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Identification of a Novel Organic Anion Transporter Mediating Carnitine Transport in Mouse Liver and Kidney

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Key Words
Organic anion transport • Zwitterions • Carnitine • Renal tubular epithelial cells

Abstract
This study identifies a novel organic anion transporter Oat9 expressed in mouse liver and kidney. Two variants were detected by screening a mouse liver cDNA library; these varients consist of 1815 (designated *Oat9S*) and 2165 (*Oat9L*) base pairs which encode 443 and 551 amino acid proteins, respectively. Oat9S has a predicted structure containing eight transmembrane domains (TMD); whereas, Oat9L possesses twelve TMD. Oat9 mRNA expression was detected in kidney and liver. This transporter was located at the apical side of the late portion of proximal tubules and at the sinusoidal side of hepatocytes. When expressed in *Xenopus* oocytes, Oat9S mediated the transport of L-carnitine (K*m* = 2.9 μM), a representative zwitterion, as well as cimetidine (K*m* = 16.1 μM) and salicylic acid (K*m* = 175.5 μM), while Oat9L did not show any transport activity. Oat9S-mediated L-carnitine uptake was inhibited by D-carnitine, acetylcarnitine, octanoylcarnitine, betaine, and other organic compounds, suggesting that quaternary ammonium cation bulkiness and relative hydrophobicity are important factors for Oat9S-substrate interactions. Among OATs, Oat9S appears to be the first member to mediate the transport of carnitine and possesses eight TMD. Overall, these new results provide added insight into the structure-activity relationship comprising the organic ion-permeation pathway.

Introduction

The liver and kidneys are vital organs in the elimination and detoxification of numerous endogenous and exogenous organic compounds [1-3]. Under physiological conditions, many organic molecules possess ionizable groups and thereby may exist as a cation, an anion or as a zwitterion. Therefore, ionic compound transport require specific carrier-mediated pathways. Molecular cloning techniques have been used to identify tissue specific organic ion transporters (*i.e.* SLC22 family). Organic ion transporters possess twelve membrane-spanning domains in their common

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architectures; on the basis of their structures and substrate selectivity, they are classified into several subfamilies [4, 5]. The first member, OCT1 (Organic Cation Transporter 1), was identified in 1994 via rat kidney; paralogues (OCT2 and OCT3) were subsequently isolated [6-8]. The OCT family members can transport many different cationic substrates and thereby called "polyspecific" transporters. Human OCT's (*i.e.* hOCT1, hOCT2, and hOCT3) have tissue specific expression, such as kidney, intestine, placenta and/or liver [5]. In 1997, the novel organic cation transporter (OCTN) family was identified [9-11]. OCTNs (hOCTN1 and hOCTN2) are structurally similar to OCTs and possess the ability to transport various prototypical organic cations, such as tetraethylammonium (TEA), 1-methyl-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and choline (2-hydroxy-*N*,*N*,*N*-trimethylethanaminium).

OCTN2, a member of the OCT/OCTN family, was initially reported to preferentially accept various organic cations [10]. However, subsequent studies revealed another functional feature; a representative molecule that possesses both cationic and anionic moieties (a zwitterion), OCTN2 mediates sodium-dependent L-carnitine (β-hydroxyl-γ-trimethylaminobutyric acid; **1**) transport [12-14]. Consequently, OCTN2 has also been termed "Carnitine Transporter 1" (CT1) [14]. Extracellular sodium has been reported to be crucial for L-carnitine/OCTN2 binding [15]; however, extensive mechanistic studies have not been described. Long-chain fatty acid β-oxidation and ATP biosynthesis require L-carnitine [16]. L-Carnitine's plasma level may be regulated from dietary uptake, liver biosynthesis, and kidney excretion/reabsorption [16, 17]. Ubiquitously expressed in liver, intestine and kidneys, OCTN2-mediated carnitine transport plays a pivotal role in carnitine homeostasis. OCTN2 gene mutations can cause systemic carnitine deficiency [On-Line Mendelian Inheritance in Man No. 212140] and may lead to cardiomyopathy, skeletal myopathy and hypoglycemia [18]. However, it has been shown that OCTN2 deficiency may be restored by carnitine supplementation; a result indicating that other biochemical processes can transport carnitine and circumvent dysfunctional OCTN2 [11]. In 2002, we identified a novel carnitine transporter (CT2) with an amino acid sequence distinctly different from OCT/OCTN [19]. Expressed in testis, CT2 mediates high-affinity L-carnitine transport.

