Glycoforms of UT-A3 urea transporter with poly-N-acetyllactosamine glycosylation have enhanced transport activity

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Su H, Carter CB, Fröhlich O, Cummings RD, Chen G. Glycoforms of UT-A3 urea transporter with poly-N-acetyllactosamine glycosylation have enhanced transport activity. Am J Physiol Renal Physiol 303: F201–F208, 2012. First published April 25, 2012; doi:10.1152/ajprenal.00140.2012.—Urea transporters UT-A1 and UT-A3 are both expressed in the kidney inner medulla. However, the function of UT-A3 remains unclear. Here, we found that UT-A3, which comprises only the NH2-terminal half of UT-A1, has a higher urea transport activity than UT-A1 in the oocyte and that this difference was associated with differences in N-glycosylation. Heterologously expressed UT-A3 is fully glycosylated with two glycoforms of 65 and 45 kDa. By contrast, UT-A1 expressed in HEK293 cells and oocytes exhibits only a 97-kDa glycosylation form. We further found that N-glycans of UT-A3 contain a large amount of poly-N-acetyllactosamine. This highly glycosylated UT-A3 is more stable and is enriched in lipid raft domains on the cell membrane. Kifunensine, an inhibitor of α-mannosidase that inhibits N-glycan processing beyond high-mannose-type N-glycans, significantly reduced UT-A3 urea transport activity. We then examined the native UT-A1 and UT-A3 glycosylation states from kidney inner medulla and found the ratio of 65 to 45 kDa in UT-A3 is higher than that of 117 to 97 kDa in UT-A1. The highly stable expression of highly glycosylated UT-A3 on the cell membrane in kidney inner medulla suggests that UT-A3 may have an important function in urea reabsorption.

N-glycan; urine concentration; urea absorption; vasopressin; inner medulla

UREA IS ONE OF THE MOST IMPORTANT solutes contributing to the medullary osmolarity gradient in the kidney. Rapid urea reabsorption in the terminal inner medulla collecting duct (IMCD) is mediated by a specialized facilitative urea transporter (UT) protein (10, 24). This process is critically important for the kidney to maintain medullary hypertonicity and enable maximal urinary concentration to occur, and therefore plays an important role in the maintenance of water and nitrogen balance (24).

The first UT gene was cloned in 1993 (38). At present, two mammalian UT subfamilies have been reported, the renal tubular-type urea transporter UT-A and the erythrocyte-vascular-type urea transporter UT-B (24). UT-A has six splicing isoforms; the longest form is UT-A1 with an open reading frame of 929 amino acids in rat. Two other forms of the transporter are also expressed, UT-A3, which represents an NH2-terminal half of UT-A1, and UT-A2, which represents a COOH-terminal half of UT-A1 (3, 16). UT-A1 and UT-A3 are both expressed in the IMCD and are believed to contribute to the very high urea permeability of the IMCD (10). UT-A2 is expressed in the descending thin limb of the loop of Henle and involved in urea recycling between the interstitium and the loop of Henle (34).

UT-A1 has been extensively investigated. It is expressed in the IMCD epithelial cells and plays a critical role in the urinary concentrating mechanism, as demonstrated by UT-A1/UT-A3 knockout mice that have a seriously impaired urinary concentrating ability (10). Vasopressin (also known as antidiuretic hormone) is the major hormone that regulates UT-A1 activity in vivo. Vasopressin rapidly regulates IMCD urea transport activity by increasing UT-A1 phosphorylation (39) and membrane trafficking (18). UT-A1 expressed in the inner medulla (IM) exhibits two different glycosylation forms that differ in size, 97 and 117 kDa; both are derived from a single 88-kDa core protein (5). It was believed that these two glycoforms of UT-A1 are the mature glycosylation forms, since they are both insensitive to endoglycosidase H treatment (5). However, our recent study shows that the 117-kDa form is more heavily glycosylated and contains poly-N-acetyllactosamine (poly-LacNAc) (8), whereas the 97-kDa form is a hybrid form containing a high amount of mannose (8). For unknown reasons, upon heterologous expression of UT-A1 in Xenopus laevis oocytes (8), Chinese hamster ovary (15), Madin-Darby canine kidney (MDCK) (8, 11), and HEK293 (14) cells, only the 97-kDa form appears.

