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mRNA distribution and membrane localization of the OAT-K1 organic anion transporter in rat renal tubules

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Abstract OAT-K1, a renal organic anion transporter, which mediates methotrexate uptake, was analyzed for mRNA distribution along microdissected nephron segments and the immunolocalization in isolated plasma membranes from rat kidney. By using a reverse transcription-coupled PCR, OAT-K1 mRNA was detected predominantly in the superficial and juxtamedullary proximal straight tubules. Western blotting with antiserum for OAT-K1 revealed that the transporter protein with the apparent molecular mass of 40 kDa was expressed exclusively in the brush-border membranes from rat kidney. These findings suggest that the OAT-K1 is localized in the brush-border membranes of the renal proximal straight tubules.

Key words: Organic anion transporter; Methotrexate; Microdissection; Brush-border membrane; Rat kidney

1. Introduction

Renal tubular cells play an important role in the reabsorption and excretion of anionic endogenous substances and xenobiotics. Methotrexate, an anionic anticancer drug, is widely used at high dosage in the treatment of malignancies and acute lymphocytic leukemia [1,2], and at relatively low doses in the treatment of arthritis [3]. In humans, methotrexate is eliminated almost entirely as the unchanged form in urine, involving glomerular filtration and active tubular secretion. The tubular secretory pathway for methotrexate is considered to be relatively nonspecific for a variety of organic anions including urate, *p*-aminohippurate and probenecid [4,5].

We recently isolated cDNA encoding a rat kidney-specific organic anion transporter, designated OAT-K1 [6], which showed 72% amino acid identity with a rat liver organic anion transporting polypeptide, oatp [7]. Methotrexate and folate, but not *p*-aminohippurate and taurocholate, were accumulated by the stably transfected renal cells expressing rat OAT-K1 [6]. The uptake of methotrexate by the rat OAT-K1-expressing cells was markedly inhibited by typical substrates for renal organic anion transport systems, such as *p*-aminohippurate, probenecid, and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), although these anions were not transported by OAT-K1 [6]. Studies concerning the distribution and localization of OAT-K1 in the kidney should suggest its physiological role in renal handling of methotrexate.

In this study, we performed reverse transcription (RT) coupled PCR to detect the mRNA expression of OAT-K1 in the microdissected nephron segments, and immunoblot analysis for its localization in the isolated renal membranes. The present data suggest that the OAT-K1 is primarily localized in the brush-border membranes of the proximal straight tubules.

2. Materials and methods

2.1. Microdissection of rat nephron

Rats (80–100 g) were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally), the abdominal aorta was ligated just above and below the left renal artery, and the left kidney was perfused via the abdominal aorta with 10 ml of solution A (130 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM Ca lactate, 2 mM Na acetate, 5.5 mM D-glucose, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4) [8]. The left kidney was then perfused with 10 ml of solution B (solution A containing 1 mg/ml collagenase (type 1, Sigma Chemical Co., St. Louis, MO), 1 mg/ml bovine serum albumin (BSA) (Sigma) and 10 mM vanadyl ribonucleotide complex (VRC) (Gibco BRL, Life Technologies Inc., Gaithersburg, MD)) and removed. Kidney slices were cut along the corticomedullary axis and incubated with solution B for 30 min at 37°C under aeration with 100% O_2. After incubation, the slices were washed three times with solution A. The tubules were microdissected to obtain the following structures: glomerulus, proximal convoluted tubule, proximal straight tubule, medullary thick ascending limb, cortical thick ascending limb, cortical collecting duct, outer medullary collecting duct and inner medullary collecting duct as described previously [8] using needles under a stereomicroscope at 10°C in solution A1 (solution A containing 10 mM VRC). Most segments were dissected from superficial nephron. Only proximal straight tubule was dissected separately from superficial or juxtamedullary nephron. Late proximal straight tubule (S3) was defined as a portion within 1 mm length from the junction to thin descending limb and was taken from juxtamedullary nephron. After microdissection, 5 glomeruli and 2 mm of each dissected tubule segment were transferred into RT-PCR tubes, which contained 10 μl of ice-cold solution A2 (solution A containing 1.7% RNase inhibitor (Boehringer Mannheim GmbH, Mannheim, Germany) and 5 mM dithiothreitol (DTT)) and then centrifuged at 15000 rpm for 5 min at 4°C. Each pellet of glomeruli or tubules was used for a single determination of OAT-K1 mRNA expression.