Next, the Organic Anion Transporters (OATs) were discovered [20]. We, and other researchers, have illustrated that OAT family members - OAT1 [21, 22], OAT2 [23], OAT3 [24], OAT4 [25], Oat5 [26], OAT6 [27], OAT7 [28], Oat8 [29] and URAT1 (a renal urate transporter) [30] - are expressed in various tissues and mediate diverse organic anion transport. To date, OAT family members have not been reported to recognize and transport carnitine. During our search for OAT family paralogues, we isolated a novel protein expressed in mouse liver and kidney; the first member identified to mediate carnitine transport as well as certain organic substrates typically transported by OAT family members. This new transporter protein has a higher sequence homology with OATs than with OCT/OCTNs. Therefore, we propose to term this novel transporter protein as 'Oat9'. In the present study, we describe Oat9's functional characteristics and its subcellular localization in mouse tissues.

Materials and Methods

Materials

[^{3}H]L-Carnitine (80 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [^{14}C]Salicylic acid (2.0 GBq/mmol) was purchased from Dupont-New England Nuclear (Boston, MA). [^{3}H]Cimetidine (673 GBq/mmol) and [^{32}P]dCTP were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals and reagents were of analytical grade.

Isolation of mouse Oat9 cDNA clones

A mouse expressed sequence tag (EST), from mouse liver cDNA libraries (GenBank™/EBML/DDBJ accession number AI098014) showing nucleotide sequence similarity to human OAT4 [25], was obtained from the Integrated and Molecular Analysis of Genomes and their Expression (IMAGE). The [^{32}P]dCTP-labeled probe was synthesized from clone AI098014 (IMAGE clone ID 1481980) and used to screen a mouse liver cDNA library. As previously described, a nondirectional cDNA library was prepared from mouse liver poly(A)$^{+}$ RNA using the Superscript Choice System (Invitrogen); the cDNAs were ligated into λZipLox EcoRI arms (Invitrogen) [21]. As described elsewhere, the cDNA library was screened using clone AI098014 [25]. The positive clone was purified and the cDNA inserts (Oat9S and Oat9L) in λZipLox phages were recovered in the expression vector, pZL1 (Invitrogen), by *in vivo* excision.

Sequence analyses

Sequence analyses were performed by web-based database searching of the National Center for Biotechnology Information (http://www.ncbi.nim.nih.gov) and the DNASIS program (Hitachi Software Engineering). Multiple sequence alignment and topology predictions were performed using the DNASIS program. Phylogenetic analyses were performed using the CLUSTALW algorithm (http://clustalw.genome.ad.jp/) and the dendrogram was constructed using the TreeView drawtree

program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Northern blot analysis

Poly(A)$^+$ RNA (3.0 μg) prepared from various mouse tissues were used for Northern blot analysis. These RNAs were electrophoresed on a 1% agarose/formaldehyde gel and transferred onto a nitrocellulose filter. We used the Oat9 cDNA fragment shared by both Oat9S and Oat9L (corresponding to nucleotide 1 - 512 and 28 - 539 of Oat9S and Oat9L, respectively) as the probe. The filter was hybridized in a hybridization solution overnight at 42 °C with the probe, which was randomly labeled with [^{32}P]dCTP. Lastly, the filter was washed in 0.1 X SSC and 0.1% SDS at 65 °C.

Antibody preparation and immunohistochemistry

We generated a rabbit anti-Oat9 polyclonal antibody directed against a keyhole limpet hemocyanin-conjugated synthesized peptide, CKGKKEDPIIKVTRF, corresponding to the 14 amino acids of the C-terminus of Oat9 (common for both isoforms). For immunohistochemical analysis, 3-μm-thick paraffin sections of mouse kidney and liver were processed as described previously [31]. The sections were incubated (overnight at 4 °C) with the affinity-purified anti-Oat9 antibody (1:500) followed by treatment with Envision (+) rabbit peroxidase (Dako) for 30 min. To detect immunoreactivity, the sections were treated with diaminobenzidine (0.8 mM). For absorption experiments, the tissue sections were treated with the primary antibodies in the presence of antigen peptides (50 μg/ml). The sections were counterstained with hematoxylin.