UT-A3 from rat kidney encodes a 460-amino acid protein comprising the NH2-terminal part of UT-A1 (16). Both UT-A1 and UT-A3 are transcribed from the same promoter (16, 21). Although both UT-A1 and UT-A3 are highly expressed in IMCD, their distribution is slightly different. In situ hybridization (27) and immunohistoassay (33) demonstrate that UT-A3 is only detectable in the deep part of the IM (IM tip), whereas UT-A1 is distributed over the entire IM (IM tip and base). Immunobots with UT-A3 antibody by Terris et al. (33) identify two bands at ~44 and ~67 kDa in the IM. Upon removal of N-glycans by treatment with PNGase, these two bands disappear and yield a single band at 40 kDa.

The exact function and cellular location of UT-A3 in the kidney remains controversial. The large abundance of UT-A3 protein in the IMCD suggests it may also be important in handling renal urea. Because UT-A1 is expressed in the apical membrane of IMCD epithelial cells and mediates urea reabsorption from the tubular lumen, one possible function for UT-A3 in the IMCD is that it could serve as the basolateral UT, permitting urea to exit from the cells (27, 29). Indeed, Stewart et al. (30, 31) showed that UT-A3 is in the basolateral membrane in mouse kidney IMCD cells, and basolateral urea flux is detected from mUT-A3-MDCK cells. However, Terris et al.
(33) showed that UT-A3 is located in the apical membrane in rat kidney IMCD cells. The double immunostaining revealed no overlap of UT-A3 with Na-K-ATPase at the basolateral surface, making it unlikely that UT-A3 represents the basolateral UT, at least in rat (33).

In freshly prepared suspensions of rat IMCD, forskolin treatment increases the UT-A3 abundance in the plasma membrane (PM) and phosphorylation, similar to UT-A1 (4). Although UT-A1 and UT-A3 are both expressed in the apical membrane of epithelial cell in rat IMCD, a study by Blount and coworkers (4) showed that these two transporters do not form a complex through a protein-protein interaction, suggesting that UT-A1 and UT-A3 function independently as separate transporters. Truly, they function independently in heterologous expression in oocytes (8, 27) and cultured cells (11, 30), indicating that their individual function does not require the presence of one another. Because UT-A1 and UT-A3 share the same promoter, it is impossible to generate solely a UT-A1 or UT-A3 knockout mouse. Deletion of UT-A1 exon 10 results in deletion of the same promoter, it is impossible to generate solely a UT-A1 or UT-A3 knockout mouse. Deletion of UT-A1 exon 10 results in deletion of the

Although UT-A3 represents only the NH2-terminal half of UT-A1, it exhibits a high urea transport activity in vitro (27). In this study, we have examined the functional role of N-glycosylation in UT-A1 vs. UT-A3 and found that differential glycosylation, and especially the content of poly-LacNAc in N-glycans in UT-A3, is associated with stabilization of UT-A3 and its increased urea transport activity.

MATERIALS AND METHODS

Plasmids. Rat UT-A1 and UT-A3 cDNAs coding the full-length protein were initially subcloned into p3XFLAG-CMV-10 vector (Sigma) in HindIII/EcoRI sites, then the genes, including the NH2-terminal fused triple FLAG tag (FLAG-UT-A1 and FLAG-UT-A3), were subcloned into mammalian expression vector pcDNA3. All of the constructs were verified by nucleotide sequence analysis.

