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# Functional and Immunochemical Characterization of a Novel Organic Anion Transporter Oat8 (Slc22a9) in Rat Renal Collecting Duct

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**Key Words**

Collecting ducts • Drug transporter • Organic anion transport • Renal tubular epithelial cells

**Abstract**

In this study, we demonstrate that a putative membrane unknown solute transporter 1 of the rat kidney (UST1r; *Slc22a9*) is a multispecific transporter of organic anions (OAs). When expressed in *Xenopus* oocytes, UST1r mediated uptake of ochratoxin A (OTA; $K_m$ = 1.0 μM) and sulfate conjugates of steroids, such as estrone-3-sulfate (ES; $K_m$ = 3.1 μM) and dehydroepiandrosterone sulfate (DHEAS; $K_m$ = 2.1 μM) in a sodium-independent manner. We herein propose that UST1r be renamed OA transporter 8 (rOat8). rOat8 interacted with chemically heterogenous anionic compounds, such as nonsteroidal anti-inflammatory drugs, diuretics, probenecid, taurocholate, and methotrexate, but not with the organic cation tetraethylammonium. The rOat8-mediated ES transport was: a) *cis*-inhibited by 4-methylumbelliferyl sulfate and β-estradiol sulfate, but not by glucuronide conjugates of these compounds, b) *cis*-inhibited by four- and five- carbon (C4/C5) dicarboxylates (succinate and glutarate (GA)), and c) *trans*-stimulated by GA, whereas the efflux of GA was significantly *trans*-stimulated by ES. By RT-PCR, rOat8 mRNA was expressed in proximal convoluted tubules and cortical and outer medullary collecting ducts, whereas in immunochemical studies, Oat8 was identified as the ~58 kDa protein that in the collecting duct colocalized with the V-ATPase in plasma membranes and intracellular vesicles in various subtypes of intercalated cells. Molecular identification of Oat8 in these cells indicates a possible novel role of OAT family in the renal secretion/reabsorption of OA and acids and bases *via* affecting the V-ATPase-dependent functions.



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## Introduction

The kidney plays an important role in the elimination of harmful endogenous compounds and xenobiotics from the body. The proximal tubule is the primary site where numerous organic anions (OAs) are taken up from the blood and excreted into urine [1-5]. The process of secretion of OAs through proximal tubule cells is achieved via unidirectional transcellular transport involving the uptake of OAs into cells from blood across the basolateral membrane followed by extrusion across the brush-border membrane into the proximal tubule fluid. To date, cDNAs encoding the OA transporter (OAT) family have been successfully cloned, including OAT1-6 and URAT1 [6]. In humans, OAT1, OAT2, and OAT3 have been shown to be localized to the basolateral side of the proximal tubule [6, 7], whereas OAT4, Oat5, and URAT1 are localized to the apical side of the proximal tubule [6-9]. Besides in the renal secretion of OAs, OAT family is involved in the reabsorptive pathway for OA in kidney, distribution of OAs in the body, drug-drug interactions and toxicity of anionic substances such as nephrotoxic drugs and uremic toxins. [10, 11].

A search of a DNA database revealed that the amino acid sequence of an unknown putative solute transporter, UST1r (*Slc22a9*), that was isolated from a rat kidney cDNA by RT-PCR [12], exhibits 69% identity to that of rat Oat5 (rOat5; *Slc22a19*) [9, 13]. UST1r possesses twelve putative transmembrane domains with an overall topology similar to that of rOat1 and rOct1 that belong to SLC22 family [14]; however, the substrate of UST1r has not yet been identified. Based on the sequence homology with rOat5, we hypothesized that UST1r is a multispecific OAT. In this study, we demonstrated that UST1r mediates the sodium-independent, multispecific OA transport, and largely localizes to the renal collecting duct intercalated cells. We renamed this newly characterized transporter as Oat8, following the most recently reported OAT isoform, OAT7 [15].

## Materials and Methods

*Materials and animals*

[$^3$H]estrone-3-sulfate (ES) (2.0 TBq/mmol) and [$^3$H]dehydroepiandrosterone sulfate (DHEAS) (520 GBq/mmol) were purchased from Perkin Elmer Life Science Products. [$^3$H]ochratoxin A (OTA) (547.6 GBq/mmol) was purchased from Moravek (Brea, CA). [$^{14}$C]glutarate (GA) (4.07 GBq/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All other chemicals and reagents were of analytical grade and purchased from either Sigma (St. Louis, MO, USA) or Fisher Scientific (New Jersey, NJ, USA).

Three months old Wistar strain rats of both sexes were from the breeding colony at the Institute in Zagreb. The studies were approved by the Institutional Ethic Committee in Zagreb, Croatia.

*Isolation of rUST1 cDNA*

Full-length UST1r cDNA (Genbank accession number Y09945) was amplified from rat kidney mRNA by RT-PCR and was subcloned into the pcDNA3.1 vector (Invitrogen) using Hind III and Bam HI. Primers used for RT-PCR amplification are shown in Table 1. UST1r cDNA was sequenced in both directions by the dye terminator cycle sequencing method using an ABI PRISM 3100 Genetic analyzer (Applied Biosystems).

*Functional characterization in Xenopus oocytes*

cRNA synthesis and radiolabeled substrate uptake measurements were performed as described elsewhere [16]. Capped cRNA was synthesized *in vitro* using T7 RNA polymerase from plasmid DNA linearized with Bam HI. A poly(A)+ tail was added using a Poly(A) Tailing Kit (Ambion, Austin, TX). Defolliculated stage IV and stage V oocytes were injected with 25 ng of capped UST1r cRNA and incubated in modified Barth's solution (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2.4 mM $NaHCO_3$ and 10 mM HEPES, pH 7.4) containing 50 µg/ml gentamicin at 18°C. After two to three days of incubation, uptake and efflux experiments were performed at room temperature (RT) using ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4).

