriched on the plasma membrane compared with the lower molecular weight band. Treatment of 2BT cells with the *N*-glycosylation inhibitor tunicamycin (0.5 μg/ml, 24 h) blocked ASBT glycosylation and shifted both ASBT bands to a lower band with a molecular mass of about 30 kDa (Fig. 1*A*). Previous studies have shown that the asparagine residue N10 located on the extracellular NH_2 terminus of ASBT is the only site responsible for ASBT glycosylation. We have generated HEK-293 cells that express ASBT-V5 fusion protein in which the N10 residue was mutated to glutamine (N10Q). As shown in Fig. 1*B*, the N10Q mutant was detected as a 30-kDa immunoreactive band similar to the band that was detected after treatment with tunicamycin. These data show that *N*-glycosylation of ASBT generates two different glycosylated proteins. Densitometric analysis presented in Fig. 1*C* shows that in response to tunicamycin treatment, total cellular expression of ASBT is significantly lower than that of the wild-type ASBT (considering the sum of densities of higher and lower molecular weight bands). The observed reduction mainly occurs because of the loss of the higher molecular weight ASBT protein. Similar to the decrease in the surface levels, ASBT function is also significantly decreased ~50% in cells treated with tunicamycin as depicted in Fig. 2.

N-glycosylation is not essential for ASBT trafficking to plasma membrane. We next investigated whether *N*-glycosylation is important for ASBT trafficking by evaluating the rate by which ASBT is targeted to the plasma membranes as

