Loss of N-Linked Glycosylation Reduces Urea Transporter UT-A1 Response to Vasopressin*

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The vasopressin-regulated urea transporter (UT)-A1 is a transmembrane protein with two glycosylated forms of 97 and 117 kDa; both are derived from a single 88-kDa core protein. However, the precise molecular sites and the function for UT-A1 N-glycosylation are not known. In this study, we compared Madin-Darby canine kidney cells stably expressing wild-type (WT) UT-A1 to Madin-Darby canine kidney cell lines stably expressing mutant UT-A1 lacking one (A1m1, A1m2) or both glycosylation sites (m1m2). Site-directed mutagenesis revealed that UT-A1 has two glycosylation sites at Asn-279 and Asn-280. Membrane fractionation of the plasma membrane, Golgi, and endoplasmic reticulum revealed that AVP or forskolin (FSK) treatment increases UT-A1 abundance in both Golgi and plasma membrane compartments in WT but not in m1m2 cells. Pulse-chase experiments showed that UT-A1 half-life is reduced in m1m2 cells compared with WT cells. Our results suggest that mutation of the N-linked glycosylation sites reduces urea flux by reducing UT-A1 half-life and decreasing its accumulation in the apical plasma membrane. In vivo, inner medullary collecting duct cells may regulate urea uptake by altering UT-A1 glycosylation in response to AVP stimulation.

Renal tubular perfusion experiments in 1987 provide functional evidence for a urea transporter (UT) protein in the rat terminal inner medullary collecting duct (IMCD) and show that it is regulated by vasopressin (AVP; also called antidiuretic hormone) (1). The first UT cDNA was cloned in 1993 (2). At present, three UT subfamilies have been reported, renal tubular-type urea transporter UT-A, erythrocyte vascular-type urea transporter UT-B, and eel renal proximal tubular-type urea transporter UT-C (3, 4). UT-A has six protein isoforms (3, 5); the longest is named UT-A1. UT-A1 is expressed in the IMCD and plays a critical role in the urine-concentration concentrating mechanism. A UT-A1/UT-A3 knock-out mouse has seriously impaired urinary concentrating ability (6).

The deduced amino acid sequence of UT-A1 shows that it is an extremely hydrophobic 929-amino-acid integral membrane protein with four potential glycosylation sites. Immunoblotting studies with normal rat renal inner medulla reveal a predominant protein band of 97 kDa and a less abundant protein band of 117 kDa (7). These two bands are not due to proteolytic cleavage of the core protein but result from different states of glycosylation. After deglycosidase PNGase F treatment, both the 97- and 117-kDa bands disappear, yielding a single 88-kDa deglycosylated UT-A1 protein (7). Unlike the potassium channel Eag1, in which the lower molecular mass band of 110 kDa is a core-glycosylated glycoprotein, the 97-kDa glycoprotein is not an immature glycosylation form for UT-A1, as its glycosylation is sensitive to endoglycosidase F (7). Therefore, a single polypeptide chain is glycosylated to two different extents to generate the 97- and 117-kDa forms. Experiments using differential centrifugation to fractionate inner medullary membranes show that both glycoproteins are expressed in the membrane and cytoplasmic fractions (9). Our previous study shows that both the 97- and 117-kDa glycoprotein forms of UT-A1 are phosphorylated in response to AVP stimulation (10).

