Molecular characterization of a novel urea transporter from kidney inner medullary collecting ducts

CHAIRAT SHAYAKUL,* HIROYASU TSUKAGUCHI,* URS V. BERGER, AND MATTHIAS A. HEDIGER

Renal Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

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Shayakul, Chairat, Hiroyasu Tsukaguchi, Urs V. Berger, and Matthias A. Hediger. Molecular characterization of a novel urea transporter from kidney inner medullary collecting ducts. Am J Physiol Renal Physiol 280: F487–F494, 2001.—In the terminal part of the kidney collecting duct, rapid urea reabsorption is essential to maintaining medullary hypertonicity, allowing maximal urinary concentration to occur. This process is mediated by facilitated urea transporters on both apical and basolateral membranes. Our previous studies have identified three rat urea transporters involved in the urinary concentrating mechanism, UT1, UT2 and UT3, herein renamed UrT1-A, UrT1-B, and UrT2, which exhibit distinct spatial distribution in the kidney. Here we report the molecular characterization of an additional urea transporter isoform, UrT1-C, from rat kidney that encodes a 460-amino acid residue protein. UrT1-C has 70 and 62% amino acid identity to rat UrT1-B and UrT2 (UT3), respectively, and 99% identity to a recently reported rat isoform (UT-A3; Karakashian A, Timmer RT, Klein JD, Gunn RB, Sands JM, and Bagnasco SM. J Am Soc Nephrol 10: 230–237, 1999). We report the anatomic distribution of UrT1-C in the rat kidney tubule system as well as a detailed functional characterization. UrT1-C mRNA is primarily expressed in the deep part of the inner medulla. When expressed in Xenopus laevis oocytes, UrT1-C induced a 15-fold stimulation of urea uptake, which was inhibited almost completely by phloretin (0.7 mM) and 60–95% by thiourea analogs (150 mM). The characteristics are consistent with those described in perfusion studies with inner medullary collecting duct (IMCD) segments, but, contrary to UrT1-A, UrT1-C-mediated urea uptake was not stimulated by activation of protein kinase A. Our data show that UrT1-C is a phloretin-inhibitable urea transporter expressed in the terminal collecting duct that likely serves as an exit mechanism for urea at the basolateral membrane of IMCD cells. 

Urinary concentration; urea transporter; vasopressin; nomenclature

*These authors contributed equally to this study.

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products of the SLC14A1 gene. Karakashian et al. (9) reported a 4th putative splice product of rat SLC14A1, but expression of this putative

The molecular properties of urea transporter isoforms are summarized in Table 1. The table lists the protein name, tissue distribution, and reference accession numbers for various isoforms of the urea transporter family. The table includes information on the tissue distribution of each isoform, such as terminal IMCD, apical membrane, tDL, terminal IMCD, basolateral?, testis?, descending vasa recta, brain, erythrocytes, and testis. The reference accession numbers range from U77971 to U81518. Additionally, the table includes information on the human and rodent species, with symbols for Rat, Human, and Mouse. The table also includes a column for GenBank accession numbers, with accession numbers ranging from AF031642 to U81518.

Amino acid alignment of members of the UrT family is shown in Fig. 1. The alignment includes sequences for rat UrT1-A, -B, and -C, as well as human UrT1-C, human UrT2, and Xenopus laevis. The alignment shows the high sequence identity between the UrT isoforms and the conservation of functional residues.

Molecular properties of urea transporter isoforms

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<td>U77971</td>
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<td></td>
<td>UrT1-B</td>
<td>tDL</td>
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<tr>
<td></td>
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METHODS

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HUGO Gene Symbol, Revised nomenclature, Alias(es), Tissue, Distribution, Species, Reference No(s.), GenBank Accession No.
RESULTS

Cloning and sequencing of rat UrT1-C cDNA. To isolate a new splice variant and avoid contamination of 4.0-kb transcripts, we constructed a size-selected cDNA library (ranging from 1.5 to 3.5 kb) by using rat kidney inner medulla mRNA and screened it by a probe corresponding to the first 652 nucleotides of rat UrT1-A (UT1) cDNA. Four positive clones of different sizes were isolated, among which the clone UrT1-C carried the largest insert of 1.989 bp. UrT1-C cDNA contains a polyadenylation sequence (ATTAAA) at position 1951, an open reading frame from nucleotide 377 to 1759, thus encoding a protein of 460 amino acids with a relative molecular mass of 50.4 kDa (Fig. 1A).