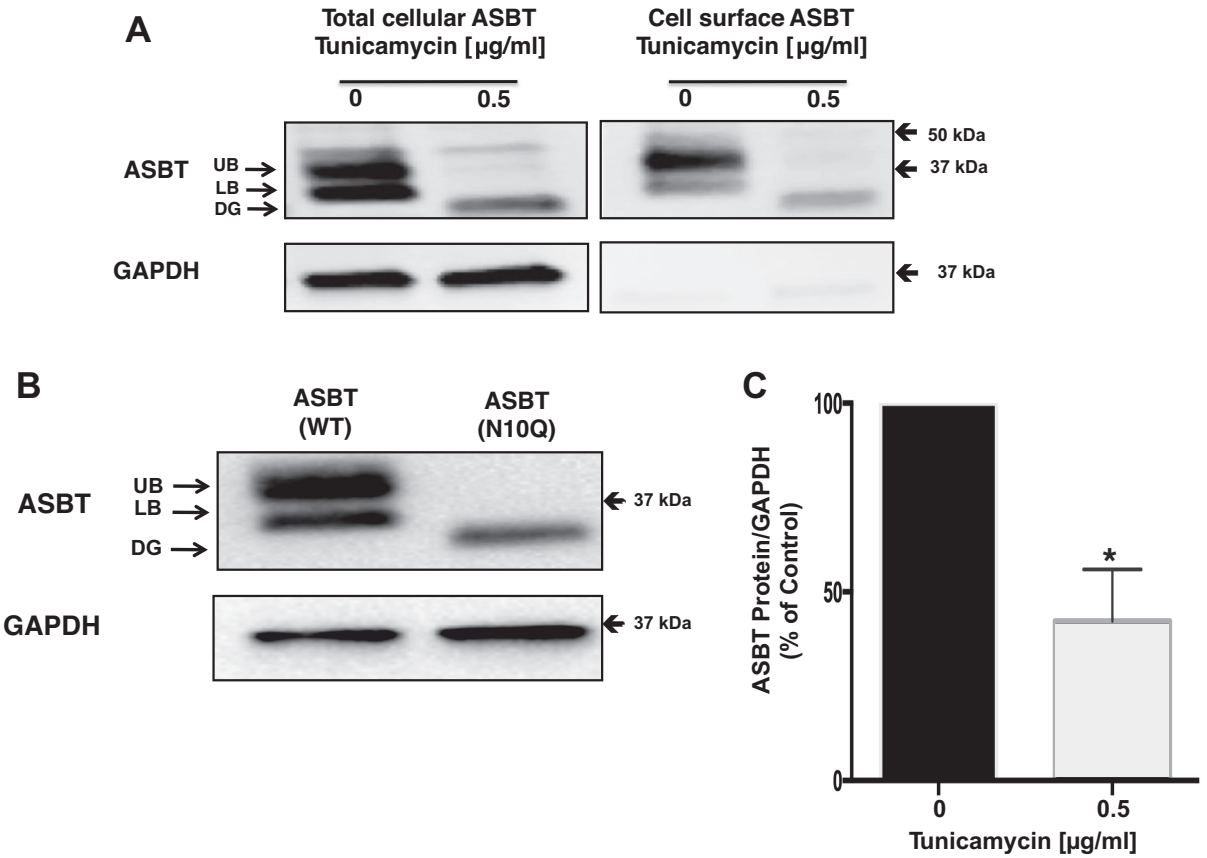

Fig. 1. Deglycosylation decreases ASBT protein levels on the plasma membrane. *A*: HEK-293 cells stably expressing wild-type ASBT-V5 fusion protein were treated with tunicamycin (0.5 μg/ml) or vehicle for 24 h followed by cell surface biotinylation. The labeled cell surface proteins were precipitated with streptavidin beads and resolved by SDS-PAGE followed by immunoblotting with anti-V5 antibody. UB, upper band (~41 kDa); LB, lower band (~35 kDa); DG, deglycosylated band (~30 kDa). The specificity of the cell surface labeling was confirmed by absence of GAPDH on the cell surface. *B*: HEK-293 cells stably expressing glycosylation deficient mutant ASBT (N10Q)-V5 total protein was extracted and separated by SDS-PAGE followed by Western blotting with anti-V5 antibody. *C*: densitometric analysis for ASBT relative expression in total cell lysates normalized to the levels of GAPDH. Values are expressed as % of control and represent means ± SE of 3–6 measurements. *$P < 0.05$ compared with control.

previously described (33). HEK-293 cells were transiently transfected with wild-type or N10Q ASBT mutant and then surface expression of the newly synthesized ASBT protein from transfected plasmid was assessed by cell surface biotinylation at different time points (6, 16, and 24 h) posttransfection. As shown in Fig. 3*A*, the two bands of ASBT were detected at the surface after 16 h from the start of transfection. Data depicted in Fig. 3*A* show that the ASBT mutant is also detected on the plasma membrane by 16 h posttransfection, and its level was increased by time. Densitometric analysis of the levels of surface ASBT over time, shown in Fig. 3*B*, demonstrates that the rate of trafficking of wild-type ASBT glycoprotein is similar to that of the nonglycosylated N10Q ASBT mutant. These data suggest that glycosylation is not essential for the trafficking of ASBT to the plasma membrane.

N-glycosylation profile of ASBT glycoprotein. As the two immunoreactive bands of wild-type ASBT represent different glycoproteins, we next examined the nature of carbohydrates that contribute to each of these two glycoproteins of ASBT. Total protein lysates from 2BT cells were treated with PNGase F and Endo H glycosidases. The Endo H recognizes high mannose carbohydrate chains, whereas PNGase F cuts the first *N*-acetylglucosamine of the glycan that is attached to asparagine residues on the protein. As expected, treatment with PNGase F shifted the two ASBT bands to the lower band of ~30 kDa similar to treatment with tunicamycin (Fig. 4). However, treatment with Endo H shifted only the low molecular weight band to 30 kDa, whereas, the high molecular weight band appears to be insensitive to Endo H treatment. These data clearly suggest that the lower band of wild type ASBT is mannose rich core glycosylated protein, whereas the higher band is the mature N-acetylglucosamine rich glycosylated ASBT protein.

To further confirm the nature of carbohydrates associated with ASBT glycoprotein, total cellular proteins from 2BT cells or from HEK-293 cells stably expressing N10Q ASBT mutant were subjected to pull-down assays with different types of lectins that interact with different carbohydrate chains associated with glycoproteins. As shown in Fig. 5, the agarose bound ConA lectin selectively enriched the mannose rich core-glycosylated ASBT, whereas the agarose bound wheat germ agglutinin (WGA) selectively pulled down the highly complex glycosylated ASBT protein (upper band). The nonglycosylated ASBT-N10Q mutant did not interact with the lectins, indicating the specificity of the assays. These results support the notion that ASBT glycosylation occurs as a core mannose rich or mature complex forms.