Kinetic parameters for the uptake of ES, DHEAS, and OTA were estimated from the following equation: $v = V_{max} \times S/(K_m + S)$, where $v$ is the rate of substrate uptake (pmol/h*oocyte), S is the substrate concentration in the medium (µM), $K_m$ is the Michaelis-Menten constant (µM), and $V_{max}$ is the maximum uptake rate (pmol/h*oocyte). These kinetic parameters were determined by the Eadie-Hofstee equation.

To examine the *trans*-stimulatory effect of both the uptake and efflux of radiolabeled substrates, 50 nl of [$^{14}$C]GA (~2 mM) or cold GA (50 mM) was injected into oocytes expressing UST1r with a fine-tipped glass micropipette as described previously [17]. The individual oocytes were then washed twice with ice-cold ND96 solution, placed on ice for 5 min, then transferred to ND96 medium with or without radiolabeled ES or cold ES, and incubated at RT for 1 h. For each data point, radioactivity was measured in both the medium and 8-10 oocytes .after a 1 h of incubation. Each experiment was performed at least twice, and the results (mean ± SEM) from representative experiments are shown in the figures.

*Antibodies*

An anti-rOat8 polyclonal immune serum was raised in rabbits against a synthetic, C-terminal peptide (amino acids 531-544: ENEGRASRQGKQND) of UST1r/rOat8 [12]. The immune serum was used as such (rOat8-IS), or the antibody was purified by an affinity column (rOat8-AP). Polyclonal antibodies for AQP2 (rabbit-raised) and V-ATPase 31 kDa

**Table 1.** PCR primers used in this study

| Construct | Directions | Sequences |
|---|---|---|
| UST1 full-length | Sense | 5'-CCCAAGCTTCCACCATGGCCTTTCAGGACCTC-3' |
| | Antisense | 5'- CGGGATCCTTAAAACTGTGTCACTTTG-3' |
| Rat Oat8 expression | Sense | 5'-GAATTCAACAGATGTCC-3' |
| | Antisense | 5'-TGGCTGGAATGGTGATTATACC-3' |

subunit (chicken-raised) were described previously [18, 19]. Secondary antibodies were purchased commercially [20, 21], and included the CY3-labeled (GARCY3), fluorescein-labeled (GARF), or alkaline phosphatase-labeled (GARAP) goat anti-rabbit IgG, and fluorescein-labeled donkey anti-chicken IgG (DACF).

*SDS-PAGE and Western blotting*

These experiments were performed with isolated total cell membranes from the pooled tissue of inner stripe and inner medulla from the rat kidney, using the protocol in our recent study of the rat renal Oat2 [21]. Briefly, after measuring their concentration by the dye-binding assay [22], the membrane proteins were denaturated at 37°C in the presence of 5% β-mercaptoethanol (β-ME), separated through 10% SDS-PAGE mini gel (80 µg/lane), and electrophoretically transferred to Immobilon (Millipore, Bedford, MA, USA). The transfer membrane was blocked in 5% non-fat dry milk, incubated in the buffer that contained rOat8-IS (1:1000) or rOat8-AP (1:100), washed, incubated in the buffer that contained GARAP, washed, and stained for alkaline phosphatase activity using the 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) method as an indicator. To block the specific labeling, the rOat8-IS or rOat8-AP was preincubated with the immunizing peptide (concentration: 0.5 mg/ml) for 4 h at RT before immunoblotting.

*Tissue fixation and immunocytochemistry*

The rat organs were fixed *in vivo* with PLP fixative (2% paraformaldehyde, 75 mM lysine, 10 mM sodium periodate), as described in details previously [18, 19, 23]. The immunostaining in 4 µm tissue cryosections was performed using the previously described experimental protocol and details for rOat2 [21], where delipidation in alcohols, microwave heating in 10 mM citrate buffer, pH 3, incubation in the rOat8-IS (1:500) at 4°C overnight, and incubation in GARCY3 at RT for 1 h, were the crucial steps.

To double stain for rOat8 and AQP2, the sections were first stained for rOat8, as described above, washed, incubated in GARCY3 at RT for 2 h in order to saturate binding sites, washed, incubated in the anti-AQP2 antibody (1:1) at RT for 2 h, washed, incubated in GARF at RT for 30 min, washed, and covered with the fluorescence fading retardant Vectashield (Vector, Burlingame, CA, USA). To double stain for rOat8 and V-ATPase, the sections were first stained for rOat8, as described above, washed, incubated in GARCY3 at RT for 1 h, washed, incubated in the anti-V-ATPase 31 kDa subunit antibody (1:20) at RT for 3 h, washed, incubated in DACF at RT for 1 h, washed, and covered with Vectashield. To block the specific staining, the rOat8-IS was preincubated with the immunizing peptide (0.5 mg/ml) for 4 h at RT, before immunostainning. The immunostaining was examined with a fluorescence microscope, photographed, and processed using the Adobe Photoshop 6.0 software [21].

Immunocytochemistry against *Xenopus laevis* oocytes injected with rOat8 cRNAs was performed as previously described [24].

