97- and 117-kDa forms of collecting duct urea transporter UT-A1 are due to different states of glycosylation

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Renal medullary urea accumulation is crucial to the urinary concentrating mechanism. The process by which the renal medulla accumulates urea depends critically on facilitated urea transport via phloretin-sensitive urea transporters in the apical and basolateral plasma membranes of the epithelial cells of the inner medullary collecting duct (IMCD) (24, 27). The apical urea transporter of the IMCD is regulated by vasopressin (antidiuretic hormone) (27). The existence of this transporter was originally identified via isolated perfused tubule experiments, which demonstrated several properties characteristic of facilitated transport, namely, saturation, competitive inhibition by structural analogs of urea, regulation by vasopressin, and inhibition by phloretin (5, 6, 22, 23). A cDNA for the transporter has been cloned (25), and the deduced amino acid sequence predicts an exceptionally hydrophobic protein with an open reading frame of 929 amino acids. The corresponding gene and its protein product have been referred to as either “UT1” (25) or “UT-A1” (24).

Antibodies prepared to synthetic peptides corresponding to the predicted COOH terminus of UT-A1 have been used to characterize the protein (20, 29). Immunocytochemistry confirmed the location of the UT-A1 protein in the apical region of IMCD cells, and immunoelectron microscopy demonstrated that the protein is present in the apical plasma membrane as well as in cytoplasmic vesicles (20). Despite the relative abundance in intracytoplasmic vesicles, regulation of UT-A1 by vasopressin apparently does not involve regulated trafficking to the plasma membrane, in contrast to the vasopressin-regulated water channel aquaporin-2 (AQP2) (11). Quantification of UT-A1 expression in rat IMCD using a fluorescence-based ELISA method revealed that UT-A1 is moderately abundant in IMCD cells with ~5,000,000 copies/cell (14). The turnover number of the transporter was estimated to be between 10^7 and 10^8 urea molecules/s, suggesting that UT-A1 functions as a urea channel (14).

In immunoblotting studies, the UT-A1 antibodies labeled an abundant membrane protein in rat renal

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inner medulla with an apparent molecular mass of 97 kDa as well as a less abundant protein with an apparent molecular mass of 117 kDa (29). Both the 97- and the 117-kDa forms have been shown to be expressed in the collecting duct (29). The 117-kDa band is increased in intensity in states associated with low medullary concentrations of urea [water diuresis (29), administration of the diuretic furosemide (29), low-protein diet (29), hypercalcemia (21)] and is decreased in intensity in states associated with high medullary concentrations of urea [antidiuresis (29), high-protein diet (29)].

To understand the regulatory significance of these responses, it is important to understand the structural basis of the two different forms of the UT-A1 protein. In this paper, we consider the following alternative explanations. First, the 97- and 117-kDa forms of UT-A may exist because of a difference in the size of the core protein, due, for example, to physiological proteolytic cleavage of a portion of the UT-A1 core protein or alternative splicing. Second, the 97- and 117-kDa forms of UT-A may exist as a result of posttranslational modification of the same core protein. The present studies were carried out by using classic protein chemistry techniques to determine the structural basis of the two collecting duct UT-A1 forms.

METHODS

Animals

The animals used for these studies were pathogen-free male Sprague-Dawley rats (Taconic Farms, Germantown, NY) and male Brattleboro rats (Harland Sprague-Dawley, Indianapolis, IN), acquired under National Heart, Lung, and Blood Institute-Animal Care and Use Committee protocol 5-KE-1. Where indicated, Sprague-Dawley rats were water loaded by being given 200 mM sucrose as the drinking solution (16) and increases the abundance of the 117-kDa UT-A protein. Where indicated, Brattleboro rats were implanted with osmotic minipumps to infuse either 1-deamino-[8-arginine]-vasopressin (dDAVP; 20 ng/h, Rhone-Poulenc, Gannat, France) or vehicle for 7 days as previously described (13).