Cell culture, transfection, and treatment. HEK293 cells were routinely cultured in DMEM supplemented with 10% FCS at 37°C in 5% CO2. Plasmids were transfected by Lipofectamine (Invitrogen). For urinary concentration, function of UT-A3 in inner medullary urea reabsorption and of a UT-A3-deficient mouse, it is not possible to evaluate the UT-A3 knockout mouse. Deletion of UT-A1 exon 10 results in deletion of the same promoter, it is impossible to generate solely a UT-A1 or UT-A3 knockout mouse. Deletion of UT-A1 exon 10 results in deletion of the

Kidney IM tissue collection and Northern blot analysis. Sprague-Dawley rats (Charles River Laboratories) weighing 125–200 g were used for evaluation of UT-A1 and UT-A3 mRNA expression. The IM tips were dissected from kidneys and cut in half as IM tip and IM base. Total RNA was extracted by TRizol (Invitrogen). The RNA integrity was examined by RNA gel with ethidium bromide stain. Ten micrograms of RNA were used for electrophoresis and blotted on a nitrocellulose membrane and probed with the denatured UT-A1 cDNA probes in Rapid-hyb Kit (Ambion). Five femtomoles of cRNA were injected into oocytes. For the kifunensine treatment, before cRNA injection, oocytes were preinjected with 23 ng kifunensine (Sigma) for 2 h. After 3 days, urea transport activity was measured by [14C]urea uptake (n = 6 oocytes/time point) as described (8). Protein expression from 10 cells/group was assessed by Western blot analysis

Western blot analysis. Western blotting was performed as described (8). Blots were probed with primary antibody at 4°C overnight, followed by secondary anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) and developed by enhanced chemiluminescence (GE Healthcare). Anti-FLAG antibody was from Sigma. Anti-UT-A1 NH2-terminal antibody was described previously (8). Western blot band densities were quantitated with the NIH ImageJ software. All animal protocols were reviewed and approved by the Emory University Institutional Animal Care and Use Committee. Sprague-Dawley rats (Charles River Laboratories) weighing 100–150 g received free access to water and standard rat chow (Purina).

RESULTS

UT-A3 exhibits a stronger activity than UT-A1 in Xenopus oocytes. Structurally, UT-A1 is two times as large as UT-A3 and contains two urea-conducting units (UT-A3 and UT-A2) (Fig. 1A). To compare their activity, we normalized the in-
jected cRNAs. Equal molar amounts (5 femtomol/oocyte) of UT-A1 (5 femtomol = 23 nl·oocyte⁻¹) or UT-A3 (5 femtomol = 2.8 ng) cRNAs were injected into the oocyte. Later (3 days), urea transport activity was measured by [¹⁴C]urea uptake for 1 and 3 min (mean ± SD, n = 6 oocytes/time point, **P < 0.01). C: analysis of protein expression. UT-A1 and UT-A3 protein expression on the cell membrane was examined by biotinylation and followed by Western blot with a UT-A1 NH₂-terminal antibody. D: quantification of C. Membrane (biotinylated) UT-A1 and UT-A3 proteins were quantified and assessed as %total UT protein from 3 independent experiments (mean ± SD, n = 3, *P < 0.05).

UT-A3 is expressed in lipid raft domains at the cell membrane. We previously reported that the highly glycosylated 117-kDa glycoforms of UT-A1 from kidney are preferably localized in lipid raft microdomains (8); thus, we examined the lipid raft association of UT-A3 expressed in HEK293 cells. FLAG-tagged UT-A3 and UT-A1 (as a control) were transiently transfected into HEK293 cells. After 48 h, lipid rafts were isolated by using the nonionic detergent Brij96. UT-A1 only shows a 98-kDa form (Fig. 2A). UT-A3 demonstrates two glycoforms at 45 and 65 kDa. The more highly glycosylated UT-A3 is solely distributed in lipid raft domains (Fig. 2A), similar to the lipid raft distribution of 117-kDa UT-A1 from kidney (8).

To confirm that the two UT-A3 bands are due to the different extents of glycosylation, we collected membrane fractions 2–4 and processed for PNGase F digestion. After removal of N-glycans, the two bands collapsed to a single band at 40 kDa (Fig. 2B). As a control, after deglycosylation treatment, the 98-kDa UT-A1 shifted to 88 kDa.