Immunocytochemistry against *Xenopus* laevis oocytes injected with Oat9 cRNAs was performed as previously described [29].

Expression and transport assay

The cRNA synthesis and transport assays were performed as described previously [32]. The capped cRNAs were synthesized *in vitro* using T7 RNA polymerase (for Oat9S) or SP6 RNA polymerase (for Oat9L) from plasmid DNAs linearized with Hind III. Defolliculated *Xenopus laevis* oocytes were injected with capped cRNA (20 - 30 ng). The injected oocytes were incubated in Barth's solution (88.0 mM NaCl, 1.0 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 10.0 mM HEPES) containing gentamicin (50 μg/ml) and pyruvate (2.5 mM), pH 7.4 at 18 °C. After incubation (2 - 3 days), uptake experiments were performed at room temperature in ND96 solution (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 5.0 mM HEPES, pH 7.4).

Uptake experiments were initiated by replacing the ND96 solution with radiolabeled ligand solution; after 1.0 h, experiments were terminated by the addition of ice-cold ND96 solution. Oocytes were washed five times with ice-cold ND96 solution and solubilized with 10% SDS; the accumulated radioactivity was determined via liquid scintillation counting (LSC; LSC6100, Aloka Co., Ltd.; Tokyo, Japan). Kinetic Oat9S uptake parameters were estimated using the following equation: $v = V\text{max}\,[S]/(K\text{m} + [S])$, where v is substrate uptake rate, [S] is substrate concentration (μM) in the medium, and K_m is the Michaelis-Menten constant (μM). These values were determined via the Eadie-Hofstee equation.

In Na^+ substitution experiments, Na^+ in ND96 solution was replaced iso-osmotically with equimolar Li^+ or *N*-methyl-D-glucamine (NMDG). When pH dependency was tested, medium pH was varied (5.5 and 8.5) by adjusting the concentrations of MES, HEPES and Tris. For the inhibition studies, [^{3}H]L-carnitine (50 nM) uptake rates in noninjected oocytes and oocytes expressing Oat9S were measured (1.0 h) in the absence or presence of test compounds (50 μM) in ND96 solution. To examine Oat9S substrate recognition, as described previously [19], we synthesized and tested various carnitine-related compounds (**2A**-**6A**); (4-carboxybutyl)-trimethyl-ammonium hydrochloride (**2A**), (4-carboxybutyl)-diethylmethyl ammonium hydrochloride (**3A**), (4-carboxybutyl)-triethyl-ammonium hydrochloride (**4A**), (4-carboxybutyl)-ethyldiisopropylammonium hydrochloride (**5A**), and (4-carboxybutyl)-tripropylammonium hydrochloride (**6A**).

Statistical analysis

The experiments were performed using three different oocyte batches. The experimental results are expressed as mean ± S.E. Statistical significance was determined using Student's *t*-test. Differences were considered significant at a level of $P < 0.05$.

Results

Structural features of Oat9

Our web-based search, using a proprietary EST database, identified a clone from a mouse liver cDNA library (GenBank accession number AI098014); the clone showed partial homology with human OAT4 [25]. Using the EST clone as our probe, we isolated two positive clones by screening 240,000 plaques from a mouse liver cDNA library. The cDNA sequences of the clones showed high similarity with each other (79%) and consisted of 2165 and 1815 nucleotides encoding 551-(designated Oat9L) and 443-amino acid (Oat9S) residue proteins, respectively (Fig. 1A). The open reading frames were flanked by identical nucleotides of 5' and 3'-untranslated regions. Characteristic of the organic anion transporter family (Fig. 1A and B), Kyte-Doolittle hydropathy studies (DNASIS) predict Oat9L to have a plasma membrane protein with twelve putative membrane-spanning domains. Resulting in putative trans-membrane domain 3 - 6 loss, Oat9S showed a 351-bp in-frame deletion within the Oat9L cDNA sequence; thus, Oat9S contains only eight putative membrane-spanning domains (Fig. 1A and B). In the Oat9S amino acid sequence, five putative N-glycosylation sites were predicted in the first hydrophilic loop; there are four predicted putative protein kinase C (PKC)-dependent phosphorylation sites in the