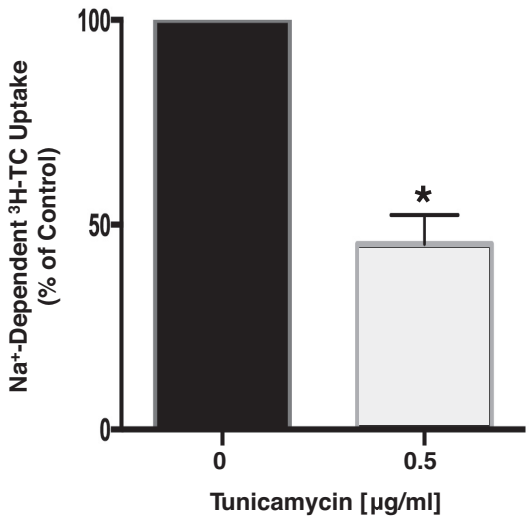

Fig. 2. Tunicamycin decreases ASBT function. HEK-293 cells stably expressing wild-type ASBT-V5 fusion protein were treated with tunicamycin (0.5 μg/ml) or vehicle (control) for 24 h and ASBT function was then assessed as described in MATERIALS AND METHODS. ASBT function was determined as Na^+ dependent [^{3}H]taurocholate ([^{3}H]TC) uptake and expressed as % of control. Data are expressed as means ± SE of 4–6 experiments. *$P < 0.05$ compared with control.

N-glycosylation of ASBT increases protein stability. Since the level of the mature glycosylated ASBT was higher than that of the mutant, we next examined whether *N*-glycosylation affects the stability of ASBT. For these studies, protein de novo synthesis in 2BT cells and HEK-293 cells stably transfected with ASBT mutant was blocked by treatment with cycloheximide (0.1 mg/ml) for different periods of time. At the end of the incubation, cell surface proteins labeled by biotin were precipitated by streptavidin beads. As shown in Fig. 6*A*, the mature glycosylated ASBT glycoprotein appears to have longer half-life compared with that of the core glycosylated ASBT when investigated with the total cell protein lysates and plasma membrane biotinylated fractions (mature glycosylated: 7 ± 0.5 h; core glycosylated: 1 ± 0.2 h; and nonglycosylated: 0.5 ± 0.1 h). Interestingly, the half-life of ASBT mutant was comparable to the half-life of core glycosylated ASBT. Densitometric analysis presented in Fig. 6*B* showed that mature *N*-glycosylation significantly increased the half-life of ASBT protein by ~7–10 fold on the plasma membrane consistent with the increase in its function.

N-glycosylation of ASBT protects against proteases. Glycosylation is known to protect plasma proteins against digestion by external proteases (30). We then examined whether N-glycosylated ASBT is more resistant to cleavage by proteases. Total protein lysates from 2BT cells and HEK-293 cells stably expressing ASBT N10Q mutant were treated with trypsin (10 μM) for 30 min and the proteolytic products were examined by Western blotting. Figure 7*A* shows that the trypsin treatment caused a decrease in the level of ASBT mutant protein more than the decrease observed in wild-type ASBT protein. The numeric representation of the data shown in Fig. 7*B* clearly demonstrates that the digestion by trypsin was significantly increased in nonglycosylated (N10Q mutant) compared with *N*-glycosylated ASBT. These data indicate that *N*-glycosylation protects ASBT against the proteolytic effects of proteases such as trypsin.