N-Glycosylation is a common post-translational modification of membrane proteins. It is a cotranslational event that occurs in the endoplasmic reticulum (ER) and Golgi apparatus in which an oligosaccharide chain is transferred to the nascent polypeptide as asparaginyl residues within the consensus N-linked glycosylation site (NX(S/T), where X ≠ proline) (11). Glycosylation has been associated with a number of glycoprotein functions, such as modulating protein biological activity, directing protein folding, regulating cell-surface expression and membrane localization, affecting substrate affinity, or destabilizing the mature protein structure (7, 12–14). Inhibition of N-linked glycosylation can cause apoptosis (15). The effect of glycosylation on the functional activity of different types of membrane transport proteins has been described previously (7, 16, 17, 12–14). N-Glycosylation is required for functional activity of many membrane transporters; however, deglycosylation does not always abrogate transporter function (18, 19). The precise role of N-linked glycosylation may vary depending upon the specific protein of interest as well as the different glycosylation sites within the glycoprotein.
UT-A1 has four potential consensus glycosylation sites at Asn-13, -279, -544, and -742, but it is not clear which site(s) are responsible for the two glycosylated forms of 97 and 117 kDa. Interestingly, the relative abundance of the two forms varies under different conditions. The 117-kDa form increases dramatically in several states associated with decreased urine concentration, such as streptozotocin-induced diabetes mellitus (20, 21), a low-protein diet (9), hypercalcemia (23), water diuresis (9), and furosemide administration (9). A tubule perfusion study of initial LMCDs from streptozotocin-induced diabetes mellitus rats shows that the appearance of the 117-kDa form in the inner medullary base is associated with increased urine transport activity (21). This finding suggests that changes in the relative abundance of the 97- and 117-kDa forms of UT-A1 may have important regulatory roles for UT-A1 function. Therefore, the goals of the present study were to determine the glycosylation sites in UT-A1 and to investigate the functional effects of glycosylation, including the response to AVP stimulation.

MATERIALS AND METHODS

Plasmid Constructs—For the generation of stably transfected UT-A1 MDCK cell lines, the UT-A1 genes were subcloned into a pcDNA5/FRT vector (Invitrogen) that contains an integrated Fpl recombination target (FRT) site for homologous recombination.

Mutagenesis of N-Glycosylation Sites—The two potential N-linked glycosylation sites were mutated with the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The asparagine (N) residue was substituted with glutamine (Q). The oligonucleotide 5′-CTGCGTCTTCCAGCGCCCGAGATCACTGTCAGAG-3′ was used to mutate the first asparagine residue at N279; oligonucleotide 5′-GCAGTTAACACACCCCAAGATCACTGTCAG-3′ was used for the second asparagine residue mutation at N742. Two single and one double mutant constructs were generated. The mutation of Asn-279 in the first large extracellular loop is denoted as A1m1, the one in the second large extracellular loop as A1m2, and the double mutant as m1m2. The glycosylated UT-A1 is denoted as wild-type (WT). The constructs were verified by nucleotide sequence analysis.

Establishment of Glycan Mutant UT-A1 MDCK Cell Line—Glycosylation mutant UT-A1 MDCK cell lines were generated using our previously described method (24, 25). We used our existing MDCK-FRT cell line and cotransfected with pOG44 (Invitrogen), a vector for transiently expressing the Flp recombinase, and with pcDNA5/FRT/UT-A1 glycan mutant constructs. The pcDNA5/FRT vector has an FRT site for homologous recombination and a hygromycin resistance gene for recombinant clone selection. Cells were grown in the presence of 800 μg/ml hygromycin (Calbiochem) medium and the protein expression verified by Western blot analysis.

Enzymatic Deglycosylation—For the peptide N glycosidase F (PNGase F) digestion, cell lysates were first denatured in 0.5% SDS and 1% β-mercaptoethanol and boiled for 10 min. After adding 1% Nonidet P-40, the samples were split into two, and 1 μl of PNGase F (500 units/μl, New England Biolabs) was added to one tube and incubated at 37 °C overnight; the control tube was incubated with water instead of PNGase F. These digested samples were then processed for Western blot analysis.