*Microdissection of the rat nephron and RT-PCR*

The nephron segments of the rats were microdissected as described previously [25] to obtain the following structures: proximal convoluted tubules (PCT), medullarly and cortical thick ascending limbs (MAL and CAL, respectively), and cortical and outer medullary collecting ducts (CCD and OMCD, respectively). Two mm of each dissected tubule were used for RT-PCR, that was performed using a cDNA synthesis kit and a PCR master kit as described previously [26]. Specific primers for rOat8 are shown in Table 1. PCR products of rOat8 were 250 bp in length. The sequence of GAPDH specific primers were reported previously [27]. The cDNA PCR amplification product of GADPH was predicted to be 308 bp in length.

*Statistical analysis*

Uptake experiments were conducted three times, each time in triplicate. The data (means ± SEM) were statistically evaluated by Student's *t*-test at the 5% level of significance. The immunochemical data represent findings in tissues/isolated membranes from 3-4 animals.

## Results

*Functional characterization*

Using the *Xenopus* oocyte expression system, we investigated the transport of OAs by UST1r. Figure 1A is an immunofluorescence of rat Oat8 (rOat8) stained with anti-rOat8 antibody. Because noninjected oocyte was not stained with the same antibody (Fig. 1B), expression of rOat8 was confirmed. The uptake rates of [$^3$H]ES (Fig. 1C) and [$^3$H]OTA (Fig. 1D) in oocytes expressing UST1r were about 8-fold higher than those in control oocytes. Because we and others already identified and functionally characterized OA transporters Oat5 [9, 13], OAT6 [28, 29] and OAT7 [15], we renamed this newly functionally characterized transporter as Oat8. rOat8 did not show any uptake of PAH, urate, salicylate, prostaglandins $E_2$ and $F_2\alpha$ estradiol-17β-glucuronide and tetraethyl ammonium (TEA) (data not shown).

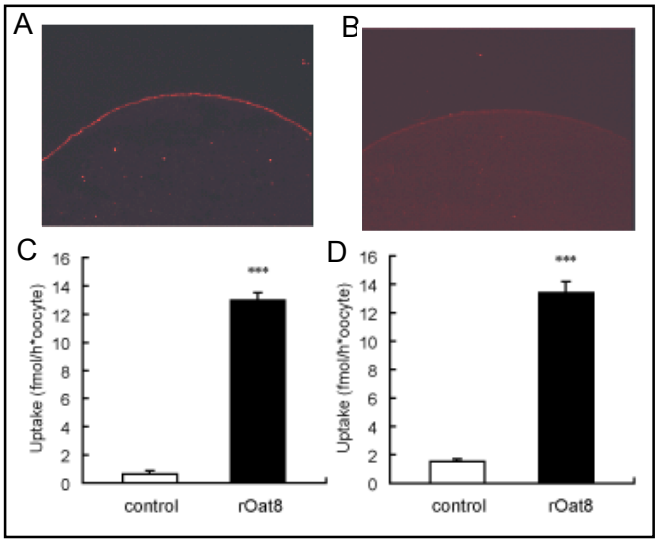


**Fig. 1.** Expression of rOat8 mRNA in Xenopus oocytes. Immunofluorescence of rat Oat8 cRNA-injected oocyte (A) and non-injected oocyte (B) using affinity-purified anti-rOatr8 antibody. Uptake of radiolabeled [$^3$H]ES (C) and [$^3$H]OTA (D) in control (open columns) and rOat8-expressing oocytes (closed columns) after 1 h-incubation at 100 nM substrates in the ambient buffer. N = 8–10. ***p<0.001.

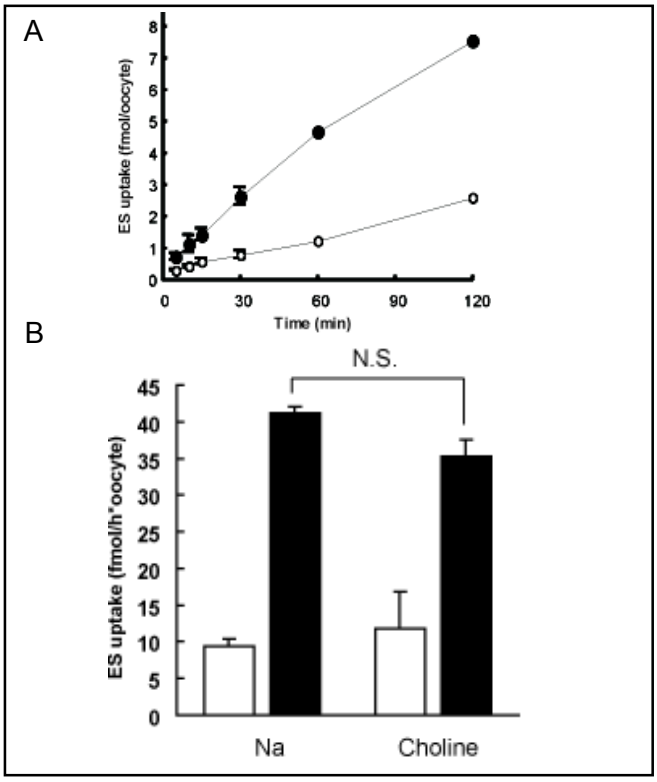


**Fig. 2.** Properties of the rOat8-mediated transport of radiolabeled ES. (A) The rates of uptake at 100 nM [$^3$H]ES in control oocytes (open circles) and rOat8-expressing oocytes (closed circles) were measured up to 2 h. (B) Effect of equimolar concentrations of extracellular cations on [$^3$H]ES uptake in control (open columns) and rOat8-expressing oocytes (closed columns) at 100 nM substrate after 1h-incubation. N = 8–10. N.S., not significant.