Crude Membrane Preparation and Immunoblotting

Tissues were homogenized in ice-cold isolation buffer so- tion, a procedure that markedly increases spontaneous water intake (16) and increases the abundance of the 117-kDa UT-A protein. Where indicated, Brattleboro rats were implanted with osmotic minipumps to infuse either 1-deamino-[8-arginine]-vasopressin (dDAVP; 20 ng/h, Rhone-Poulenc, Gannat, France) or vehicle for 7 days as previously described (13).

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In addition to UT-A1 antibodies, this study also employed rabbit polyclonal antibodies to AQF2 (LI27) (7) and to the type 2 bumetanide sensitive Na-K-2Cl cotransporter NKCC2 (LI24) (9).

Immunoprecipitation of UT-A Protein

Preparation of antibody-laden beads for immunoprecipitation of UT-A proteins. To prepare antibody-laden beads for immunoprecipitation, 100 μl of a Dynal bead suspension (Dynabeads M-280 sheep anti-rabbit IgG; 10 mg beads/ml; 7 × 10^9 beads/ml) were transferred to a 1.5-ml Eppendorf tube. The beads were pelleted on a Dynal MPC-M magnetic particle concentrator (Dynal, Oslo, Norway) for 3 min, and the supernatant was removed by aspiration. The beads were washed twice in 1,000 μl of wash buffer (PBS, 0.1% BSA, pH 7.4). After the beads were re-suspended to 1,000 μl with wash buffer, 318.7 μl (3.187 mg; 2.2 × 10^9 beads) of the suspension were delivered to a 1.25-ml Sarstedt screw-cap microtube cup (Sarstedt; Newton, NC). Again, the beads were pelleted on the Dynal MPC-M for 3 min to aspirate off the fluid. The anti-urea transporter primary antibody [9.56 μg of L194 (IgG concentration = 0.224 μg/ml)] was delivered to the Sarstedt cup and diluted to 100 μl with wash buffer containing 0.02% azide (3.0 ml of 100 mM sodium azide/100 ml wash buffer). The mixture was incubated overnight at 4°C with moderate agitation to allow the antibody to become bound to the beads.

To prepare for cross-linking the primary antibody to the beads with dimethylpimelimidate (DMP), the beads were pelleted as described above, resuspended to 1 ml with wash buffer, and washed four times (for 30 min each time) at 4°C on a Dynal bidirectional sample mixer (Dynal; Long Island, NY). The bead pellets were then resuspended in 1 ml of 0.2 M triethanolamine buffer (14.8 g/400 ml H₂O, pH 8.2) and washed twice with triethanolamine buffer. Next, the 1 ml of bead volume was quantitatively transferred to a 15-ml Sarstedt conical tube. The beads were divided into two 500-μl aliquots, and the volume of each one was brought up to 10 ml with triethanolamine buffer. The beads were pelleted on a Dynal MPC-6 magnetic particle concentrator (Dynal) for 3 min to aspirate off the fluid, washed once with 10 ml of triethanolamine buffer, and resuspended to 10 ml with triethanolamine buffer. Afterward, 130 μl of DMP stock solution (200 mg/500 ml triethanolamine buffer) were added to each tube, and the beads were bidirectionally mixed for 45 min at room temperature. To quench the cross-linking reaction, the beads were pelleted on the Dynal MPC-6 as above, and the supernatant was discarded. At this point, the beads were resuspended in 10 ml triethanolamine buffer and incubated for a further 2 h at room temperature. For removal of all noncovalently coupled antibody from the beads, the tubes were incubated by bidirectional mixing for 2 h at 4°C. Afterward, the beads were washed three times with wash buffer as described above with 1 ml of wash buffer and pelleted after each wash. The protein was eluted from the beads with 200 μl of 1× Laemmli sample buffer at 60°C for 15 min. At this point, the beads were pelleted as described above, and the supernatant was recovered into 1.5-ml Eppendorf tubes. After 50 μl of 5× Laemmli sample buffer were pipetted into both the supernatant and the immunoprecipitated eluate, both samples were incubated at 60°C for 15 min. Immunoblots from the immunoprecipitated material and the supernatant were probed with the COOH-terminal anti-urea transporter antibody L194.