2.2. RT-PCR

After removal of solution A2, 3.5 μl of 0.2% Triton X-100, containing >1 U/μl of RNase inhibitor and 5 mM DTT, was added to solubilize the cells. The RT was performed using a cDNA synthesis kit (Boehringer Mannheim) [9]. The synthesized cDNA was used for subsequent PCR using the PCR Master (Boehringer Mannheim) with a set of rat OAT-K1 primers for the nucleotide sequence (sense strand, 5′-GTAGACACAGGGTCTGTGAATACA-3′ (bases 664–687), antisense strand, 5′-AAGGTACTGCAGCTTGTTGGTGCA-3′ (bases 1516–1539)). The predominant PCR product was predicted to be 876 bp in length. The tubes were placed in a Programmed Tempcontrol System (Astec, Tokyo, Japan) programmed as described [6].

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Abbreviations: oatp, organic anion transporting polypeptide; PCR, polymerase chain reaction; RT, reverse transcription; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride

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2.3. PCR product analysis

After ethanol precipitation, the PCR products were separated by 2% agarose gel electrophoresis. Following electrophoresis and ethidium bromide staining, DNA bands were visualized with an ultraviolet transilluminator (Funakoshi, Tokyo, Japan). For Southern blot analysis, gels were blotted onto a nylon membrane Hybond-N^+ (Amersham International, Buckinghamshire, UK) as described [9]. Whole OAT-K1 cDNA was labeled with [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham), and prehybridization/hybridization was performed as described [6].

2.4. Polyclonal antibodies against rat OAT-K1

Polyclonal antibodies were raised against the synthetic peptide corresponding to the intracellular domains near the COOH-terminal (TDVHRNPKFKNDGEL) of rat OAT-K1. The peptide was synthesized with cysteine for its NH_2-terminal (purity of 97.8% by high performance liquid chromatography; Peptide Institute, Osaka, Japan). After obtaining pre-immune serum, rabbit antiserum was raised against the peptide conjugated to keyhole limpet hemocyanin (Calbiochem-Behring, La Jolla, CA). A male New Zealand White rabbit (2.5 kg) was immunized with 1 ml of conjugates (1 mg of the peptide) emulsified with Freund's complete adjuvant. The emulsified conjugates were injected for boosts every 2 weeks until the antibody was obtained. After each boost, blood was collected and the antibody production was determined by enzyme-linked immunosorbent assay.

2.5. Affinity purification of antibody and Western blot analysis

The antiserum used in Western blot analysis was purified by immunoadsorption on Immobilon membrane strips according to the method reported by Sambolic et al. [10] with some modifications. The synthetic antigen peptide was separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the Immobilon membrane by semi-dry electroblotting for 30 min. A horizontal strip of membrane containing the peptide was verified by Western blotting, excised, washed with Tris-buffered saline containing Tween 20 (TBS-T, 20 mM Tris/HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.5), and then incubated in the immune serum overnight at 4°C to adsorb anti-OAT-K1 antibody. The strips were then washed with TBS-T buffer, and the immunoaffinity-adsorbed antibody was released by incubation in 0.1 M citrate buffer (pH 2.0) for 1 min under constant vortexing, followed by neutralization to pH 7.4 with 1 M Tris/HCl buffer (pH 10.5). The affinity-purified anti-OAT-K1 antibody was diluted 1:50 for Western blotting.

Crude plasma membrane fractions were prepared from several tissues of rats (200–220 g) as reported previously [11]. Brush-border and basolateral membranes were purified simultaneously from rat renal cortex and medulla as described [12]. To carry out Western blot analysis, the membrane fractions were solubilized in a solubilizing buffer (2% SDS, 125 mM Tris, 20% glycerol in the absence and presence of 5% β-mercaptoethanol), and heated at 100°C for 3 min. The samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Hybond-PVDF; Amersham) by semi-dry electroblotting for 30 min. The blots were blocked and washed as described [13]. The blots were incubated with the purified antiserum preabsorbed with the synthetic antigen peptide (0.5 μg/ml) or the primary purified antibody (1:50) overnight at 4°C. The blots were washed three times with TBS-T, and the bound antibody was detected on X-ray film by enhanced chemiluminescence with a horseradish peroxidase-conjugated anti-rabbit IgG antibody and cyclic diacylhydrazides (Amersham).