Figure 2 shows the properties of ES transport via rOat8. The cell-associated count of [$^3$H]ES increased linearly until 2 h after the start of incubation in rOat8-expressing oocytes, indicating that rOat8 not only binds but also translocates ES into the cytoplasm (Fig. 2A). The rate of ES uptake via rOat8 was not affected by the replacement of extracellular sodium with choline (Fig. 2B).

We then examined the concentration-dependent uptake of [$^3$H]ES, [$^3$H]DHEAS and [$^3$H]OTA via rOat8 (Fig. 3). The rOat8-mediated uptake of these three compounds manifested saturable kinetics and followed the Michaelis-Menten equation. The Eadie-Hofstee equation yielded $K_m$ values of 3.1 ± 0.9, 2.1 ± 0.2 and 1.0 ± 0.3 μM and $V_{max}$ values of 1.8 ± 0.33, 5.6 ± 1.3 and 0.918 ± 0.18 pmol/h*oocyte for ES, DHEAS and OTA, respectively, which were determined using the same batch of oocytes.

To determine the substrate selectivity of rOat8, the *cis*-inhibitory effects of various compounds at 100 μM on rOat8-mediated [$^3$H]ES (100 nM) uptake were investigated: unlabeled ES and DHEAS strongly inhibited, whereas furosemide, salicylate, probenecid, taurocholate, and methotrexate (MTX) showed a moderate inhibitory effect (Fig. 4A). Furthermore, the interaction studies with several sulfate and glucuronide conjugates showed that the unlabeled ES, DHEAS, α-naphthyl sulfate and β-estradiol sulfate strongly inhibited the rOat8-mediated uptake of [$^3$H]ES, while *p*-nitrophenyl sulfate, minoxidil sulfate, and various glucuronide conjugates,, which are the well-known substrates for organic anion-transporting polypeptides (OATPs) and multidrug resistance-associated proteins (MRPs) (Fig. 4B). Finally, to test if rOat8 functions as an organic anion/dicarboxylate exchanger similar to OAT4 [8] and Oat5 [9], and to define its counterion candidate(s), we performed inhibition experiments in which the uptake of 100 nM [$^3$H]ES was measured in the presence of various dicarboxylates (1 mM) ranging from C2 oxalate to C9 azelate [30], where C3 to C5 dicarboxylates exist as endogenous OAs in the cells. As shown in Fig. 4C, the rOat8-mediated [$^3$H]ES uptake was partially inhibited by succinate (C4) and GA (C5) and by longer dicarboxylates (C8 and C9).

We then tested the *trans*-stimulatory effects of GA and ES on the uptake and efflux of radiolabeled substrates via rOat8 after injecting the cold or hot anionic compounds

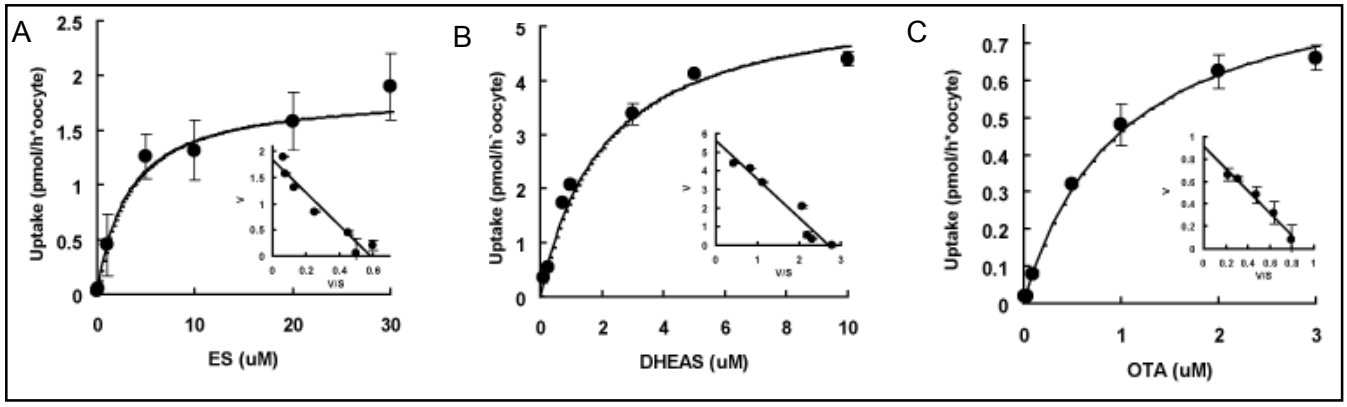


**Fig. 3.** Representative rates of the rOAT8-mediated, concentration-dependent uptake of [$^3$H]ES (A), [$^3$H]DHEAS (B), and [$^3$H]OTA (C). The rates of uptake were measured in both control and rOat8-expressing oocytes after 1 h-incubation at various concentrations of these substrates, and the rOat8-mediated transport was determined by subtracting transport velocity in control oocytes from that in rOat8-expressing oocytes. Shown are the data for one, out of three typical experiments (n = 8-10). The inset shows the Eadie-Hofstee blot of the data.

directly into oocytes as previously described [17]. The uptake of [$^3$H]ES was significantly *trans*-stimulated by unlabeled GA injected into oocytes (Fig. 5A), and the efflux of injected [$^{14}$C]GA was significantly *trans*-stimulated by unlabeled ES in the medium (0.1 mM) (Fig. 5B).