Cell Urea Flux Measurements—Cells were grown on collagen-coated Costar Transwell inserts (Corning). The transepithelial resistance was measured daily using an epithelial resistance meter (EVMX-G, World Precision Instruments). We used only cells with 800 ohms or higher resistance for urea flux measurements. The method we used for transepithelial 14C-urea flux has been described previously (24, 25). The urea flux medium contained Hanks’ balanced salt solution without bicarbonate supplemented with 20 mM HEPES and 5 mM urea. For the flux measurements, we replaced the medium on the apical side with 400 μl of prewarmed flux medium containing 0.4 μCi 14C-urea. The inserts were then transferred at 3-min intervals from well to well, each well containing 1.5 ml of flux medium at 37 °C. We then collected the medium for liquid scintillation counting. Ten nanomolar AVP (Sigma) or 10 μM forskolin (FSK, Sigma) were applied to the basolateral side to stimulate urea transport.

Cell Surface Biotinylation—After treatment, the cells were placed on ice to minimize protein trafficking and endocytosis and washed twice with ice-cold phosphate-buffered saline. The cells were labeled twice with freshly prepared 0.5 mg/ml EZ-Link Sulfo-N-hydroxyssunimide disulfide-Biotin (Pierce) in borate buffer for 20 min in a cold room with gentle shaking. The biotin reaction was quenched for 20 min with 0.1 M L-lysine (Sigma) in 10% fetal calf serum/Dulbecco’s modified Eagle’s medium, followed by two phosphate-buffered saline washes. The cells were lysed in 700 μl of radioimmune precipitation assay buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors). The homogenates were centrifuged at 8,000 revolutions/min for 10 min to remove cell debris. Fifty microliters of supernatant was saved for Western blot as pre-peak total proteins, whereas 600 μl of supernatant was incubated with 20 μl of Immunopure immobilized streptavidin-agarose beads (Pierce) overnight at 4 °C with gentle shaking. The beads were washed five times with radioimmune precipitation assay buffer, and biotin-labeled proteins were resuspended in 60 μl of Laemmli sample buffer, boiled, and analyzed by Western blot.

ER, Golgi, and Plasma Membrane (PM) Isolation—Sucrose gradient ultracentrifugation was performed according to the method of Kaplan and colleagues (26, 27) with some modification. Cells were grown in 10-cm plates to confluence and treated with 10 nm AVP or 10 μM FSK for 15 min and then frozen at −20 °C for 1 h. The cell pellets were collected and resuspended in HB (250 mM sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4). Cells were disrupted via Dounce homogenization, and the cell mixture was centrifuged for 15 min at 1,000 × g to remove intact cells and debris. ER, Golgi, and PM compartments were separated by loading supernatant on a five-step sucrose gradient (2.0, 1.6, 1.4, 1.2, and 0.8 M sucrose) and ultracentrifuged in a SW28 rotor at 25,000 revolutions/min for 2 h. After centrifugation, ER, Golgi, and PM were collected from density interfaces (1.6/1.4, 1.4/1.2, 1.2/0.8 M interfaces, respectively) and diluted (1:3) in HB for further ultracentrifugation with an SW50.1 rotor at 30,000 revolutions/min for 2 h.
pellets were resuspended in HB containing protease inhibitors for Western blot analysis.

**Pulse-Chase Stability Assay**—We performed the pulse-chase assay according to the method of Hendriks et al. (13). Cells were grown in 6-cm tissue culture dishes, washed with phosphate-buffered saline, and then preincubated for 30 min with methionine- and cysteine-free minimum Eagle’s medium (Sigma). The cells were labeled for 1 h at 37 °C with 0.2 mCi/ml [35S]methionine/cysteine (Amersham Biosciences), washed with Dulbecco’s modified Eagle’s medium, and then incubated with Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 1 mM methionine, and 1 mM cysteine for complete protein translation. The cells then were chased for the indicated times in 10% fetal calf serum/Dulbecco’s modified Eagle’s medium and collected in 700 μl of lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 1 mM EDTA, and a mixture of protease inhibitors) on ice. The cells were sonicated and centrifuged at 10,000 revolutions/min for 10 min to remove insoluble debris. Supernatants were precleared by incubation with bovine serum albumin-coated protein-A beads for 1 h at 4 °C and then transferred to a fresh tube and incubated with UT-A1 antibody overnight at 4 °C. The next day, 10 μl of protein-A-agarose beads (Santa Cruz Biotechnology) were added and incubated for 1 h. After complete washing with 0.05% Triton X-100, 0.1% SDS, 0.3 mM NaCl, 10 mM Tris-HCl, pH 8.6, the samples were resuspended in 50 μl of Laemmli sample buffer and then resolved by SDS-PAGE. The gel was dried and analyzed by autoradiography.