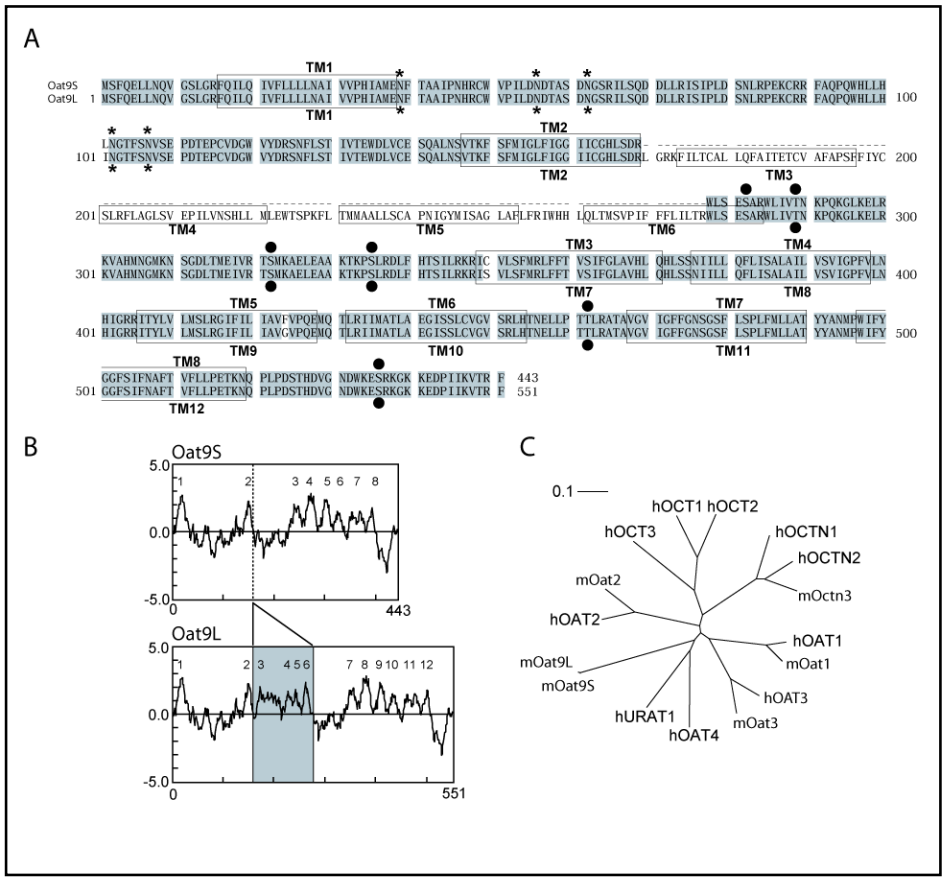

Fig. 1. Structural features of Oat9. A, mouse Oat9S and Oat9L amino acid sequences. Identical sequences are shaded. Underlines indicate the transmembrane domains in Oat9S (TM1 - TM8) and Oat9L (TM1 - TM12). Putative *N*-linked glycosylation sites (asterisks), and putative protein kinase C phosphorylation sites (closed circles) are indicated above or below the sequences. B, Oat9S and Oat9L Kyte-Doolittle hydropathy analyses (DNASIS). Predicted membrane-spanning regions are numbered. C, phylogenetic tree of organic ion transporter families. The entire peptide sequences were aligned with the CLUSTAL algorithm (gap penalty, 5; open gap cost, 10; unit gap cost, 10), and the dendrogram was constructed using TreeView software. h, m and r in the figure indicate that the transporters were isolated from human, mouse and rat, respectively. Branch lengths are proportional to the number of residue substitutions.

hydrophilic loop between the second and third transmembrane domains. In Oat9L, five putative N-glycosylation sites are predicted in the first hydrophilic loop, and three putative PKC-dependent phosphorylation sites in the hydrophilic loop between the sixth and seventh transmembrane domains (Fig. 1A). Overlapping known mammalian transporters with putative trans-membrane domains, the Oat9 N- and C-termini were likely oriented toward the cytoplasmic side. The amino acid sequence analysis showed Oat9L and Oat9S to have 41% identity to OAT1 [21], 44% to OAT4 [25], 52% to Oat5 [26], 45% to URAT1 [30], and remote similarity with OCT/OCTN family members (Fig. 1C).