2.6. Materials

HEPES, Tris and Tween 20 were purchased from Nacalai Tesque (Kyoto, Japan). DTT was obtained from Sigma. All other chemicals used for the experiments were of the highest purity available.

3. Results

With reverse transcription and subsequent PCR, two distinguishable bands, which were the predicted size of 876 bp for OAT-K1 and the smaller size of ~800 bp for an unknown product, were detected from the proximal straight tubule from superficial nephron, and the proximal straight tubule and late

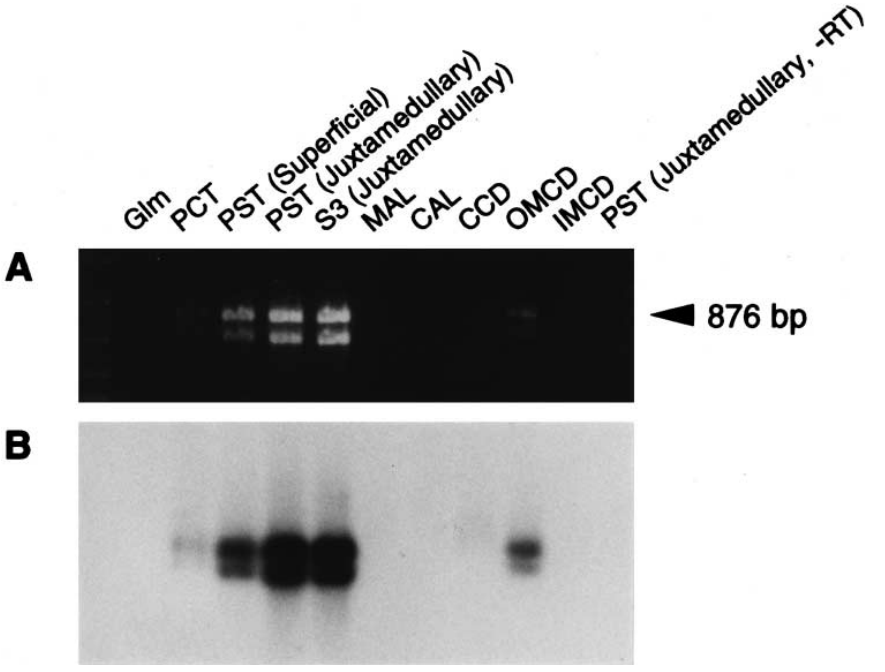

Fig. 1. Detection of OAT-K1 mRNA in microdissected renal nephron segments by RT-PCR by agarose gels (A) and autoradiograms of corresponding Southern blots (B). Each PCR amplification (30 cycles) was performed using either 5 glomeruli or a 2 mm length of renal tubule. A: After microdissection, 5 glomeruli and 2 mm of each dissected tubule segment were reverse-transcribed, and the cDNA synthesized was amplified using a set of primers for OAT-K1 as described in the text. The PCR products were separated by electrophoresis through 2% agarose gels and stained with ethidium bromide. B: The agarose gels were transferred onto a nylon membrane and hybridized with the [^{32}P]dCTP-labeled rat OAT-K1 cDNA as a probe at high stringency. Glm, glomerulus; PCT, proximal convoluted tubule; PST, proximal straight tubule; MAL, medullary thick ascending limb; CAL, cortical thick ascending limb; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct.