**RESULTS**

**Determination of UT-A1 N-Linked Glycosylation Sites**—The amino acid sequence of UT-A1 contains four consensus sites for N-glycosylation. Based on the predicted membrane topology of UT-A1, Asn-13 and -544 are on the cytoplasmic side of the membrane. Thus, these two are unlikely to be the sites of glycosylation. It appears that the two most likely sites of glycosylation are Asn-279 located in the first large extracellular loop and Asn-742 in the second large extracellular loop (Fig. 1A). We performed site-directed mutagenesis of these two sites by substituting glutamine (Q) for asparagine (N) in the consensus sequence and generated two single glycosylation site mutants of UT-A1, A1m1 (N279Q) and A1m2 (N742Q), and a double mutant m1m2 (N279Q/N742Q) and created stably transfected UT-A1, A1m1 (N279Q) and A1m2 (N742Q), and a double mutant m1m2 (N279Q/N742Q) and created stably transfected UT-A1 MDCK cell lines. As can be seen in Fig. 1B, all three of these UT-A1 mutants (A1m1, A1m2, and m1m2) have reduced molecular size; the double mutant m1m2 further reduces the molecular size to a band with a molecular mass of ~88 kDa. These data suggest that both the Asn-279 and -742 sites are involved in N-linked glycosylation for UT-A1.

This conclusion is further supported by glycosidase digestion. As shown in Fig. 1C, PNGase F treatment brought down all bands to 88 kDa. The double mutant m1m2 has the same size band as the deglycosylated form of UT-A1 treated by PNGase F, showing that UT-A1 has only the two glycosylation sites at Asn-279 -742. The single mutant bands A1m1 and A1m2 are larger than the deglycosylated UT-A1 protein, but the size of these two bands are different. The Asn-742 site in the second extracellular loop appears to have a greater extent of glycosylation than the Asn-279 site in the first extracellular loop.

**Unglycosylated UT-A1 Expressed in MDCK Cells Has a Delayed Response to AVP**—We then tested whether elimination of the two glycosylation sites affected urea transporter function by examining the effect of AVP and FSK on urea transport in stably transfected MDCK cells expressing the UT-A1 protein. We have previously shown that both AVP and FSK stimulated transepithelial urea transport in UT-A1-MDCK cells (24, 25). In agreement with these findings, Fig. 2 shows that both in the absence of a urea transport activator and in the presence of a urea transport inhibitor, the transepithelial urea flux in these cells is low, corresponding to the lipid bilayer permeability of the epithelium. In wild-type cells, transepithelial urea fluxes were increased nearly 5-fold in response to AVP. However, in the mutant cells, this response was attenuated by >80%. Furthermore, the time course of urea flux activation differed between wild-type and mutant cells. In wild-type cells, urea flux activation appeared to have two components, a small early component that reached maximum within ~10 min and a slower component that required about 30 min to reach maximum and that constituted ~70% of the total response amplitude. In the mutant cells, the slow component was attenuated by 80%, and the early activation component (in response to AVP) appears to be completely eliminated. A similar effect was observed in response to FSK, which is a more potent stimulator of urea transport and where the late component makes up a much larger portion of the overall activation. For an unknown reason, the resistance of the two single mutant cells (A1m1, A1m2) could not reach 800 ohms. Therefore, we were unable to measure transepithelial urea flux in these two cell lines; hence,