Tissue distribution and localization of Oat9 expression

To elucidate Oat9 tissue-specific expression, we employed highly stringenent Northern blots; equivalent amounts of poly(A)$^+$ RNA isolated from various mouse tissues were probed with an Oat9 DNA fragment (Fig. 2). To detect the two variant transcripts, we used a fragment shared by Oat9S and Oat9L (corresponding to nucleotide 1 - 512 and 28 - 539 of Oat9S and Oat9L, respectively) as the probe. Two transcript sizes (1.8 and 2.1 kilobases) were abundantly detected in mouse kidney,

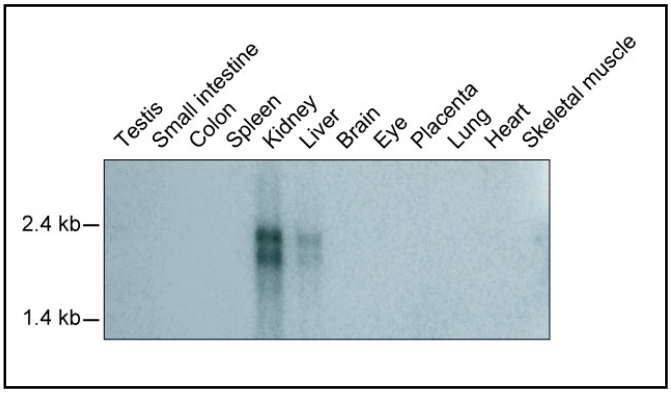

Fig. 2. Localization of Oat9 poly(A)$^+$ RNA in mouse tissues by Northern blot analysis. poly(A)$^+$ RNA (3.0 µg) from various mouse tissues were probed with the [^{32}P]-labeled Oat9 cDNA fragment. *kb*, kilobases.

and moderately detected in mouse liver. The two transcripts appear to be consistent with Oat9L and Oat9S cDNAs nucleotide length. No hybridization signals were detected from poly(A)$^+$ RNA isolated from other mouse tissues, including brain, heart, skeletal muscle, small intestine, colon, spleen, lungs, placenta, testes, and eyes (Fig. 2).