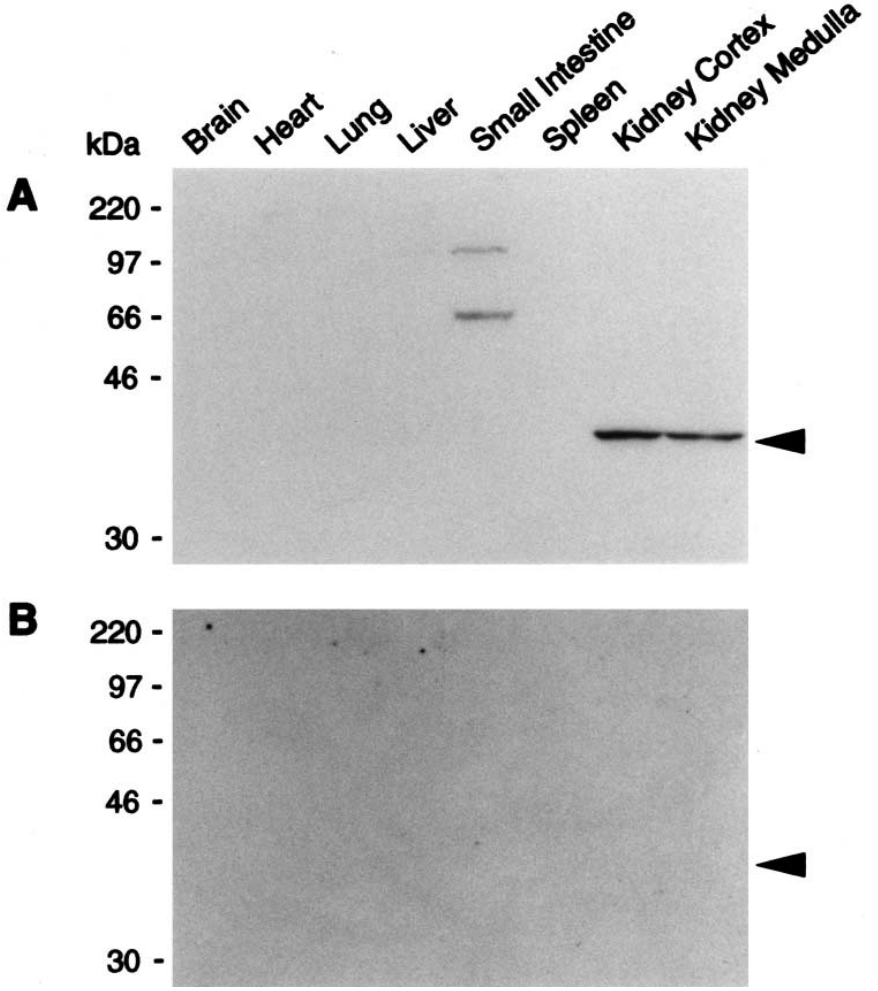

Fig. 2. Western blot analysis of rat tissue membranes with antiserum for rat OAT-K1. 50 μg of each membrane was separated on SDS-PAGE (10%) under reducing conditions and blotted on PVDF membrane. A: The affinity-purified antiserum (1:50 dilution) for rat OAT-K1 was used as primary antibody. B: The affinity-purified antiserum (1:50 dilution) preabsorbed with the antigen peptide (0.5 μg/ml) of rat OAT-K1 was used. A horseradish peroxidase-conjugated anti-rabbit IgG antibody was used for the detection of bound antibodies, and the strips of blots were visualized by chemiluminescence on X-ray film. The arrows indicate the position of OAT-K1.

proximal straight tubule (S3) from the juxtamedullary nephron (Fig. 1A). The same bands were slightly detected in the proximal convoluted tubule and the outer medullary collecting duct (Fig. 1A). When the PCR procedure was carried out in the absence of reverse transcriptase, neither band was detected from the juxtamedullary proximal straight tubule, indicating that both PCR products originated from mRNA, not from genomic DNA. The Southern blots of the gels demonstrated that the OAT-K1-specific probe hybridized to both PCR products, and there was no other detectable band (Fig. 1B).

By Western blot analysis with the affinity-purified antibody against rat OAT-K1, an immunoreactive protein with the apparent molecular mass of ~40 kDa was detected in the crude membrane preparations of renal cortex and medulla but not in those of other tissues (Fig. 2A). The immunoreactive bands were completely abolished when the antibody was preabsorbed with the antigen peptide (0.5 μg/ml) (Fig. 2B).

Next, we performed Western blot analysis of rat renal brush-border and basolateral membranes. As shown in Fig. 3A, the ~40-kDa immunoreactive proteins were detected in the brush-border but not in the basolateral membranes of both renal cortex and medulla, under reducing conditions with β-mercaptoethanol. Furthermore, under nonreducing conditions in which β-mercaptoethanol was absent in the solubilizing buffer for SDS-PAGE, the immunoreactive proteins with the same size of ~40 kDa appeared in the brush-border membranes (Fig. 3C). The ~40 kDa bands were abolished when the preabsorbed anti-serum for OAT-K1 was used (Fig. 3B,D), suggesting that the positive bands observed were specific for the antigen peptide.