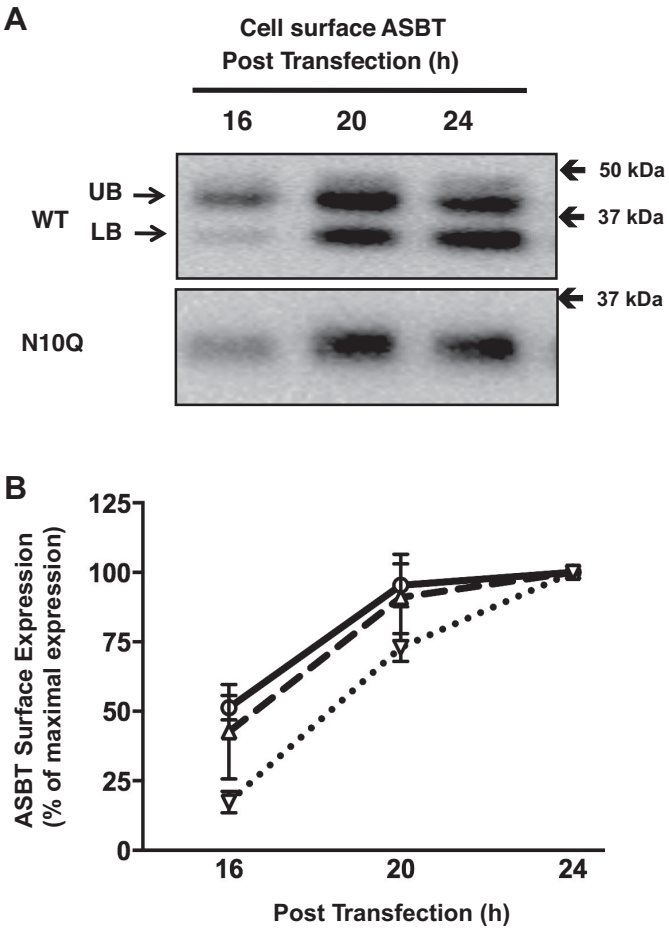

Fig. 3. *N*-glycosylation is not essential for ASBT trafficking to plasma membrane. *A*: transiently transfected cells were biotinylated at various time points posttransfection, and cell surface proteins were precipitated with streptavidin beads followed by Western blotting with anti-V5 antibody. UB, upper band; LB, lower band. *B*: densitometric analysis for the surface expression of wild-type ASBT (up triangles, UB; down triangles, LB) and ASBT-N10Q (circles) at various times posttransfection. Values are expressed as % of maximal expression and represent means ± SE of 3 experiments.

High glucose increases ASBT glycosylation and function. Recent studies in animal models and humans demonstrated that diabetes mellitus and hyperglycemia modulate *N*-glycosylation by increased glucose flux through the hexosamine pathway (9, 23, 28). To examine whether high glucose in the cell culture media alters ASBT *N*-glycosylation levels, 2BT cells were incubated with medium containing 5 mM concentration of

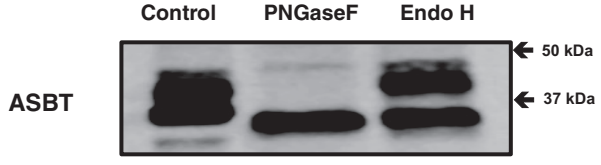

Fig. 4. ASBT *N*-glycosylation profile. Total cell lysates extracted from wild-type ASBT-V5 fusion protein were treated with peptide-*N*-glycosidase F (PNGase F) and endoglycosidase H (Endo H), and then samples were solubilized in Laemmli sample buffer and resolved by SDS-PAGE followed by Western blotting with anti-V5 antibody.

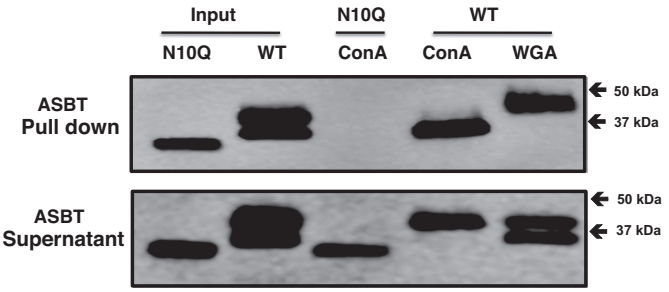

Fig. 5. Lectin pull-down assays for wild-type ASBT and glycosylation deficient N10Q mutant ASBT protein samples. Equal amounts protein from total cellular lysate were incubated with agarose-bound lectins (4°C) overnight along with protease inhibitor cocktail. After washing with RIPA buffer, bound samples were eluted by boiling with 2X Laemmli buffer and used for Western blotting with anti-V5 antibody. Top panel shows the pull-down fractions and bottom panel shows unbound proteins in the supernatant.

glucose (normal) or 25 mM concentration of glucose (high) for 48 h. Figure 8 shows that ASBT activity, judged by the Na^+-dependent [^{3}H]taurocholate uptake, was significantly increased when cells where incubated with 25 mM compared with 5 mM glucose. Western blot analysis presented in Fig. 9, *A* and *B*, showed that the expression of the fully mature glycosylated ASBT protein was significantly increased in response to incubation with 25 mM glucose. These findings suggest that high levels of glucose increase ASBT glycosylation, leading to an increase in the expression of the fully mature glycoprotein and an increase in ASBT function.