*Intrarenal localization of Oat8 in the rat*

To ascertain the specificity of anti-rOat8 antibody, total cell membranes from the renal inner stripe and inner medulla were immunoblotted with the rOat8-IS or rOat8-AP. As shown in Fig. 6A, the total immune serum (TIS) labeled several protein bands, and two of them (~50 kDa and ~58 kDa) were peptide blockable (not shown), whereas the rOat8-AP (AP) labeled a single protein band of ~58 kDa (-Pep), which was blocked by the immunizing peptide (+Pep).

Immunofluorescence staining of tissue cryosections with the rOat8-IS revealed that the staining was predominantly positive and peptide-blockable in the individual cells of terminal distal tubule, connecting segment and collecting duct in the cortex (Fig. 6B, CT), and in the collecting ducts of outer and inner stripe (not shown) and inner medulla (Fig. 6B, IM). A somehow stronger baseline fluorescence of proximal tubules, which usually occurs after this kind of antigen retrieval technique, remained even after blocking the primary antibody with the peptide, and was also present in the sections that had been incubated only with the secondary antibody or in the absence of both antibodies (not shown), thus precluding any conclusion on possible localization of the specific staining in these nephron segments.

To identify the positively-stained individual cells, we performed double immunostaining for rOat8 and AQP2. As shown in the cortical and inner medullary collecting duct (Fig. 6C), AQP2 was localized to the apical domain and intracellular vesicles in principal cells (arrows), whereas the rOat8-IS strongly stained the apical domain and intracellular vesicles of the AQP2-negative, intercalated cells (arrowhead). The vesicular nature of rOat8-positive intracellular staining in intercalated cells is clearly indicated in the enlarged images of the cells in cortical (CCD) and inner medullary collecting ducts (IMCD).

To determine subtypes of the rOat8-positive intercalated cells, we performed double staining with the antibodies for rOat8 and V-ATPase, and looked for localization of these epitopes in CCD and IMCD. As shown in Fig. 7 (Cortex, -Peptide), in CCD both stainings completely colocalized apically in A (arrowhead), basolaterally in B (arrow), and diffusely in C (double small arrows) type intercalated cells. The rOat8-immunizing peptide blocked the rOat8-related staining (Cortex, +Peptide) without affecting the V-ATPase staining. Similar data were observed in the the IMCD (Fig. 7, Inner Medulla), where in the absence of peptide rOat8 and V-ATPase colocalized in the apical membrane and intracellular organelles of the A type intercalated cells. After the rOat8-IS was inactivated by the immunizing peptide, the staining of Oat8 was removed without affecting the staining of V-ATPase.

In order to exclude the possibility that the rOat8-antibodies label the B1 and/or B2 subunits of the V-ATPase, whose molecular weight in Western blots is also 56-58 kDa, we have performed an alignment of the peptide sequence used to generate immune serum against