The amino acid sequence is identical to UT-A3 cDNA (GenBank AF041788) isolated from rat kidney on the basis of PCR amplification by other investigators (9), except for two amino acids. Pro at position 17 (Leu in UT-A3) and Ala at position 95 (Gly in UT-A3). Compared with UrT1-A cDNA, UrT1-C cDNA is identical at the 5' ends from position 1 to 1754, whereas it differs at the 3' end, 219 nucleotides from position 1755 to 1973. The amino acid sequence of UrT1-C is therefore identical to the NH2-terminal half of UrT1-A and has 70 and 62% identity to rat UrT1-B and UrT2, respectively (Fig. 1A). The hydropathy plot of UrT1-C is in accordance with the characteristic pattern of members of the urea transporter protein family, which includes two extended hydrophobic membrane stretches interspersed with hydrophilic regions (Fig. 1, B and C). UrT1-C polypeptide contains one potential N-glycosylation site at Asn279, two potential phosphorylation sites for protein kinase A (PKA; Ser84 and Ser91), and three potential protein kinase C phosphorylation sites (Ser23, Ser79, and Thr447).

Tissue distribution and localization of UrT1-C mRNA in rat kidney. Northern analysis using full-length UrT1-C as probe revealed a strong hybridization signal of ~2.2 kb and a weak signal of 4.0 kb in rat kidney inner medulla, in particular in the deeper portion (Fig. 2). No signal was detected in the regions of the kidney or in other organs, indicating that the UrT1-C is specific to the kidney inner medulla. Karakashian et al. (9) also observed signals in testis; however, our Northern analysis revealed no signal for testis when using high-stringency washing conditions. To further localize UrT1-C mRNA in rat kidney, we performed in situ hybridization and compared its distribution with the other kidney UrT isoforms, UrT1-A and UrT1-B, using adjacent sections. Because the UrT1-C nucleotide sequence is identical to that of the NH2-terminal domain of UrT1-A, we prepared a cRNA probe corresponding to 219 bp of 3' untranslated region of UrT1-C cDNA, which can distinguish between UrT1-C and UrT1-A transcripts. Hybridization of the antisense URT1-C cRNA probe revealed the strongest signals in the deep portion of the IMCD (Fig. 3), consistent with the distribution observed by Northern analysis. In comparison, hybridization signals obtained with the probe that recognizes both UrT1-A and UrT1-C extended into the basolateral part of the inner medulla (Fig. 3). Because the UrT1-A probe used is three times longer than the UrT1-C probe, these data do not
reveal the relative proportion of UrT1-A and UrT1-C expression in the IMCD. However, they indicate that the expression levels of both UrT1-A and UrT1-C increase toward the deep IMCD, as has been shown for UrT1-A by quantitative RT-PCR in microdissected kidney tubules (22). Hybridization with UrT1-B-specific cRNA probes showed that the UrT1-B transcript is located in the inner stripe of outer medulla and the outermost portion of the inner medulla. These data indicate that UrT1-C mRNA expression is strongest in the terminal IMCD and that its tubular expression coincides with that of UrT1-A. Its distribution is in good agreement with the regions previously shown by perfusion studies to have extremely high urea permeability (13, 25).

**Functional properties of UrT1-C in X. laevis oocytes.** We examined the functional characteristics of UrT1-C in the X. laevis oocyte expression system. When expressed in the oocytes, UrT1-C mediated an ~15-fold increase in [14C]urea uptake (1 mM) compared with water-injected control oocytes (Fig. 4A). Urea transport mediated by UrT1-C reached a maximum after incubation for 30 min (Fig. 4B). The level of transport activity of UrT1-A and UrT1-C was compared in the same batch of oocytes (Fig. 4D). Urea transport mediated by UrT1-C was about three- to fourfold higher than that mediated by UrT1-A, 20–30 vs. 5–10 × 10⁻⁶ cm/s. Reducing the amount of UrT1-C cRNA for microinjection to as low as 10 ng/oocyte did not significantly affect UrT1-C transport activity and gave consistently higher uptake levels than UrT1-A. Furthermore, this uptake was about fivefold higher than that mediated by AQP3 (20–30 vs. 5–10 × 10⁻⁶ cm/s; Ref. 29). UrT1-C-mediated urea uptake was not changed by replacing 200 mM mannitol with 96 mM NaCl, indicating that transport is not sodium dependent (Fig. 4A). Uptake of urea (1 mM) was inhibited by preincubation for 30 min with phloretin (0.2–0.7 mM) in a dose-dependent fashion (Fig. 4A). The level of phloretin sensitivity was similar to those observed for UrT1-A and UrT1-B (23, 24) and consistent with the properties of urea transport characterized in perfusion studies of the IMCD (3, 25).