DISCUSSION

Ileal ASBT activity has previously been shown to decrease in response to deglycosylation (34). However, the mechanisms by which glycosylation affects ASBT function remains unclear. Data presented in the current study for the first time demonstrate that *N*-glycosylation is essential for ASBT protein stability on the plasma membrane and for the protection against digestion with proteases.

We investigated ASBT glycosylation utilizing human embryonic kidney (HEK)-293 cells stably transfected with ASBT-V5 fusion protein that has been previously established by us as an excellent model to examine posttranslational regulation of ASBT (7). ASBT-V5 protein in 2BT cells was detected by Western blotting as two bands. In the current studies, the nature of the ASBT bands was scrutinized by two complementary approaches: digestion with glycosidases and lectin pull-down assay. The peptide-*N*-glycosidase F (PNGase F) cleaves the bond between the asparagine residue on the glycoprotein and the first *N*-acetylglucosamine (GlcNAc) sugar molecule in the attached polysaccharide releasing the full glycan chain from all types of the *N*-glycosylated proteins. PNGase F treatment to

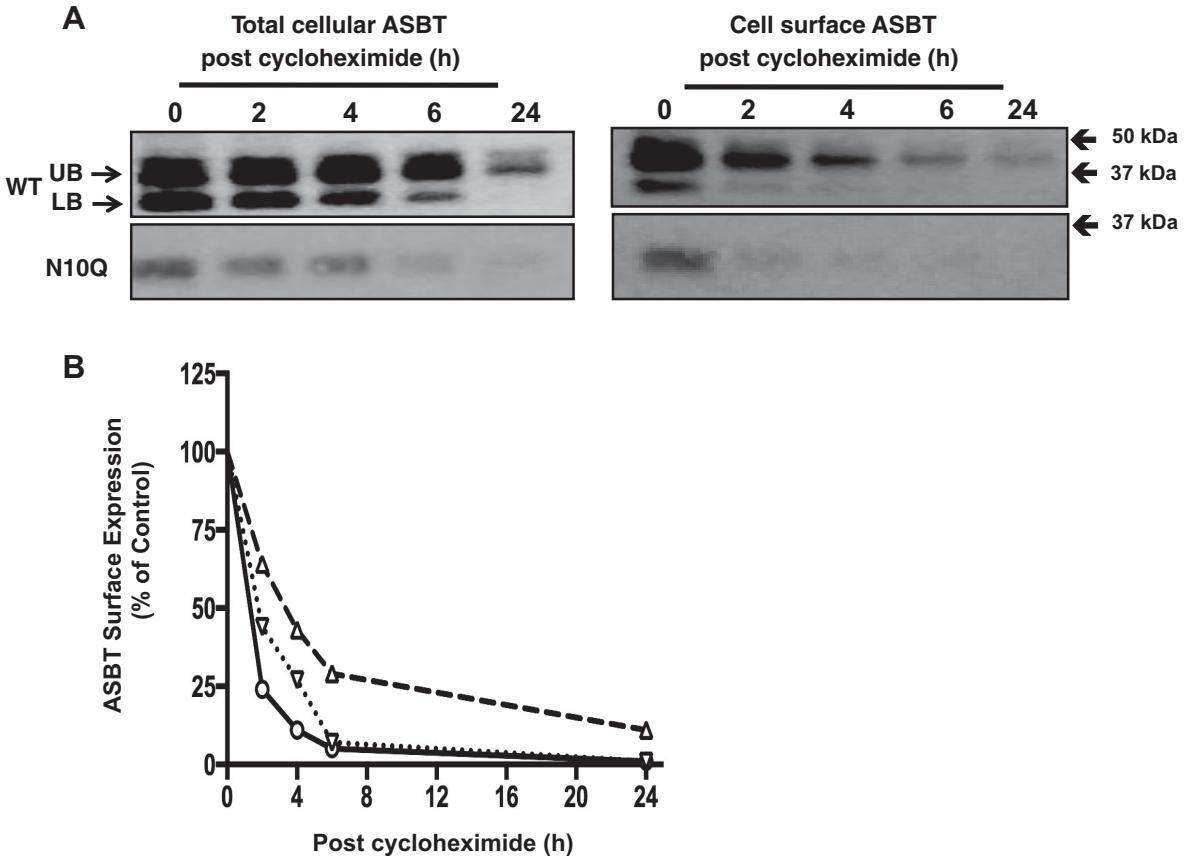

Fig. 6. *N*-glycosylation increases ASBT protein stability. *A*: HEK-293 cells stably expressing wild-type ASBT or glycosylation deficient N10Q mutant ASBT were treated with 0.1 mg/ml of cycloheximide for different periods of time and then cell surface proteins were biotinylated. The labeled cell surface proteins were precipitated with streptavidin beads and resolved by SDS-PAGE followed by Western blotting with anti-V5 antibodies (UB, upper band; LB, lower band). *B*: densitometric analysis of surface levels of wild-type ASBT and glycosylation deficient mutant ASBT-N10Q (up triangles, UB; down triangles, LB; circles, N10Q mutant). Values are expressed as % control (values at 0 h).

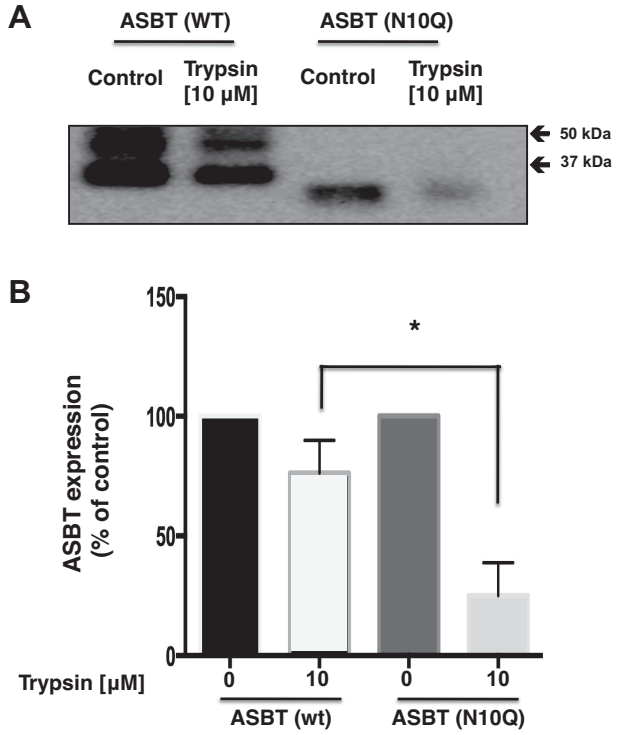