Deglycosylation of immunoprecipitated UT-A1 proteins. Deglycosylation was accomplished by exposing the beads, after immunoadsorption but before elution, to commercially available glycosidases specific to N-linked glycosylation, namely, endoglycosidase F (Endo F) and N-glycosidase F (PNGase F), both from Boehringer Mannheim (Indianapolis, IN). The protocols recommended by the manufacturer were followed. Controls followed the same protocols, except that the enzymes were not added. The incubations with the glycosidases were carried out for 48 h at room temperature in sealed Eppendorf tubes. The reaction was quenched, and the beads were eluted with 1:4 vol of 5× Laemmli buffer at 60°C for 15 min before magnetic removal of beads, SDS-PAGE of the supernatant, and immunoblotting.

Deglycosylation of UT-A1 Protein in Inner Medullary Homogenates

Forty-five microliters of inner medullary homogenate prepared as described above (1 μg/μl protein) were denatured by heating to 100°C for 10 min after addition of SDS to 0.5% and β-mercaptoethanol to 1% (vol/vol). After addition of NP-40 detergent to 1% and addition of 2,500 U PNGase F (catalog no. 704S, New England Biolabs, Beverly, MA), the mixture was incubated at 37°C for 60 min. The reaction was quenched by the addition of an equal volume of 2× Laemmli sample buffer. These samples were heated again to 60°C for 15 min before SDS-PAGE and immunoblotting.

Deglycosylation of UT-A1 Protein From Crude Membranes

A crude membrane fraction was prepared from inner medullary homogenates as described above (Crude Membrane Preparation and Immunoblotting), and the deglycosylation was conducted by using a commercially available glycosidases specific to N-linked glycosylation, namely, Endo F (catalog no. 903–329, Boehringer Mannheim) and endoglycosidase H (Endo H; catalog no. 100–117, Boehringer Mannheim). The protocols recommended by the manufacturer were followed. Controls followed the same protocols, except that the enzymes were not added. The incubations with the glycosidases were carried for 5 days at 37°C in sealed Eppendorf tubes. The reaction was quenched with 1:4 vol of 5× Laemmli buffer, and the samples were incubated again at 60°C for 15 min before SDS-PAGE and immunoblotting.
Limited Proteolysis of UT-A1 in Intact Membrane Vesicles

A low-density membrane fraction was prepared from homogenates of rat inner medulla. In brief, the supernatant from a 20-min centrifugation at 17,000 g was centrifuged at 200,000 g for 60 min. The pellet from the 200,000 g centrifugation, which contains intracellular vesicles but not plasma membranes (8, 18, 28), was resuspended in 500 μl of MOPS buffer, pH 7.0 (50 mM MOPS, 50 mM KCl, 0.5 M EGTA). One hundred and nineteen-microliter aliquots of this solution were incubated in 0, 5 × 10⁻³, 5 × 10⁻², and 5 × 10⁻¹ mg/ml of chymotrypsin (a-chymotrypsin type VII; Sigma; St. Louis, MO) in a final volume of 200 μl. The incubation was carried out at 4°C for 5 min with vigorous shaking. The reaction was quenched with 50 μl of 5× Laemmli sample buffer and incubated at 60°C for 15 min to solubilize membranes. The proteolytic products were visualized via SDS-PAGE and immunoblotting as described above.

Cross-Linking of Membrane-Associated Proteins

Inner medullary crude membrane fractions were prepared as described above. Before solubilization, 1 ml of the membrane fraction was incubated with 3 mM of each of three commercial H₂O-soluble, membrane-impermeant homobifunctional cross-linkers: sulfo-BSOOCOE (Pierce; formula weight (FW) = 640.46; linker arm length, 13 Å); DMP (Sigma; FW = 259.2; linker arm length, 9.2 Å); and sulfo-ethylene glycol bis (sulfosuccinimidylsuccinate) (sulfo-EGS; Pierce; FW = 666.47; linker arm length, 16.1 Å). The control sample included the remaining volume of the membrane fraction incubated without any cross-linker. All samples were incubated for 30 min at room temperature with bidirectional mixing. The reaction was quenched with Tris buffer (ultrapure Tris adjusted to pH 7.4 with 12 M HCl), such that the final concentration of the buffer in the reaction mixture was 50 mM, and incubated for an additional 30 min at room temperature with bidirectional mixing. A 200-μl aliquot was removed and incubated with 50 μl 5× Laemmli sample buffer at 60°C for 15 min before SDS-PAGE and immunoblotting.