4. Discussion

In humans, an administered dose of methotrexate is recovered mostly unchanged in the urine, with the renal elimination involving glomerular filtration, active tubular secretion and reabsorption. However, the renal handling mechanisms of methotrexate have not yet been elucidated. Recently, we cloned a rat kidney-specific organic anion transporter, OAT-K1, and characterized its function using the stable transfected renal epithelial cells, suggesting that OAT-K1 mediates basolateral

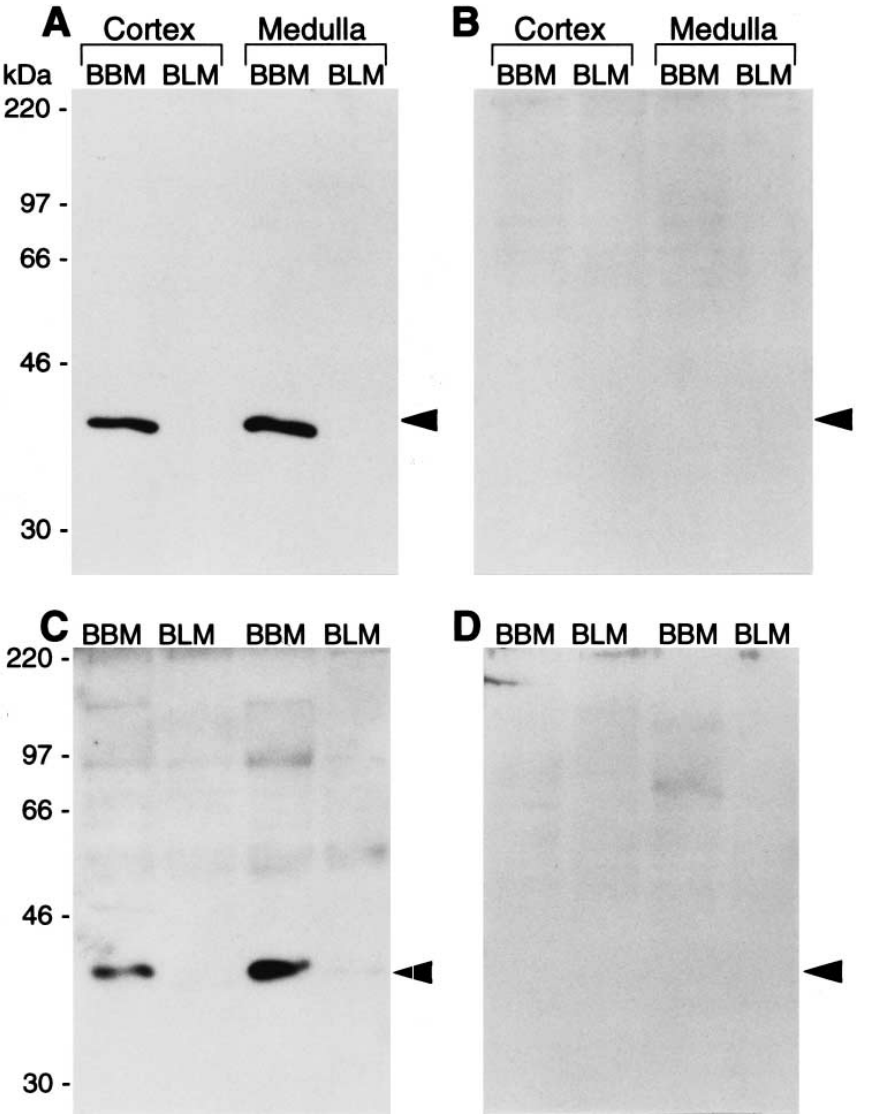

Fig. 3. Western blot analysis under both reducing (A, B) and nonreducing (C, D) conditions of brush-border and basolateral membranes purified from either rat kidney cortex or medulla with antiserum for rat OAT-K1. 50 μg of each membrane was separated on SDS-PAGE (10%) and blotted on PVDF membrane. A and C: The affinity-purified antiserum (1:50 dilution) for rat OAT-K1 was used as primary antibody. B and D: The affinity-purified antiserum (1:50 dilution) preabsorbed with the antigen peptide (0.5 μg/ml) of rat OAT-K1 was used. A horseradish peroxidase-conjugated anti-rabbit IgG antibody was used for the detection of bound antibodies, and the strips of blots were visualized by chemiluminescence on X-ray film. The arrows indicate the position of OAT-K1. BBM, brush-border membrane; BLM, basolateral membrane.

uptake of methotrexate and folate in the transfectant [6]. The tissue distribution and localization of OAT-K1 in the kidney have been remained to be identified.