**FIGURE 1.** **A** shows two glycosylation sites at Asn-279 and -742. A, schematic representation of the potential glycosylation sites in UT-A1 and the three UT-A1 mutants. B, Western blot analysis of the MDCK cells stably expressing wild-type (WT) UT-A1 as well as three types of mutant UT-A1 lacking one (A1m1, A1m2) or both glycosylation sites (m1m2). C, PNGase F treatment. Cell lysates were incubated with (+) or without (–) PNGase F at 37 °C overnight as described under “Materials and Methods. Samples were then processed for Western blot analysis of UT-A1.
Unglycosylated UT-A1 Has Reduced Ability to Move to the Cell Surface—To investigate whether the loss of glycosylation affects the ability of UT-A1 to reach the cell surface, we performed cell surface biotinylation. A 15-min treatment with 10 nM AVP or 10 μM FSK significantly increased UT-A1 cell surface expression in WT cells but not in m1m2 cells (Fig. 3A). However, a 15-min exposure to AVP or FSK did not significantly increase total UT-A1 protein abundance in either WT or m1m2 cells (Fig. 3B). The cell surface expression of UT-A1 (biotinylation assay) was confirmed using immunohistochemistry, where AVP and FSK increased UT-A1 staining in the apical cell surface of WT cells but not in m1m2 cells (Fig. 3C).

Unglycosylated UT-A1 Is Retained in ER and Golgi Complex—To explore whether unglycosylated UT-A1 can exit the ER or Golgi complex, we performed ultracentrifugation to isolate the PM, Golgi apparatus, and ER (26, 27). Except for those UT-A1 molecules in the cytoplasm trafficking between ER to Golgi or Golgi to PM, the largest amount of UT-A1 in subcellular compartments was in the ER in WT cells. These molecules include folded, unfolded, and misfolded UT-A1. A 15-min treatment with AVP or FSK markedly enhanced UT-A1 export, both from ER to Golgi and Golgi to the cell surface (Fig. 4). However, in m1m2 glycan mutant cells, there is no such redistribution of UT-A1 molecules among these three compartments with a 15-min treatment with AVP or FSK. Interestingly, when comparing untreated WT and m1m2 cells, we observed that relatively more unglycosylated UT-A1 was retained in the Golgi apparatus in the m1m2 cells, reflecting the possibility that glycosylation may have an important role in directing UT-A1 movement out of the Golgi to the cell surface.

Unglycosylated UT-A1 Is Unstable—To address whether the N-linked oligosaccharide chain could stabilize the UT-A1 protein, we pulsed WT and glycan mutant m1m2 cells with [35S]labeled methionine/cysteine and then chased with unlabeled medium to assess protein stability at different time points. As seen in Fig. 5, unglycosylated UT-A1 (m1m2) demonstrates decreased stability when compared with WT UT-A1.

DISCUSSION

The urea transporter UT-A1 plays a key role in the urine-concentrating mechanism. UT-A1 is a glycoprotein with two different glycosylated forms of 97 and 117 kDa; both are derived from the same 88-kDa core protein (7). The major findings in the present study are 1) UT-A1 is glycosylated at two sites located individually in the two large extracellular loops at Asn-279 and -742, 2) unglycosylated UT-A1 stably expressed in MDCK cells has a delayed and decreased stimulation in response to AVP and FSK treatment compared with WT UT-A1, 3) less unglycosylated UT-A1 protein is delivered to the cell membrane in the first 15 min after AVP and FSK stimulation than WT UT-A1, 4) unglycosylated UT-A1 is mainly retained in the Golgi apparatus even after stimulation by AVP and FSK, and 5) unglycosylated UT-A1 has decreased stability than WT UT-A1.