RESULTS

Effect of Chronic Vasopressin Treatment on Electrophoretic Mobility of UT-A1

The Brattleboro rat is a rat strain that lacks circulating vasopressin and therefore undergoes a chronic water diuresis. Figure 2 shows an immunoblot probed with an antibody directed to the COOH-terminal tail of UT-A1 (antibody L403) demonstrating the effect of a 7-day infusion of the vasopressin analog dDAVP on the electrophoretic mobility of UT-A1 in the inner medullas of Brattleboro rats. The blot compares the bands observed in six vehicle-treated Brattleboro rats (left) with those observed in six dDAVP-treated rats (right). As can be seen, the antibody recognizes two UT-A forms in the vehicle-treated rats, with apparent molecular masses of 117 and 97 kDa. Infusion of dDAVP for 5 days resulted in a decrease in the density of the 117-kDa band, with a concomitant increase in the density of the 97-kDa band. This result confirms our previous observations (29). The appearance of the 117-kDa form was seen in a variety of conditions with increased water excretion and correlated with an increase in urea permeability in isolated perfused tubes taken from diuretic rats (12, 29).

Immunoprecipitation of UT-A1

To confirm that the 97- and 117-kDa proteins are indeed different forms of UT-A, we carried out immunoprecipitation/immunodepletion experiments. In preparation for immunoprecipitation of UT-A1, a series of detergents was tested using homogenates from untreated rats to determine the detergents’ abilities to solubilize UT-A1 protein for immunoprecipitation. Figure 3A shows a single immunoblot loaded with rat inner medullary membrane fractions that were ini-
tially solubilized using different detergents and probed with the COOH-terminal antibody L403. When the membranes were solubilized in the strong ionic detergent SDS (left lane), the protein ran at ~97 kDa as previously reported, although there was a weak band at ~206 kDa on this deliberately overexposed blot. However, immunoprecipitations cannot be carried out in SDS-solubilized material because SDS denatures IgG. The remaining lanes show results with a series of detergents that do not denature IgG. When the membranes were solubilized initially with the nonionic detergents Triton X-100 or NP-40, a broad band centered at 206 kDa was obtained with a concomitant decrease in the density of the 97-kDa band. The 206-kDa band is presumably a protein complex containing UT-A1 (see below). The density of the broad upper band was reduced, and that of the 97-kDa band was increased when the NP-40-solubilized sample was treated with 8 M urea, a strong denaturing agent. Solubilization with a commonly used detergent mixture, RIPA (1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate), also yielded both the broad 206- and the 97-kDa band but increased the amount of the latter relative to single nonionic detergents. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate failed to solubilize the UT-A protein (not shown). The results suggest that UT-A1 is found in a high-molecular-weight complex, which is dissociated by SDS but not by nonionic detergents. Figure 3B shows a UT-A immunoblot of an inner medullary membrane fraction solubilized with 1% NP-40 and 8 M urea. This blot was loaded with relatively small amounts of protein and exposed to film for a relatively short period to estimate the size of the high-molecular-weight complex. As can be seen, at the lowest loading, the UT-A antibody labeled a 206-kDa band. On the basis of these solubilization studies, we chose to carry out immunoprecipitations in RIPA-solubilized inner medullary membranes to maximize the amount of monomeric UT-A1.

Figure 4 shows the results of immunoprecipitation experiments using the COOH-terminal antibody L194. Increasing the amount of antibody progressively increased the urea transporter protein yield (top) whereas the amount of urea transporter protein in the supernatant from the bead incubation was steadily depleted (bottom). The immunoprecipitated material included the 97-kDa band, the 117-kDa band, and the 206-kDa oligomeric complex. All three forms were immunodepleted from the supernatant in proportion to the amount of immunoprecipitating antibody, providing additional evidence that all three represent alternative forms of UT-A proteins. These findings suggest that the COOH-terminal antibody was able to recognize the three bands with approximately equal affinity.