RT-PCR for mRNA from the rat microdissected nephron segments with specific primers for OAT-K1 resulted in two PCR products: one with the expected length (876 bp) for OAT-K1 and another with a short length (~800 bp). Both PCR products were found in the proximal straight tubule from the superficial nephron, and the proximal straight tubule and late proximal straight tubule (S3) from the juxtamedullary nephron. The same bands were detected at a fainter level in the proximal convoluted tubule and the outer medullary collecting duct. Partial nucleotide sequencing of the shorter band indicated that the product was derived from oatp (unpublished data). Methotrexate is accumulated extensively in both the kidney cortex and medulla [6]. Therefore, we assume that OAT-K1 localized in the superficial and juxtamedullary proximal straight tubules is involved in the accumulation of methotrexate in the kidney. However, the possibility that other organic anion transporters localized in the proximal tubule participate in the renal secretion and reabsorption of methotrexate cannot be excluded.

Western blot analysis showed that an immunoreactive protein with the apparent molecular mass of 40 kDa was detected preferentially in the renal crude plasma membranes. Previously, we found that OAT-K1 mRNA was expressed exclusively in the renal cortex and medulla by both Northern blotting and RT-PCR analyses [6]. Therefore, we assumed that the 40-kDa protein was a translation product of the OAT-K1 mRNA. The apparent molecular mass of 40 kDa was relatively smaller than the calculated molecular mass of 74 kDa (without four potential sugar chains) of the OAT-K1, suggest-

ing proteolytic processing in the molecule. A similar event was reported in immunoblotting of the oatp, an organic anion transporting polypeptide expressed in both the liver and kidney, and two alternative cleavage sites have been postulated in the oatp [14]. Interestingly, the proteolytic cleavage of oatp was observed in the kidney but not in the liver, suggesting differential biosynthetic processing of oatp [14]. In the case of oatp protein detected in the kidney, two cleaved portions of oatp might be linked by a disulfide bond after the internal processing [14]. In the present study, however, Western blotting showed that the OAT-K1 transporter protein appeared as 40 kDa under both the reducing and nonreducing conditions (Fig. 1A,C). Therefore, it was suggested that the OAT-K1 transporter protein does not exist as oligomeric form linked by a disulfide bond. Nevertheless, we detected the immunoreactive protein of ~70 kDa in the transfected LLC-PK_1 cells (unpublished data). The difference in molecular weight of OAT-K1 between renal tubular cells and the transfected LLC-PK_1 cells is unknown. The physiological significance and precise mechanism for the proteolytic processing and membrane sorting of OAT-K1 should be further studied.

The transfected LLC-PK_1 cells expressing OAT-K1 showed the enhanced uptake of methotrexate from basolateral side, but not from the apical side [6]. In contrast, the OAT-K1 appeared in the brush-border membranes but not in the basolateral membranes from both the renal cortex and medulla (Fig. 3A). Under the nonreducing condition, these immunoreactive bands did not shift to a higher molecular mass compared to that observed under reducing conditions (Fig. 3C). These findings indicate that the membrane sorting mechanisms in rat kidney tubular cells differ from those in the transfected LLC-PK_1 cells. Facilitative glucose transporters (e.g. GLUT-1, -2, -3, -4, and -5) stably transfected in polarized Madin-Darby canine kidney cells have been reported to differ in membrane targeting [15]. GLUT-1 and -2 were found in the basolateral membranes, but GLUT-3 and -5 were detected in the apical membranes, and insulin-regulatable GLUT-4 was in the intracellular tubulovesicular structures. Comparison of functional characteristics of OAT-K1 in renal brush-border membranes with those in the transfected cells deserves future investigations. At present, whether OAT-K1 transporter contributes to secretion and/or reabsorption of methotrexate in renal tubules is unknown.

In conclusion, the OAT-K1 organic anion transporter is suggested to be localized to the brush-border membranes of proximal straight tubules along rat nephron.

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