The amino acid sequence of UT-A1 contains four potential N-linked glycosylation consensus sites. However, it is not known whether some or all of these four are the actual sites of UT-A1 N-linked glycosylation. A previous study using limited protein enzyme digestion shows only one possible glycosylation site for UT-A1 in the N-terminal half of the molecule, namely Asn-279 (7). In this study, we determined that the two sites, Asn-279 and -742, residing in the two large extracellular loops are responsible for UT-A1 N-linked glycosylation and that these two sites have different extents of glycosylation. The site at Asn-742 appears to have a greater extent of glycosylation than the site at Asn-279, suggesting differential glycan synthesis and trimming (Fig. 1B). Our data show that both of the glycosylation sites contribute to UT-A1 glycosylation. However, we cannot determine whether, in vivo, the 97-kDa protein form shifts to 117 kDa due to an extended carbohydrate chain in one site or in both sites. We also cannot explain why the cells transfected with UT-A1 cDNA only produce a single 97 kDa size protein rather than the two bands of 97 and 117 kDa detected in rat inner medulla. However, enzymatic deglycosylation can reduce the 97-kDa UT-A1 to its core molecular mass of 88 kDa. This suggests that UT-A1 glycosylation may vary within the...
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FIGURE 3. Unglycosylated UT-A1 has impaired cell surface expression. A, cell surface biotin labeling. Cells were grown in a 6-well plate to confluence and then treated with 10 nm AVP or 10 μM FSK or without any treatment for 15 min. Cell surface proteins were biotinylated and analyzed by Western blot. The left panel shows membrane proteins collected on streptavidin beads. The right panel shows pre-bead samples (total proteins). B, densitometric quantification of the Western blot signals was performed with the NIH Image J program. The control (ctrl) untreated cell bands of WT and m1m2 UT-A1 were set to 100% (*, p < 0.05; **, p < 0.01). C, immunofluorescence microscopy. Cells were grown on a Transwell insert filter for 3 days to allow the cells to develop polarity and then treated with 10 nm AVP and 10 μM FSK for 15 min. The cells were fixed, permeabilized, and then incubated with UT-A1 antibody and fluorescein isothiocyanate-conjugated secondary goat anti-rabbit antibody. The cells were further incubated with Texas Red-X Phalloidin (Molecular Probes) to visualize F-actin. Cells are shown with apical membrane (top) and basolateral membrane (bottom) as a cross-section through the cell (x-z section). The image was obtained by confocal microscopy.

The function of a transport protein relies on its presence in the plasma membrane. Compared with glycosylated forms, the fully deglycosylated UT-A1 has reduced urea transport activity. One of the mechanisms might be that loss of the oligosaccharide chain affects the movement of the UT-A1 molecule to the cell surface. In many cases, N-linked glycosylation is critical for membrane protein intracellular movement and its eventual delivery to the cell surface. Most membrane proteins targeted to the plasma membrane possess N-linked glycosylation (12, 13, 16, 17, 28). One type of hereditary AQP2 mutant (T125M), which causes recessive nephrogenic diabetes insipidus, is not glycosylated because the N-linked glycosylation motif is disrupted (29). Glycosylation has also been shown to be essential for the efficient function and surface expression of the NaCl cotransporter (14), the cardiac potassium channel HERG (30), and the Oatp1 organic anion transporter (16). We have previously shown that AVP and FSK can stimulate urea flux in MDCK cells stably expressing UT-A1 (24, 25). In contrast, m1m2 cells have a delayed and significantly reduced maximally stimulated urea flux. As shown in Fig. 2, mutant UT-A1 lost the early response to AVP and FSK stimulation; one possible reason could be that mutant UT-A1 lost its ability to rapidly target to the cell membrane. Consistent with the flux results, WT UT-A1 translocates to the cell surface both in the presence of either AVP or FSK. However, the double mutant m1m2 has significantly less abundance in the cell surface, as judged from experiments with biotinylation, ultracentrifugation, and confocal microscopy. Hendriks et al. (13) report that the glycan mutant AQP2 is unable to exit the Golgi apparatus and be delivered to the cell membrane. Our results from fractional ultracentrifugation revealed that unglycosylated UT-A1 is mainly trapped in the Golgi apparatus, and it significantly lost its ability to move to the cell surface in response to AVP and FSK (for 15 min). This suggests that loss of glycosylation will affect UT-A1 exiting both the ER and Golgi, especially when moving from the Golgi to the cell surface. Unlike AQP2, which completely lost its ability to move to the cell surface in response to AVP and FSK treatment (13), we still could see some mutant UT-A1 able to reach the cell surface in the biotinylation assay (Fig. 3A) and ultracentrifugation (with long exposure, data not shown), which may explain why unglycosylated UT-A1 still had some urea transport activity in the later phase. We currently do not know how the small amount of unglycosylated UT-A1 could escape from the ER quality control system and exit the Golgi complex to move to the cell surface.