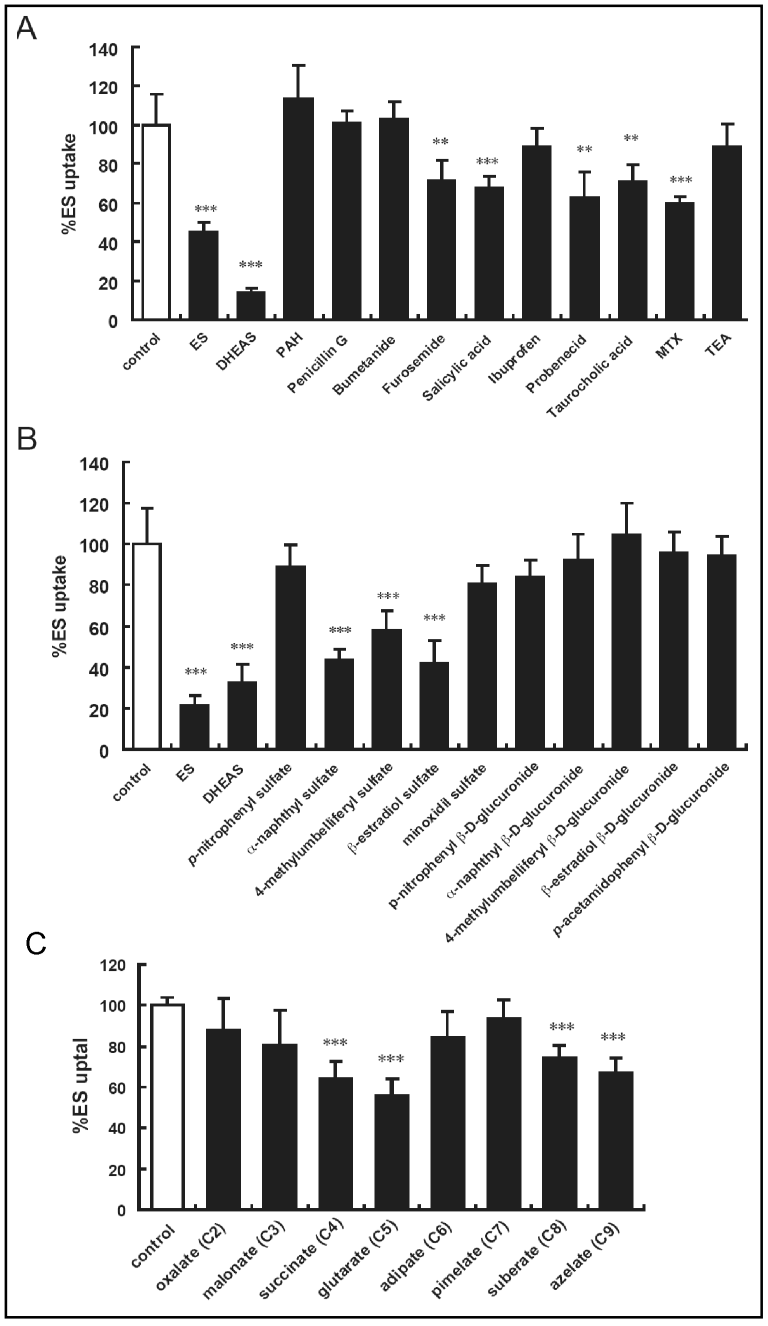


**Fig. 4.** Interaction of various non-conjugated anions (A), sulfate- and glucuronate-conjugated anions (B), and dicarboxylates (C) with the rOat8-mediated [$^3$H]ES uptake. The relative rates of [$^3$H]ES uptake (at 100 nM ES) in rOat8-expressing oocytes (subtracted for the rates in control oocytes) were determined after 1 h-incubation in the absence (open columns = 100%) and presence of 100 μM (A and B) or 1 mM (C) indicated compounds (closed columns). N = 8-10. **p<0.01; ***p<0.001.

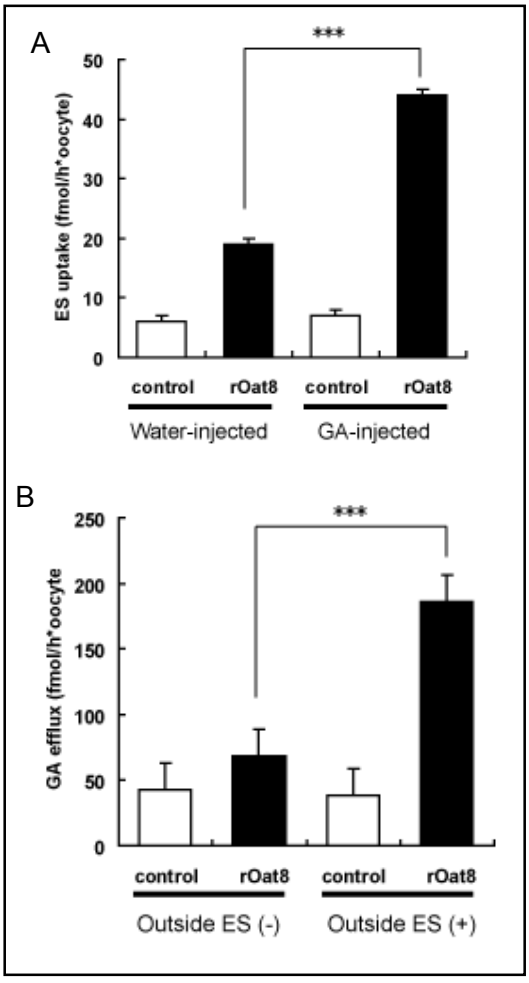


**Fig. 5.** The rOat8-mediated trans-stimulation of ES uptake (A) and glutarate efflux (B). (A) Control (open columns) and rOat8-expressing oocytes (closed columns) were injected with 50 nl water (water-injected) or 50 mM unlabeled glutarate (in 50 nl water; GA-injected), incubated for 1 h at room temperature, and then incubated for another 1 h with [$^3$H]ES (100 nM) for determination of the [$^3$H]ES uptake. (B) Control (open columns) and rOat8-expressing oocytes (closed columns) were injected with ~2 mM [$^{14}$C]glutarate (in 50 nl water), incubated at room temperature for 1 h, and then incubated for another 1 h in the buffer without (Outside ES-) or with 0.1 mM unlabeled ES (Outside ES+) for determination of the [$^{14}$C]glutarate efflux. Shown are typical, out of two separate experiments (n = 8-10). ***p<0.001.

rOat8 (UST1r in database) and of the whole UST1r protein with the amino acid sequences of B1 and B2 V-ATPase subunits. A search of the relevant database (PubMed, Protein) showed that the sequences for B1 and B2 V-ATPase subunits exist for mice but not for rats. The multiple protein sequence alignment search using