The 65-kDa glycoform of UT-A3 contains high amounts of poly-LacNAc. Because the UT-A3 glycoform has a single predicted N-glycosylation site at Asn²⁷⁹, the high apparent molecular weight of that form is likely to be due to a large-sized N-glycan. Typically, such very large N-glycans contain long repeats of the disaccharide [3Galβ1→4GlcNAcβ1→]n, which is known as poly-LacNAc. To explore the N-glycan structure of UT-A1 and UT-A3, we collected UT-A1 and UT-A3 lipid raft fractions 2–4 and performed pulldown assays.
by using different lectins that recognized different structural determinants in glycans. As shown in Fig. 3, the 97-kDa UT-A1 is bound by both ConA and WGA. These lectins bind glycans with high contents of mannose or N-acetylglucosamine/sialic acid, respectively (6, 2, 20, 23, 1). By contrast, the 45-kDa glycoforms of UT-A3 were mainly pulled down by ConA, indicating a significant amount of mannose, whereas the 65-kDa glycoforms of UT-A3 were pulled down by WGA and tomato lectin. After kifunensine treatment, both UT-A1 and UT-A3 were precipitated only by ConA, and, notably, the high-molecular-weight form of UT-A3 seen in the absence of kifunensine was not observed (Fig. 4B). These results demonstrate that UT-A3, but not UT-A1, when expressed in oocytes acquired poly-LacNAc chains and has more highly processed N-glycans compared with those in UT-A1.

Kifunensine reduces UT-A3 urea transport activity. To confirm whether the mature glycosylation is important for the increased UT activity of UT-A3, we preinjected Xenopus oocytes with kifunensine for 2 h. Kifunensine inhibits ER α-mannosidase I (9), which prevents further maturation of N-glycans, resulting in accumulation of high-mannose-type N-glycans. As seen in Fig. 4A, kifunensine treatment significantly reduced UT-A3, as well as UT-A1, urea transport activity. The effect of kifunensine on UT-A1 and UT-A3 glycan structure was examined by lectin pulldown. UT-A1 expressed in oocytes was efficiently precipitated by ConA, and weakly by WGA, and not by other lectins (Fig. 4B). The relatively immature N-glycosylation of UT-A1 when expressed in oocytes could be one of the reasons why it has lower activity. By contrast, UT-A3 glycosylation was hardly recognized by ConA, indicating that the N-glycans were highly processed, as confirmed by the efficient precipitation with both WGA and tomato lectin. After kifunensine treatment, both UT-A1 and UT-A3 were precipitated only by ConA, and, notably, the high-molecular-weight form of UT-A3 seen in the absence of kifunensine was not observed (Fig. 4B). These results demonstrate that UT-A3, but not UT-A1, when expressed in oocytes acquired poly-LacNAc chains and has more highly processed N-glycans compared with those in UT-A1.

![Fig. 3. Lectin pulldown assay.](http://ajprenal.physiology.org/)

![Fig. 4. Kifunensine (Kifu) treatment.](http://ajprenal.physiology.org/)
Fully glycosylated 65-kDa UT-A3 has extended half-life time. One of the important roles of N-glycosylation is to increase protein stability (7). To investigate this possibility in relation to UT-A3, FLAG-UT-A3 and UT-A1 were transfected into HEK293 cells. After 2 days, the cells were treated with cycloheximide to block synthesis of new protein, and the total protein expression of UT-A3 was chased for 18 h. As shown in Fig. 5, the lower glycosylated 45-kDa UT-A3 exhibited faster turnover with a half-life time of ~10 h, the same as UT-A1; by contrast, the high glycosylated 65-kDa UT-A3 was more stable with an extended half-life time >18 h.

Differential mRNA and protein expression of UT-A3 in IM. To compare these results on in vivo expressed glycoforms of UT-A1 and UT-A3, we examined native UT-A3 expression in the kidney. Northern blots demonstrated that UT-A3 is only present in the IM tip, whereas UT-A1 exists in both IM tip and base. This is consistent with prior studies using in situ hybridization (27). We quantified UT-A1 and UT-A3 mRNA expression in the IM tip. UT-A3 is only about one-fifth as abundant as UT-A1 (Fig. 6A). At the protein level, the total UT-A3 abundance (65 + 45 kDa) appears to be only slightly lower than that of UT-A1 (117 + 97 kDa) (Fig. 6B). Unlike the heterologously expressed UT-A1, the native UT-A1 from kidney tissue appears to be two major glycoforms, including the highly glycosylated form of 117 kDa that is bound by both WGA and tomato lectin, and thus has a high content of both GlcNAc/sialic acid and poly-LacNAc (8) (Fig. 6C). We prepared cell PM and then carefully examined UT-A3 and UT-A1 expression on the cell PM. The UT-A3 ratio of 65 to 45 kDa is higher than that of 117–97 kDa in UT-A1 (Fig. 6B), indicating that UT-A3 on the cell membrane is heavily glycosylated. As expected, the 65-kDa UT-A3 on the cell membrane is efficiently pulled down by both WGA and tomato lectin, which indicates that the 65-kDa glycoform contains high amounts of both GlcNAc/sialic acid and poly-LacNAc (Fig. 6C).