Fig. 7. *N*-glycosylation protects against proteases. *A*: equal amount of protein from total cell lysates prepared from wild-type ASBT or glycosylation deficient mutant ASBT-N10Q were digested with 10 μM trypsin for 10 min and then reaction was stopped with trypsin inhibitor. Samples were then solubilized in Laemmli buffer and used for Western blotting with anti-V5 antibody. *B*: densitometric analysis of wild-type ASBT and glycosylation deficient mutant ASBT-N10Q is presented. Data are presented as % of respective controls and are shown as means ± SE of 4 experiments. $*P < 0.05$ compared with control.

protein lysates of 2BT cells shifted both the high and low molecular weight bands of ASBT to a lower molecular weight band (~30 kDa), indicating that both the bands represent glycosylated ASBT. This pattern was similar when the cells were incubated with the glycosylation inhibitor tunicamycin. The observation that the band generated by treatment with PNGase F and tunicamycin is a nonglycosylated ASBT was further confirmed by showing that ASBT band generated by the treatment has the same molecular weight as the glycosylation deficient N10Q-ASBT mutant. The other glycosidase used was endoglycosidase H (Endo H) that selectively cleaves the oligomannose-type of glycans attached to glycosylated proteins. The low molecular weight, but not the high molecular weight, glycosylated ASBT was sensitive to Endo H and was cleaved to a nonglycosylated ASBT. Collectively, these data suggested that the low molecular weight ASBT band represents a glycoprotein with a mannose rich glycan, whereas, the high molecular weight band represents a fully mature glycosylated protein. It is worth noting that Dawson et al. (15) previously demonstrated the presence of glycosylated ASBT protein in brush-border membrane vesicles prepared from mouse intestine that was not sensitive to digestion by Endo H indicating a mature glycosylated protein. This previous observation implies that ASBT protein expressed on the apical membrane of native intestinal epithelial cells is a mature glycosylated protein. Therefore, investigating the functional aspects of the mature glycosylated ASBT in 2BT cells is relevant to the native intestinal epithelial cells.