As shown in Fig. 5, the anti-COOH-terminal antibody L194 successfully immunoprecipitated the urea transporter proteins (97-, 117-, and 206-kDa UT-A forms), whereas an equal amount of preimmune IgG from the same rabbit did not (left). On preadsorption of the anti-urea transporter antibody with the synthetic peptide, there was complete ablation of the urea transporter bands (right). The data in Figs. 4 and 5 provide strong evidence that the 97-, 117-, and 206-kDa bands all represent alternative forms of UT-A protein rather than non-UT-A proteins nonspecifically recognized by the UT-A antibodies.

Preparation of Polyclonal Antibodies to Multiple UT-A1 Epitopes

The coding sequence for UT-A1 is 929 amino acids in length, corresponding to a predicted molecular mass of 102 kDa for the core protein. With the assumption that UT-A1 is glycosylated (see below), it seemed possible that the full-length UT-A1 is the 117-kDa form, and that the 97-kDa form is derived from the 117-kDa form by deletion of an ~20-kDa portion of the core protein. To test this, we raised peptide-directed polyclonal antibodies to five sites from the NH2 terminus through the COOH terminus of the reported amino acid sequence (Fig. 1) and used these antibodies to look for deletions by immunoblotting of rat inner medullary homogenates. The immunoblots (Fig. 6) revealed that all antibodies recognized both the 97- and 117-kDa
bands. Thus we were unable to identify major deletions of the peptide sequence in either the NH2-terminal tail [amino acids (aa) 1–122], the middle loop (aa 421–587), or the COOH-terminal tail (aa 884–929) that would account for the two molecular masses. These immunoblots, however, do not rule out deletions in the hydrophobic regions (aa 123–420 or 588–883).

Deglycosylation of UT-A1 Protein

To test whether UT-A1 is N-glycosylated and to address further whether the core molecular weight for the 97- and 117-kDa UT-A1 forms is different, inner medullary homogenates from three water-loaded rats (with prominent expression of the 117-kDa form) were pooled and incubated with PNGase F or vehicle. Figure 7A shows the resulting immunoblot probed with the L403 antibody directed to the COOH-terminal tail of UT-A1. The electrophoresis was run on a 10% polyacrylamide gel. After PNGase F treatment, there was a single 88-kDa deglycosylated UT-A1 protein with the disappearance of the 97- and 117-kDa UT-A1 bands. A similar result was found in a repeat experiment, in which the electrophoresis was run on a 12% gel, and preadsorption with an excess of the immunizing peptide completely ablated the 88-, 97-, and 117-kDa bands (Fig. 7B). These results demonstrate 1) that UT-A1 is N-glycosylated; 2) that the 97- and 117-kDa forms of UT-A1 are derived from the same core protein; and 3) that the 97- and 117-kDa bands represent two states of UT-A1 glycosylation, perhaps representing N-linked glycosylation at two distinct sites. Similar results were found with two additional deglycosylation experiments not shown here.

Limited Proteolysis of UT-A1 Protein

There are several asparagine moieties in the UT-A1 sequence that are potential N-glycosylation sites (see Fig. 1). To determine whether the site of variable glycosylation is in the first half or the second half of the UT-A1 sequence, we carried out limited proteolysis of UT-A1-bearing intracellular vesicles with chymotrypsin to cleave UT-A1 in the middle loop region (aa 421–587). On the basis of the membrane-spanning models of Shayakul et al. (25) and Sands et al. (24), the hydrophilic middle loop is believed to be located on the cytosolic side of the membrane. This loop contains several potential cleavage sites for chymotrypsin. The strategy therefore is to cut the membrane-bound UT-A1 molecule in half by partial proteolysis with chymotrypsin and determine the molecular sizes of the fragments. In these experiments, a low-density membrane fraction containing intracellular vesicles (8, 17, 18, 28) was isolated from the inner medullas of water-loaded rats by differential centrifugation and exposed to varying concentrations of chymotrypsin (Fig. 8). Immunoblots loaded with the products of the chymotrypsin incubation were probed with antibodies to the NH2-terminal tail (L446), to the COOH-terminal tail (L403) (Fig. 8A), and to the middle loop (L448 and L455) to determine the size of the products.