DISCUSSION

Glycosylation is a key posttranslational modification that can affect the structure and function of glycoproteins. We (8, 7) and others (36) have shown that the state of glycosylation is related to UT-A1 activity. UT-A1 from kidney IM is fully glycosylated and appears as two glycoforms: 97 and 117 kDa (5, 8). Animal experiments show that these two forms are regulated differentially in different pathological conditions. The 117-kDa form increases dramatically in several states associated with decreased urea concentration, such as streptozotocin-induced diabetes mellitus (17), low-protein diet (32), hypercalcemia (25), water diuresis (32), and furosemide administration (32). These changes could reflect the fact that the kidney increases urea transport activity in response to the specific needs under crucial situations. Indeed, a tubule perfusion study of IMCD from diuretic rats shows that the appearance of the 117-kDa isoform is associated with increased urea permeability (36). Therefore, the change in the relative abundance of the 97- and 117-kDa forms of UT-A1 may play important roles in regulating UT-A1 function in vivo. Direct evidence demonstrates that inhibition of glycosylation by tunicamycin or elimination of the two glycosylation sites within UT-A1 at Asn279 and Asn742 by mutation significantly affects UT-A1 membrane trafficking and lipid raft expression and reduces UT-A1 protein stability and urea transport activity (8, 7).

Both UT-A1 and UT-A3 are highly expressed in the terminal IMCD. Because UT-A1 is the longest form among the three kidney isoforms of UT-A1, UT-A2, and UT-A3, it has been more extensively studied and is believed to play a key function in urea reabsorption from the apical membrane of IMCD cells (10, 22). By contrast, the role of UT-A3 in kidney urea reabsorption and intrarenal urea recycling has not been well explored. A decade ago, Shayakul et al. (27) observed that urea transport mediated by UT-A3 has higher uptake levels than that by UT-A1 in oocyte expression. However, they did not examine the protein expression of these two transporters, and the possible mechanism was not addressed (27). Structurally, UT-A1 is two times as large as UT-A3. It is not clear at present how UT-A3, which represents the NH2-terminal half of UT-A1, has more urea transport activity compared with UT-A1. In this study, we further compared these two transporters by comparing their transport functions at similar expression levels and found that the single UT-A3 molecule had a higher activity than that of UT-A1, which is consistent with the finding reported by Shayakul et al. (27). Strikingly, protein analysis by Western blot shows that, in addition to a 45-kDa band, UT-A3 has a heavily glycosylated broad band around 65 kDa. UT-A1
has only the low glycosylated 97-kDa band as seen before (8). It is well known that maturation of glycosylation is linked to proper function in many glycosylated proteins (12, 13, 35, 26). We propose that the mature glycosylation form of UT-A3 could be contributing to the increased activity. This was confirmed by treatment with kifunensine (Fig. 4), which shows that blocking glycosylation maturation significantly reduces UT-A3 urea transport activity.