UrT1-C-mediated urea uptake was inhibited by urea analogs (150 mM) including dimethylurea (4.7 ± 0.6% of control), thiourea (44.4 ± 5.9% of control), and acetamide (58.6 ± 5.5% of control) (Fig. 4C). The inhibition was in good agreement with the studies of the perfused IMCD showing that thiourea and methylurea inhibit urea permeability more effectively than acetamide (3). One important characteristic of urea transport on the apical membrane of terminal IMCD segments is its activation by vasopressin via a cAMP-dependent pathway. We demonstrated in previous studies that UrT1-
A-mediated urea uptake in *X. laevis* oocytes can be stimulated by acute activation of protein kinase A, consistent with the effect of vasopressin stimulation. Preincubation of 30 min with cAMP agonists, however, did not alter transport activities of UrT1-C. These studies indicated that UrT1-C displays functional characteristics of the urea transport previously described in the terminal IMCD with three- to fourfold higher transport activity than UrT1-A but does not appear to be regulated by acute activation of protein kinase A.

**DISCUSSION**

In the present study, we show that another splice variant of the SLC14A1 gene, UrT1-C, is primarily expressed in the terminal IMCD. Sequence analysis indicates that UrT1-C has a hydrophobic transmembrane protein structure similar to the other isoforms UrT1-B (UT2) and UrT1-A (UT1). *X. laevis* oocytes expressing UrT1-C facilitate transport of urea with high capacity, and transport is inhibited by urea analogs and phloretin. The transport characteristics of UrT1-C are consistent with those previously described in the terminal IMCD with three- to fourfold higher transport activity than UrT1-A but does not appear to be regulated by acute activation of protein kinase A.

We have summarized the properties of urea transporter family members in Table 1. Most of the nucleotide sequences of UrT1-C cDNA correspond to the 5' part of UrT1-A cDNA. On the basis of genomic analysis (23), we have previously showed that UrT1-A arises by splicing of exon group I and group III, which are located about 7 kb apart (Fig. 1D). UrT1-C mRNA presumably arises as a splice product of exon group I and group Ia. Other investigators recently reported the cloning of two splicing variants of the SLC14A1 gene, UT-A3 and UT-A4, by PCR from rat kidney cDNA (9). Of these isoforms, the physiological role of UT-A4 (UrT1D) remains unclear because its transcript was too low to be detected by Northern analysis. The sequence of UT-A3 cDNA is 99% identical to UrT1-C cDNA and induced phloretin-inhibitable urea transport when expressed in human kidney HEK-293 cells, similar to our expression data in the oocytes.

Our localization and functional characterization data suggest that UrT1-C may be responsible for urea transport on the basolateral membrane of the kidney IMCD cells. Urea reabsorption in the terminal part of the IMCD is an important aspect of urinary concentration (Fig. 5). To ensure maximal urinary concentrating capacity, urea absorption is delayed to the most terminal part of the IMCD, where effective blood flow is low. This process prevents the escape of urea, through the vasa recta, in the upper part of the inner medulla, where blood flow is much higher (1, 13). Urea permeability measurements in isolated perfused rat and rabbit IMCD segments showed that the urea permeability of the IMCD in the initial part is at least one order of magnitude lower than that in the terminal portions (19). Because transepithelial urea permeability is extremely high in the terminal IMCD, urea movement across the IMCD cells is considered to occur via urea transporters present on both apical and basolateral membranes. Previous perfusion studies indicated that...
It is possible that basolateral urea exit is mediated by a pathway other than UrT1-C. Several water channels have been reported to be permeable to water as well as urea (6, 7, 29). Among those, aquaporin-3 (AQP3) was shown to be expressed on the basolateral membranes of cells throughout the IMCD (5, 8). However, AQP3 does not appear to play an important role in basolateral exit of urea, because it is expressed most abundantly in the outer half of the inner medulla and it is absent in the terminal part of the IMCD (5).