Figure 8A shows an immunoblot run with the products of the limited proteolysis probed with the L446 antibody directed to the NH2-terminal hydrophilic tail. In the absence of chymotrypsin, the NH2-terminal antibody recognized the usual two bands at 97 and 117 kDa. Increasing the enzyme concentration to $5 \times 10^{-4}$ mg/ml yielded two products of ~55 and 75 kDa, presumably corresponding to the products of the 97- and 117-kDa forms, respectively. This result is compatible with the expected sizes if the protein were cleaved at a...
single site somewhere in the middle loop. The ability of
the NH2-terminal antibody to detect the cleavage prod-
ucts attested to the intactness of the NH2-terminal tail.
The highest enzyme concentration, $5 \times 10^{-3}$ mg/ml,
yielded two products which ran at 48 and 68 kDa,
consistent with a second cleavage site in the middle
loop ~7 kDa upstream from the first site. Preadsorp-
tion controls using the immunizing peptide for the
L446 antibody resulted in ablation of all bands (data
not shown).

Figure 8B shows an immunoblot of the same samples
as used for Fig. 8A but probed with the COOH-termi-
nal antibody L403. In the absence of chymotrypsin, the
usual 97/117-kDa bands were seen. After chymotryp-
sin incubation at a concentration of $5 \times 10^{-4}$ mg/ml, a
predominant 40-kDa band was seen. The size of this
fragment is again compatible with an initial cleavage
site along the proposed cytosolic loop. With the highest
enzyme concentration, no product was discernible, pos-
sibly because of proteolysis at the COOH terminus.
Preadsorption controls using the immunizing peptide
for the L403 antibody resulted in ablation of all bands
(data not shown).

Figure 8C shows an immunoblot probed with anti-
body L448 directed against the middle hydrophilic re-
gion of UT-A1. Chymotrypsin treatment eliminated
the 97/117-kDa doublet, but no lower molecular weight
bands appeared. This suggests that the chymotrypsin
treatment obliterated the epitope recognized by L448.
However, when a blot using the same samples were
run with antibody L455 (corresponding to an earlier
portion of the middle hydrophilic loop), the pattern was
virtually identical to that seen for L446 (shown in Fig.
8A), indicating that the chymotrypsin cleavage sites
are downstream from aa 463–485 (see Fig. 1).

In summary, the finding that only the NH2-terminal
antibody L446 recognizes two bands that differ by ~20
kDa suggests that the structural difference (additional
glycosylation) that accounts for the presence of two
UT-A1 bands (97 and 117 kDa) exists in the first half of
the UT-A1 protein.

**Stabilization of UT-A1 Protein Complexes by
Chemical Cross-Linking**

If UT-A1 is complexed either with itself or with other
proteins as suggested by the immunoprecipitation
studies, the use of homobifunctional cross-linking
agents have the potential of stabilizing such com-
plexes. To test for the presence of high-molecular-
weight UT-A1 complexes in native IMCD membranes,
we carried out cross-linking studies in a crude mem-
brane fraction from rat inner medulla using a series of
homobifunctional cross-linking agents. Figure 9 shows
immunoblots of the cross-linked samples using the
COOH-terminal antibody L403. In the absence of
cross-linking, the 97- and 117-kDa bands were most
prominent, whereas a faint band just above the 201-
kDa marker was barely visible. (Note that we have
observed that the apparent mobility of monomeric UT-

A1, relative to the globular proteins used as molecular weight markers, is increased with decreases in the percent polyacrylamide used for the electrophoresis. In Fig. 9, on 4–12% polyacrylamide gradient gels the nominal 97-kDa band is shifted downward, somewhat relative to the molecular weight markers, to ~90 kDa. When cross-linking was carried out with sulfo-BSOCOES or DMP, a strong broad band centered just above the 201-kDa marker was obtained. Note that the sulfo-EGS incubation resulted in disappearance of both the monomeric and oligomeric bands, perhaps because of stabilization of an even larger complex. On the basis of this analysis, we conclude that UT-A1 is normally present in renal membranes as a high-molecular-weight complex, possibly a homodimer. Parallel blots probed for AQP2 (a water channel expressed in the collecting duct) showed no evidence of AQP2 in the 206-kDa complex (not shown).