A previous study reports that AVP does not regulate UT-A1 trafficking to the cell membrane (31). However, that conclusion is based on studies of Brattleboro rats, which do not have AVP. We have data in normal Sprague-Dawley rats showing that acute AVP or FSK administration significantly increases UT-A1 accumulation in the plasma membrane of rat IMCD suspensions and WT UT-A1-MDCK cells (32). The present study further supports the hypothesis that AVP increases UT-A1 accumulation in the apical plasma membrane and suggests that it is one of the important mechanisms for regulation of urea transport by UT-A1.

Stabilization of the mature protein is critical to its proper function. The mechanism for glycosylation-regulating transporter activity may also contribute to glycoprotein stabilization. Removal of the glycosylation site in AQP2 produces a protein with a reduced half-life (13). Our pulse-chase metabolic
labeling study revealed that glycosylated UT-A1 is more stable than its non-glycosylated counterpart. Unglycosylated immature protein tends to stay longer in the ER (13). The prolonged residence in the ER may facilitate UT-A1 breakdown by the ER quality control mechanisms. Even if an unglycosylated protein escapes from the ER, it is more susceptible to proteolytic attack and has a greater chance of being degraded before being inserted into the plasma membrane (33). Additionally, post-ER quality control mechanisms, such as the ubiquitin-proteasome pathway, may also exist. Li et al. (33) also found that the non-glycosylated dopamine transporter was less stable at the cell surface, as demonstrated by apparently enhanced endocytosis. It is very likely that the decreased urea flux amplitude for glycan mutant UT-A1 is caused by an unstable UT-A1 protein in the cytoplasm or inserted into the membrane due to the lack of its N-glycan chain.

Phosphorylation of UT-A1 has been shown to be one of the important mechanisms by which vasopressin rapidly increases urea permeability in vivo (10). Therefore, phosphorylation could be another reason that mutant UT-A1 lost the early response to AVP and FSK stimulation. Wall et al. (22) in 1992 observed that vasopressin increases urea permeability in rat terminal IMCDs within 5–10 min. Previous work from our laboratory (10) demonstrates that vasopressin treatment increases UT-A1 phosphorylation at 2 min. The peak is at 5–10 min, and the phosphorylation remains elevated for up to 30 min. We also examined whether the phosphorylation of UT-A1 could be one of the mechanisms for unglycosylated UT-A1 to lose its responsiveness to vasopressin in the early phase. However, we did not see any difference in protein phosphorylation between WT and glycan mutant UT-A1 (data not shown).

In summary, the urea transporter UT-A1 has two glycosylation sites at Asn-279 and -742. These two sites make different contributions to the glycosylation of UT-A1. The Asn-742 site in the second extracellular loop appears to have a greater extent of glycosylation than the Asn-279 site in the first extracellular loop. Removing these two glycan sites affects urea transport activity, especially reducing AVP-stimulated activity. We further found that the reduced activity of the unglycosylated forms in response to AVP treatment is at least partially because of its impaired trafficking to the cell membrane and reduced molecule stability. Our study provides new insight that, in vivo, under certain circumstances, IMCD cells may be able to regulate urea transport by changing the extent of UT-A1 glycosylation and thereby increasing or decreasing (or even losing) its responsiveness to AVP stimulation.
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