The complex structure of the glycan attached to the high molecular weight band of ASBT was further confirmed by the ability of wheat germ agglutinin (WGA) lectin, which binds to glycoproteins rich with GlcNAc and sialic acid, to pull down the upper ASBT band. Notably, WGA lectin did not completely pull down the upper ASBT band suggesting the presence of multiple types of complex glycan species forming the mature glycosylated ASBT protein. Incubation with lectin ConA that binds to α-linked mannose glycan, pulled down only the lower ASBT band further supporting the notion that the low molecular weight ASBT band represents mannose rich ASBT glycoprotein.

N-glycosylation plays important roles in protein folding and trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus as well as targeting of proteins to the apical membrane of epithelial cells (32). In this regard, a polysaccharide chain is usually attached to a newly synthesized plasma membrane protein in the ER that is then transported to the Golgi apparatus for further processing (32). Some proteins, however, traffic through the Golgi and post-Golgi without any processing and reach the plasma membrane as mannose-rich glycoproteins (17). Our data showed that the core glycosylated, mannose rich ASBT glycoprotein (the low molecular weight band) is expressed on the plasma membrane indicating that a pool of ASBT protein could be targeted to the plasma membrane through the Golgi apparatus without any additional processing. Both the two glycosylated ASBT proteins and the glycosylation-deficient ASBT mutant (N10Q) were targeted to the plasma membrane with similar efficiencies after the start of transient transfection of their corresponding expression vector in HEK-293 cells. Our data show that glycosylation is not essential for ASBT targeting. These data are consistent with the previous studies of Zhang et al. (34) showing comparable

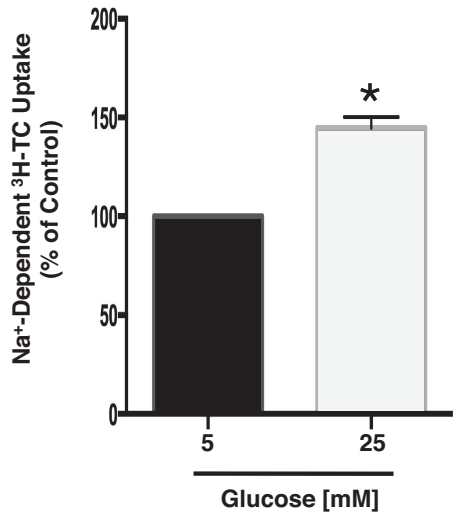

Fig. 8. High glucose increases ASBT function. *A*: HEK-293 cells stably expressing wild-type ASBT were incubated with media containing 5 or 25 mM glucose for 48 h, and then ASBT function was assessed as described in MATERIALS AND METHODS. ASBT function was determined as Na^+ dependent [^{3}H]TC uptake and expressed as % of control (5 mM glucose). Values are shown as means ± SE of 4 experiments. $*P < 0.05$ compared with control.

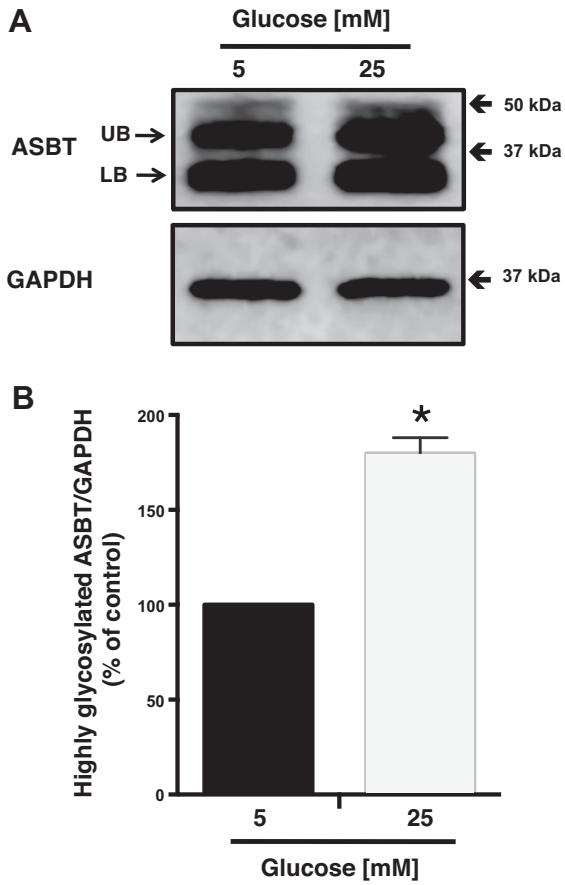

Fig. 9. High glucose increases ASBT glycosylation. *A*: HEK 293 cells stably expressing wild-type ASBT were incubated with media containing 5 or 25 mM glucose for 48 h. Total cellular proteins were extracted and equal amounts of protein were resolved by SDS-PAGE, and Western blotting was performed with anti-V5 antibody. *B*: densitometric analysis of the mature glycosylated for ASBT in 25 mM glucose treated cells and expressed as % of control (5 mM glucose). $*P < 0.05$ compared with control.