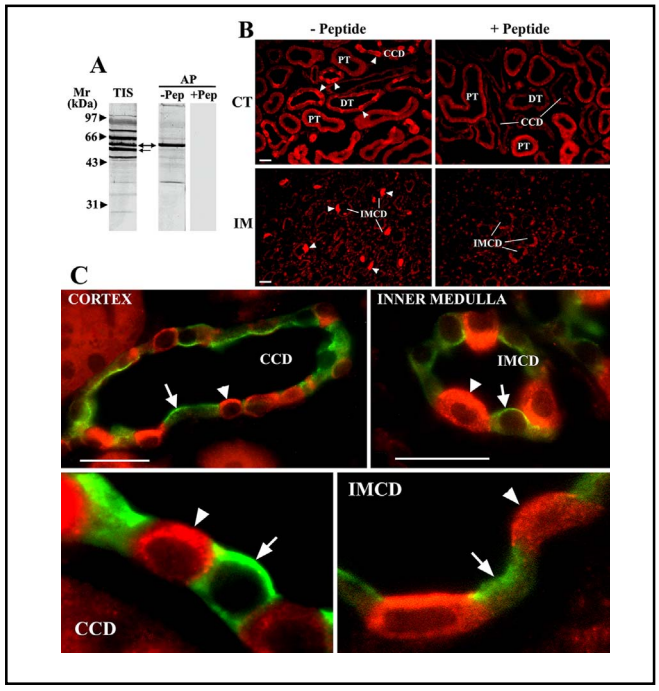


**Fig. 6.** Oat8 in the rat kidney: immunoblot of isolated total cell membranes (A) and immunolocalization along the nephron (B and C). (A) Immunoblot of total cell membranes from the inner stripe and inner medulla with the total immune serum (TIS) or affinity purified antibody (AP) that had been pretreated without (-Pep) or with (+Pep) the immunizing peptide. The TIS labeled numerous bands, of which two (~50 kDa and ~58 kDa, arrows) were blocked by the immunizing peptide (not shown), whereas the AP labeled only one band (~58 kDa) that was blocked by the peptide. (B) By immunocytochemistry, in the cortex (CT, -Peptide) the antibody strongly stained individual cells in the terminal distal tubule (DT), connecting segment and collecting duct (CCD), in the outer and inner stripe collecting duct (not shown) and the inner medullary collecting duct (IM, -Peptide, IMCD). This staining was absent after blocking the antibody with the immunizing peptide (+Peptide). The proximal tubules (PT) in the cortex exhibited a relatively high, peptide-resistant background staining. (C) By double immunostaining of the cortex and inner medulla for rOat8 and water channel AQP2, the rOat8-IS strongly stained the apical domain and intracelular vesicles in intercalated cells (red, arrowheads) whereas the adjacent, AQP2-positive principal cells were stained with green flourescence (arrows). The dot-like intracellular staining in the enlarged images of intercalated cells in CCD and OMCD clearly indicate the localization of rOat8 in intracellular vesicles/organelles (arrowhead). The pattern of Oat8 staining was similar with the affinity purified antibody, but much weaker (not shown). Bar = 20 µm.

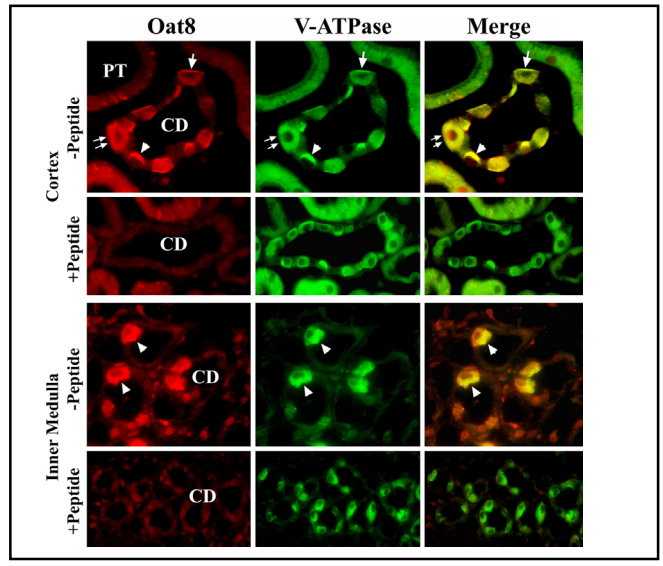


**Fig. 7.** Colocalization of Oat8 (red fluorescence) and V-ATPase (green fluorescence) in subtypes of intercalated cells in the cortical and inner medullary collecting ducts. In various types of intercalated cells of the cortical collecting duct (Cortex, -Peptide, CD), the rOat8-related immunostaining was localized apically (arrowhead), basolaterally (arrow) or diffusely (double small arrows). This pattern completely colocalized with the pattern of V-ATPase staining in the A (apical), B (basolateral), and C (diffuse) subtypes of intercalated cells, giving a yellow color in a merged image (Merge). The rOat8-immunizing peptide blocked the staining of Oat8, whereas the staining of V-ATPase remained intact (Cortex, +Peptide). In the inner medulla, the A type intercalated cells were positive for both rOat8 (-Peptide, CD, arrowheads) and V-ATPase, exhibiting a complete colocalization in the merged image. Again, the rOat8-immunizing peptide blocked the staining of Oat8, leaving the staining of V-ATPase intact (+Peptide). Bar = 20 µm.

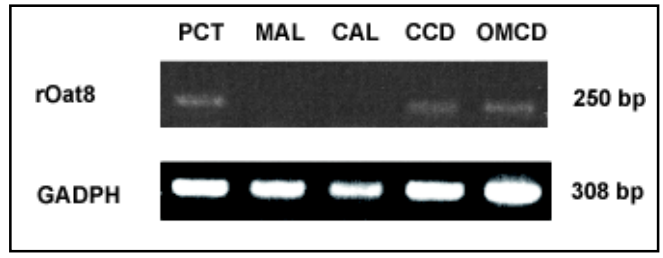


**Fig. 8.** Intranephron distribution of Oat8 in the rat kidney. rOat8 mRNA was expressed in PCT, CCD, and OMCD. PCT: proximal convoluted tubules, MAL: medullarly thick ascending limbs, CAL: cortical thick ascending limbs, CCD: cortical collecting duct, OMCD: outer medullary collecting duct.

CLUSTAL W (1.83) indicated that neither the immunizing peptide nor the whole protein of UST1r [Rattus norvegicus] (NP_620263.1) have a significant homology with the sequences of V-ATPase subunits B1 (NP_031535.2) and B2 (NP_598918.1) in mice [Mus musculus]. We therefore conclude that the rOat8 antibody, used in this study, did not crossreact with the rodent V-ATPase B1 and B2 subunits.