Deglycosylation of UT-A1 Complex

To further address the glycosylation state of the UT-A1 protein, we tested the effects of PNGase F and Endo F on the apparent molecular weight of the high-molecular-weight UT-A1 complex immunoprecipitated from inner medulla (Fig. 10). The apparent molecular weight of the complex was shifted downward, and the spread of the broad high-molecular-weight UT-A1 was markedly reduced by both Endo F and PNGase F, confirming that the high-molecular-weight complex contains N-glycosylated proteins. In addition, both agents shifted the 97-kDa monomeric band downward by ~10 kDa, confirming the results reported above.

Endo F vs. Endo H Sensitivity

Glycosylation of integral membrane proteins occurs in both the endoplasmic reticulum and Golgi apparatus. High-mannose-type glycan structures typical of glycosylation in the endoplasmic reticulum are sensitive to endoglycosidase H (Endo H), whereas mature, complex glycan structures typical of glycosylation in the Golgi apparatus are sensitive to endoglycosidase F (Endo F). Figure 11 shows immunoblots for AQP2, UT-A1, and NKCC2 carried out with inner medullary membranes from nondiuretic rats after incubation with Endo H and Endo F. As can be seen in Fig. 10, top, the broad 38-kDa band of AQP2 [previously demonstrated to be the glycosylated form of AQP2 (2)] was completely ablated by Endo F incubation, but not Endo H incubation. Endo F shifted the molecular mass of UT-A1 downward from 97 to 88 kDa, but Endo H incubation did not alter the mobility of UT-A1. Similarly, the apparent molecular mass of NKCC2 was shifted from 163 to 110 kDa (consistent with its expected core molecular weight) by incubation with Endo F, whereas Endo H was without effect. [Note that in addition to its expression in the outer medulla, we have found that NKCC2 is expressed at moderate levels in the inner medulla (M. Knepper, unpublished observations). The physiological role of NKCC2 in the inner medulla is under investigation.] A similar deglycosylation pattern was found for NKCC2 in the outer medulla (not shown). Thus we conclude that the N-linked glycosylation of UT-A1 is primarily of the mature, Endo F-sensitive type.

DISCUSSION

UT-A1 is a urea transporter with channel-like properties that is believed to mediate the apical component of transepithelial urea transport in the inner medul-
Our previous studies established that the IMCD of the rat kidney expresses two putative UT-A1 forms with electrophoretic mobilities compatible with distinct proteins of 97 and 117 kDa (29). Experiments using differential centrifugation to fractionate inner medullary membranes suggested that the two isoforms have similar intracellular distributions. The relative abundance of the two UT-A forms appears to be regulated in response to maneuvers associated with altered urine flow or medullary urea concentration (29). Specifically, diuretic states (furosemide administration and water diuresis) were associated with an increase in the abundance of the 117-kDa form and a concomitant decrease in the abundance of the 97-kDa form. The finding that the appearance of the 117-kDa isoform is associated with increased urea permeability in isolated perfused collecting ducts from diuretic rats (12) suggests that both forms are N-glycosylated and that the difference between the two is the presence of an additional degree of glycosylation in the 117-kDa form. There are only four potential N-linked glycosylation sites in the hydrophilic regions of the sequence: at Asn-13, Asn-279, Asn-544, and Asn-742 (25). Membrane-spanning models of UT-A1 (24, 25) assume that the NH2-terminal tail, the site of Asn-13, is on the cytoplasmic side of the membrane, based in part on the absence of a hydrophobic signal sequence at the NH2 terminus. Thus Asn-13 is unlikely to be a site of glycosylation. Immunogold studies at an electron microscopic level with an antibody directed to the COOH-terminal tail of UT-A1 (L194) showed predominant labeling of the cytoplasmic side of the plasma membrane, placing the COOH-terminal tail in the intracellular milieu (20). Cleavage of the middle loop of UT-A1 (aa 421–587) with chymotrypsin in an intracellular vesicle-derived membrane fraction from inner medulla confirmed the assumption made in prior models (24, 25) that the middle loop is located on the cytosolic side of the membrane. Thus Asn-544 is unlikely to be N-glycosylated. Therefore, it appears that the two most likely sites of glycosylation are Asn-13 and Asn-742. Experiments that utilized limited proteolysis to cleave the middle loop indicated that the extra glycosylation responsible for the difference between the 97 and 117 forms is in the first half of the molecule, suggesting variable glycosylation at Asn-279 (Fig. 8).