Furthermore, on the basis of studies in X. laevis oocytes, the calculated urea permeability mediated by AQP3 was relatively small ($-5 \times 10^{-6}$ cm/s). Finally, on the basis of the perfusion studies with isolated IMCD, separate pathways were shown for urea and water transport, because the urea reflection coefficient was $\approx 1$ and because distinct phloretin sensitivities were observed for water and urea permeability (13, 25).

Recently, Kato and Sands (10) showed evidence for a secondary active, sodium-dependent urea transport in the oocytes, making it less likely that UrT1-C might be crucial for the IMCD cells to respond promptly to changes in interstitial urea concentration without many changes in cell volume, especially when vasopressin is stimulated during water restriction. Immunolocalization of UrT1-C will be the ultimate approach to confirm the basolateral localization of UrT1-C. However, these studies have been hampered due to the amino acid sequence identity of UrT1-C to UrT1-A at the NH$_2$ terminus and difficulties in raising specific antibodies against its NH$_2$ terminus. This study requires comparison of immunocytochemical staining using 1) antibodies made from the NH$_2$-terminal domain of UrT1-A that also recognize UrT1-C and 2) antibodies that recognize only UrT1-A at the COOH-terminal domain, in parallel with in situ hybridization studies using specific probes for UrT1-A and UrT1-C.

![Fig. 5. Proposed pathway of urea transport in IMCD cells. Schematic representation of the rat kidney and role of urea transporters in urinary concentration are shown. The corticomedullary osmotic gradient, which is a primary driving force for water reabsorption, is maintained mainly by the Na-K-2Cl cotransporter and urea transporters UrT1 and UrT2. Arginine vasopressin (AVP) increases the expression of aquaporin-2 (AQP2) in the early part of collecting ducts via a vesicle-trafficking mechanism, thereby permitting water entry from the luminal side to the blood. In contrast, collecting ducts (CD) in the cortex, outer medulla, and basal part of inner medulla are essentially impermeable to urea. Thus luminal urea is concentrated during water reabsorption in these segments. However, the terminal IMCD is highly permeable to urea and becomes even more permeable in the presence of AVP. When the luminal fluid reaches this segment, the bulk of urea is transported to and trapped in the medullary interstitium, thereby providing urea accumulation in the deepest part of the medulla. UrT1-A is expressed in the apical surface of terminal IMCD and involved in a vasopressin-regulated urea reabsorption. UrT1-C is expressed in the terminal IMCD and probably functions as a basolateral transporter, thereby allowing rapid transcellular transport of urea in this segment. UrT1-B is located in the late part of thin descending limb of short loop and participates in urea recycling. UrT2 is expressed in the descending vasa recta (DVR) and allows efficient countercurrent exchange between ascending vasa recta (AVR) and DVR. TDl thin descending limbs of the loop of Henle; TAL, thick ascending limb.](http://ajprenal.physiology.org/)
might involve the multiple transport steps, including apical urea uptake by UrT1-A and basolateral urea exit by UrT1-C. Further studies are necessary to clarify whether and how UrT1-C is regulated.

In summary, we have isolated and characterized an additional urea transporter isoform, UrT1-C; also known as UTA-3, from the rat kidney inner medulla. UrT1-C mRNA is exclusively expressed in the terminal IMCD, and its transport properties are consistent with those observed in perfusion studies of isolated IMCDs, i.e., in terms of phloretin sensitivities and inhibition by urea analogs. We propose that UrT1-C is the urea transporter on the basolateral side of the IMCD cells allowing basolateral exit of urea and maintenance of medullary hypertonicity that are essential to ensure maximal urinary concentration.

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Present address of C. Shayakul: Renal Unit, Dept. of Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol Univ., Bangkok, 10700 Thailand.

REFERENCES