The observed pattern of Oat8 immunostaining along the nephron and immunoblotting in isolated membranes was comparable in male and female rats (data not shown), thus indicating an absence of gender differences in the expression of Oat8 protein, which clearly differs from the well known gender-dependent expression of rOat1, rOat3, and rOat2 [20, 21].

Finally, we performed RT-PCR analysis to determine the intranephron distributions of rOat8 mRNA. As shown in Fig. 8, rOat8 mRNA was expressed in proximal convoluted tubules, and cortical and outer medullary collecting ducts.

## Discussion

In this study, we identified the putative rat renal membrane transport protein UST1r [12] as a new organic anion transporter, rOat8 (Mr ~58 kDa), that transports ES, DHEAS and OTA. These three compounds are also substrates for some other apical OAT isoforms, e.g. , OAT4 [31, 32] and rOat5 [9]. In addition, rOat8 was identified as an organic anion/dicarboxylate exchanger, similar to OAT4 and rOat5. Since rOat8 and rOat5 exhibit a 69%-homology in their amino acid sequences, the resemblance of the transport properties between these two OATs is not surprising. Therefore, rOat8 seems to be a counterpart of the proximal tubular apical rOat5, that in the collecting ducts may be involved in the reabsorption of some OA, driven by an outwardly directed dicarboxylate gradient, as proposed for OAT4 [8]. However, although the transport properties are similar, the circumstances surrounding rOat8 and rOat5 are different due to differences in their intrarenal localization. It has been reported that the filtered α-ketoglutarate (α-KG) is abundantly reabsorbed from the proximal tubule lumen [33], and that portion of the reabsorbed α-KG may contribute to the basolateral uptake of OAs via organic anion/α-KG exchangers OAT1 and OAT3 [34]. The luminal transport process for dicarboxylates may be mediated by $Na^+$-dicarboxylate transporter NaDC1 [35], whose expression in the $S_2$ and $S_3$ segments in rats [36] resembles that of rOat5 [9]. A coordinated function of two different transporters NaDC1 and Oat5 may thus determine the net reabsorption of dicarboxylates in proximal tubules, as we proposed previously [37]. In contrast, in collecting ducts, NaDC1 expression was not detected [36], but the principal cells in these ducts in rats and mice exhibit Oat3 at their basolateral membrane [20, 38].

What could be the role of rOat8 that colocalizes with the V-ATPase in the collecting duct intercalated cell apical domain? Since in the rat collecting duct the rOat8-positive intercalated cells exhibit no overlapping expression of NaDC1 [36] and rOat3 [20], the contribution of Oat8 in the net transtubular reabsorption of dicarboxylates, similar to that in proximal tubules, seems unlikely. Furthermore, in our hands rOat8-expressing oocytes did not accumulate, among various other substances (PAH, urate, salicylate, estradiol-17β-glucuronide, TEA), $PGE_2$ and $PGF_{2\alpha}$, and are unlikely involved in the reabsorption of physiologically-important PGs in the signaling system in the distal parts of the nephron, as proposed for the mouse Oat3 [39]. However, the apical Oat8 in intercalated cells, as well as the basolateral Oat3 in principal cells may be involved in the transport pathway for sulfate conjugates that are generated by the distal tubules; although proximal tubules are thought to be more metabolically active compared to the distal tubules in conjugating various xenobiotics [40], Ng *et al.* recently reported on sulfate conjugation in, and subsequent transport of a number of sulfated conjugates out of Madin-Darby canine kidney (MDCK) type II distal tubular cells, and they proposed that the distal tubule could provide a backup system for proximal tubular functions [41]. Besides a possible role in the transport of endogenous detoxication products, recent demonstration of the functionally-important G-protein-coupled receptors (GPR) for succinate in proximal tubules (GPR91) and α-KG in distal tubules (GPR99) in the mouse kidney [42] raise a remote possibility that rOat8 participates in this specific signaling system; the same report showed that succinate increases blood pressure in mice and that the succinate-induced hypertension involves the renin-angiotensin system. These findings suggest that the level of dicarboxylates, that surrounds tubular cells and depends on the uptake via NaDCs and/or OATs, including via Oat8, may be some factor that regulates renovascular hypertension [6]. Next, having a relatively high affinity for mycotoxin OTA (1 μM), rOat8 may also function as an apical uptake pathway for OTA, as Schwerdt *et al.* reported in MDCK-C11 cells, which resembles intercalated cells of the canine collecting duct [43]. Finally, a strict colocalization of Oat8 and V-ATPase in various subtypes of intercalated cells in the respective plasma membrane domains and intracellular organelles clearly indicates that Oat8 and V-ATPase may be functionally related, possibly in regulating acidification of intracellular recycling vesicles [44] and/or secretion and reabsorption of protons and chloride by the α- (apical colocalization) and β-intercalated cells (basolateral

colocalization), respectively, in normal and deranged acid/base conditions [45-48]. Since Oat8 operates as an (electroneutral) anion exchanger, as demonstrated in this study, it is unlikely that in intracellular organelles it serves for charge compensation of the electrogenic V-ATPase, as shown for chloride and a few other inorganic anions in cortical endosomes [49], nor it could be related to the specific chloride channel isoform and water channel AQP6; the apical recycling organelles in acid-secreting α-intercalated cells contain ClC-5 and AQP6 [50], whereas the acid-reabsorbing β-intercalated cells express the kidney-specific ClC-K2 at their basolateral domain [51]. Rather, the Oat8 transporter may rather use unknown substrates that serve specific functions, common to all types of intercalated cells, that may affect/regulate the intracellular vesicle recycling via endo- and exocytosis.

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