levels of surface membrane expression of wild-type ASBT and the glycosylation-deficient ASBT. Our results and the previous findings indicate that glycosylation is not essential for ASBT targeting to the plasma membrane.

Protein glycosylation was shown to increase the half-life of plasma membrane transporters like the potassium channel kv1.4 and the receptor potassium channel TRPP2 as well as receptor potential melastatin 4b* protein (31, 33). Our studies showed that the half-life of the mature glycosylated ASBT is significantly higher than the half-life of the core glycosylated and the deglycosylated forms of ASBT, thus providing novel evidence showing that complex glycosylation of ASBT increases its protein stability on the plasma membrane.

ASBT protein on the plasma membrane of ileal epithelial cells is exposed to a milieu that is rich with digestive enzymes including proteases. Indeed, glycosylation was shown to be one of the important mechanisms protecting intestinal epithelial proteins from degradation by digestive enzymes (30). This was shown to be the case for the H^+/K^+-ATPase proton pump and intestinal SLC26A3 chloride transporter (13, 20). Similarly, our data showed that mature glycosylated ASBT is resistant to digestion by trypsin suggesting that glycosylation is essential for the protection of the ASBT protein from digestion by luminal proteases.

Changes in the *N*-glycans attached to proteins were altered in diseases such as diabetes mellitus (26). Hyperglycemia associated with diabetes mellitus may lead to an increase in the flux of glucose through the hexosamine pathway resulting in an increase in the production of UDP-GlcNAc and hence affecting protein glycosylation (9, 23, 28). For example, studies showed that the carbohydrate structure of the N-glycan attached to renal urea transporter UT-A1 was altered in diabetes mellitus causing an increase in the total level of the protein (12). Our data demonstrated a significant increase in ASBT function in 2BT cells in response to prolonged incubation (48 h) with high levels of glucose. This increase in the function is not due to transcriptional regulation since an exogenous promoter in 2BT cells drives ASBT expression. Indeed, the level of the mature glycosylated, but not the core glycosylated, ASBT protein was significantly increased by exposure to glucose. These data suggest that high levels of glucose enhanced ASBT glycosylation leading to an increase in ASBT protein levels, likely via promoting its protein stability.

In conclusion, our data for the first time show that ASBT half-life on the plasma membrane is significantly increased by mature *N*-glycosylation. Also, *N*-glycosylation of ASBT provides protection against digestion with luminal proteases that may have access to the brush-border membrane of intestinal epithelial cells. Interestingly, high levels of glucose increased the glycosylation of ASBT and enhanced its function. In this regard, we have previously shown that ASBT mRNA and protein expression were significantly elevated in rat model of diabetes mellitus (5). Our current studies provide novel evidence for a posttranslational mechanism that contributes to an increase in ASBT protein levels in response to hyperglycemia associated with diabetes mellitus. Indeed, elucidating the mechanisms by which ASBT glycosylation is altered by high glucose may unravel novel pathways that could be targeted to inhibit bile acid absorption in diabetes mellitus. Future studies are needed that focus on investigating the nature of the changes in the *N*-glycans attached to ASBT in diabetic patients and in animal models of diabetes mellitus.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.M., S.S., R.K.G., P.K.D., and W.A.A. conception and design of research; S.M., P.M., M.H., A.K.D., and S.B. performed experiments; S.M., P.M., M.H., A.K.D., S.B., S.S., R.K.G., P.K.D., and W.A.A. analyzed data; S.M., P.M., M.H., A.K.D., S.B., S.S., R.K.G., P.K.D., and W.A.A. interpreted results of experiments; S.M., R.K.G., and W.A.A. prepared figures; S.M., R.K.G., and W.A.A. drafted manuscript; S.M., S.S.,

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