We observed that UT-A3, which possesses the same amino acid sequence as the NH2-terminal half of UT-A1, is glycosylated differently from UT-A1. Only a lower glycosylated 97-kDa UT-A1 has been identified in heterologous expression (8, 15, 11, 14), whereas UT-A3, as shown in this study, can get fully glycosylated in both oocytes and HEK293 cells. We previously reported that N-glycosylation facilitates UT-A1 lipid raft expression (8). Consistently, we observed that the highly glycosylated 65 kDa of UT-A3 shows preferential association with lipid raft domains, similar to the lipid raft distribution of the 117-kDa UT-A1 from kidney IM (8). Targeting to lipid raft microdomains is important for membrane protein apical trafficking in polarized epithelial cells (8). Thus the high glycosylation of UT-A3 may enhance its membrane expression by facilitating transporter targeting to lipid rafts. We compared the glycan structures of UT-A1 and UT-A3 from cell membrane lipid raft fractions. The 97-kDa UT-A1 glycan mainly contains less mature N-glycans rich in mannose and GlcNAc/sialic acid as reported in MDCK cells (8). The low band of UT-A3 is primarily a mannose-rich glycoform that is only bound by ConA. As expected, the larger band of 65 kDa UT-A3 contains a large amount of GlcNAc/sialic acid and particularly poly-LacNAc, as it is bound efficiently by both WGA and tomato lectin, respectively. However, UT-A1 also has a significant amount of fucose, a feature not seen in UT-A3. The mechanism of differential processing of N-glycans in these different transporter forms is unclear and should be the subject of future studies.
In the current study, we also observed that the N-glycosylation differs between oocytes and HEK293 cells for both UT-A1 and UT-A3. In HEK293 cells, UT-A3 shows clearly the two different size bands at 45 and 65 kDa (Fig. 2, A and B), which is similar to the sizes observed in kidney IM. UT-A3 expressed in oocytes has a 45-kDa band but with a broader smear of heterogeneous glycosylated forms. They are different glycosylation forms, since the high glycosylated UT-A3 smear disappeared after kifunensine treatment (Fig. 4B). Also, the 97-kDa UT-A1 expressed in MDCK cells contains significant amounts of GlcNAc/sialic acid (8), but it has very low GlcNAc/sialic acid in oocytes (Fig. 4B), much more immature than in MDCK (8) and HEK293 cells (Fig. 3). This may be related to the very low transport activity of UT-A1 compared with UT-A3, especially in oocytes.

The finding that UT-A3 has enhanced activity linked to a high level of glycosylation and poly-LacNAc content may have important physiological significance. Although both UT-A1 and UT-A3 are expressed in IM, UT-A3 is more concentrated in the terminal IMCD. Interestingly, at the mRNA level, we found that UT-A3 is only one-fifth of UT-A1 in the IM tip, but UT-A3 protein abundance is only slightly lower than that of UT-A1. There are several possibilities for this, including that UT-A3 mRNA may be more stable and/or UT-A3 protein is more stable. In support of the latter, the 65-kDa UT-A3 has a significantly extended half-life time in this study (Fig. 5). Another interesting finding is that the highly glycosylated 65-kDa UT-A3 in the PM is more abundant than the 117-kDa UT-A1, strongly suggesting that UT-A3 may have more impact on controlling the final urea reabsorption in the terminal part of the kidney collecting duct.

In summary, the large abundance and codistribution of UT-A3 with UT-A1 in the terminal IMCD suggests that UT-A3 may play an important role in renal urea handling. Because of its small size (one-half of UT-A1) and being less explored, the functionality of UT-A3 may have been underappreciated for a long time. In this study, we found that the single UT-A3 molecule has much stronger transport activity than UT-A1. The mature glycosylation of UT-A3, with high amounts of poly-LacNAc, could contribute to the increased urea transport activity of UT-A3. Interestingly, relative to the low level of mRNA, we found that kidney IMCD actually has a high UT-A3 protein expression level, particularly the 65-kDa UT-A3 in PM fractions. Taken together, our study strongly suggests that UT-A3 may act as an effective UT and play a much more important role in kidney than thought to date. Further in vivo investigations are required to reevaluate the role of UT-A3 in kidney urea reabsorption and the urinary concentrating mechanism.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: H.S. and C.B.C. performed experiments; H.S., O.F., R.D.C., and G.C. analyzed data; H.S., O.F., R.D.C., and G.C. interpreted results of experiments; H.S. and G.C. prepared figures; G.C. conception and design of research; G.C. drafted manuscript; G.C. edited and revised manuscript; G.C. approved final version of manuscript.

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