The functional significance of the added glycosylation in the 117-kDa UT-A1 form is unknown. In previous studies, we have demonstrated that chronic diuresis associated with furosemide administration is...
associated with an increase in the molecular weights of AQP2, Tamm-Horsfall protein, and NKCC2 in the renal medulla due to increased glycosylation (9). This finding prompted us to speculate that Golgi-mediated glycosylation could have been accelerated by the metabolic alkalosis associated with the furosemide treatment. Indeed, furosemide treatment also increases the abundance of the 117-kDa form of UT-A1, indicating an increase in the extent of UT-A1 glycosylation (29). However, Brattleboro rats are not known to manifest chronic metabolic alkalosis. Thus another explanation must be sought for the increased glycosylation seen in vasopressin-deficient vs. vasopressin-replete Brattleboro rats (Fig. 2).

What role could altered glycosylation play in regulation of UT-A1 function? N-linked glycosylation of integral membrane proteins like UT-A1 plays a role in quality control of folding in the endoplasmic reticulum and may play other roles in membrane trafficking. Assuming that glycosylation is important in membrane targeting, it seems possible that the alteration of the UT-A1 glycosylation pattern would signal some change in trafficking to the plasma membrane. Immunocytochemistry, surface biotinylation studies, and differential centrifugation studies were unable to detect changes in UT-A1 distribution in the IMCD of Brattleboro rats in response to acute treatment with vasopressin (11). However, the effect of long-term vasopressin administration on UT-A1 distribution in the IMCD cell of the Brattleboro rat has not yet been examined.

**UT-A1 Exists in the Native Membrane as Part of a Protein Complex**

Solubilization of UT-A1 in nonionic detergents revealed a high-molecular-weight complex seen as a broad band centered at 206 kDa (Fig. 3). When membrane fractions were subjected to homobifunctional cross-linkers, a similar complex was stabilized (Fig. 9). Immunoprecipitation with the COOH-terminal antibody yielded predominantly the 206-kDa broad band centered at 206 kDa (Fig. 3). When membrane fractions were subjected to homobifunctional cross-linkers, a similar complex was stabilized (Fig. 9). These results suggest that UT-A1 exists in the native membranes of IMCD cells as a complex, although the present studies do not establish whether the complex is simply a dimer of UT-A1 or may be a heterooligomer. A number of transporters have been demonstrated to assemble in the plasma membrane as higher molecular complexes, in-dex AQP1 as a tetramer (26), AQP4 as a large crystalloidal hexagonal array (30), the Na/H exchangers NHE1 and NHE3 as dimers (10), NKCC2 as a dimer (19), the Cl/HCO3- exchanger AE1 (3), and the amiloride-sensitive sodium channel ENaC as a heterotetramer (4). Thus, as previously emphasized by Klingenberg (15), the formation of multimeric complexes is a common property of transport proteins. The formation of complexes may be necessary for stabilization of independently functioning protein monomers in the membrane, as is seen for AQP1 (1), or may allow individual subunits to interact to form a solute-permeable channel across the membrane, as appears to be true for ENaC (4).

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