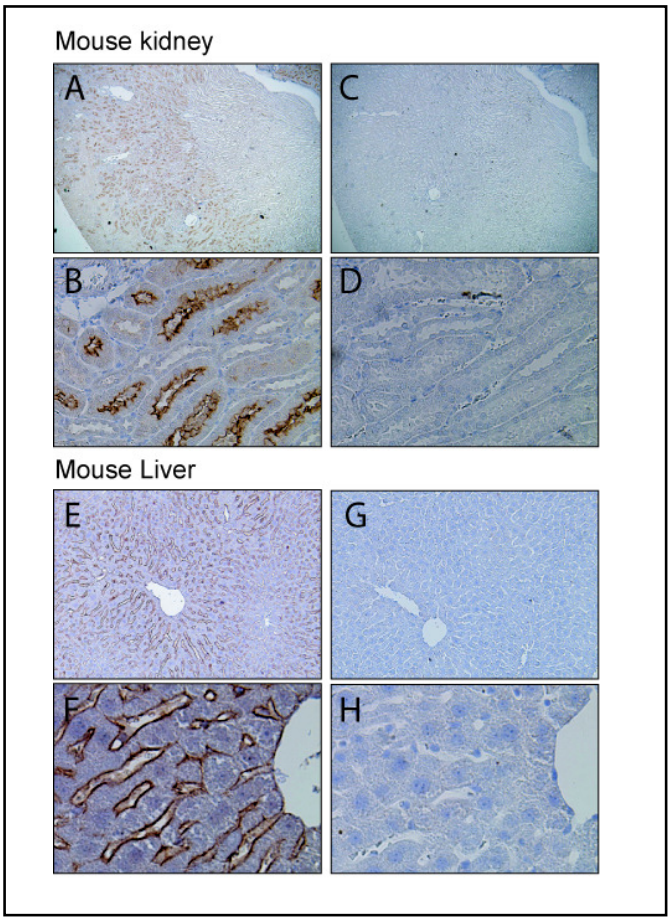

Fig. 3. Subcellular localization of Oat9 protein in mouse kidney (A - D) and liver (E - H). Immunohistochemistry results: A, Oat9 immunostaining detected in the proximal tubules of the outer stripe of outer medulla. B, high magnification view of the renal cortex. The Oat9 protein was present in the apical membrane of the proximal tubules. No positive staining was present in distal tubules. E, Oat9 protein was detected in mouse hepatocytes. F, high magnification view. The Oat9 protein was located at the plasma membrane of hepatocytes adjacent to the sinusoidal capillaries in mouse liver. C, D, G and H are immunohistochemistry results via primary antibodies preabsorbed with the Oat9 oligopeptide (50 μg/ml) and serve as negative controls. These figures are representative of typical section samples. Magnifications, X20 (A, C), X200 (B, D), X100 (E, G) and X400 (F, H).

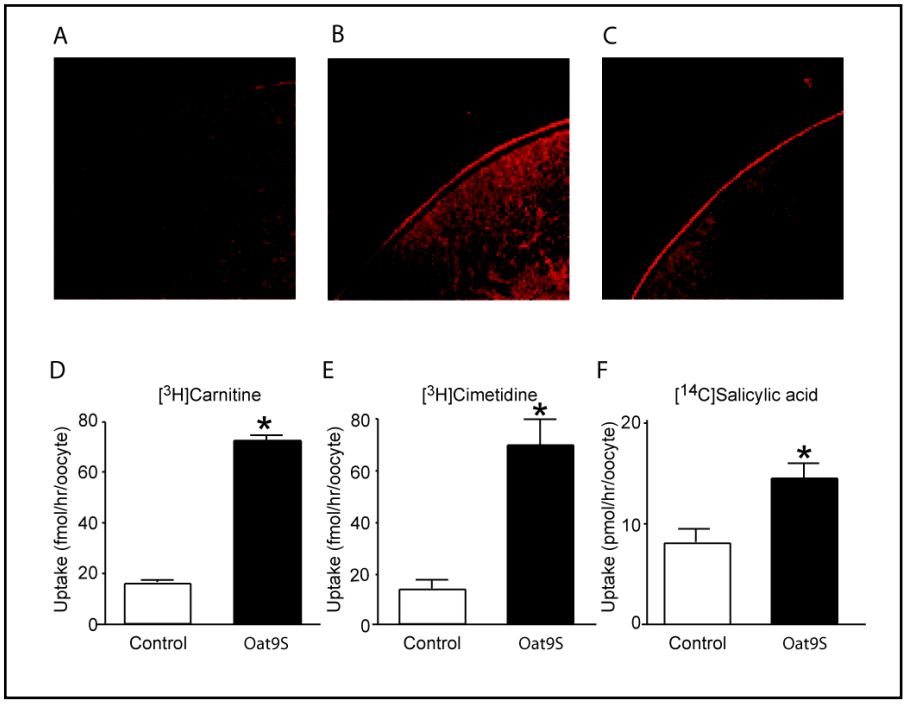

Fig. 4. Oat9S-mediated uptake of radiolabeled organic compounds. Immunofluorescence of non-injected oocyte (A), mouse Oat9S (B) and Oat9L cRNA-injected oocyte (C) using affinity-purified anti-mOat9 antibody. Radio-labeled compound uptake rates: [^{3}H]L-carnitine, 50 nM (D); [^{3}H]cimetidine, 200 nM (E); and [^{14}C]salicylic acid, 20 μM (F) in control (open columns) and Oat9S-expressing (closed columns) oocytes measured after 1.0 h incubations (mean ± S.E.; n = 8 - 10). *P < 0.05 versus control.

To determine Oat9S protein subcellular localization, we generated a rabbit polyclonal antibody directed against Oat9S's C-terminus and subsequently performed immunohistochemistry (Fig. 3) studies. In mouse kidney, Oat9S immunoreactivity was observed in the proximal tubules (S_2 and S_3 segments; Fig. 3A). Under high

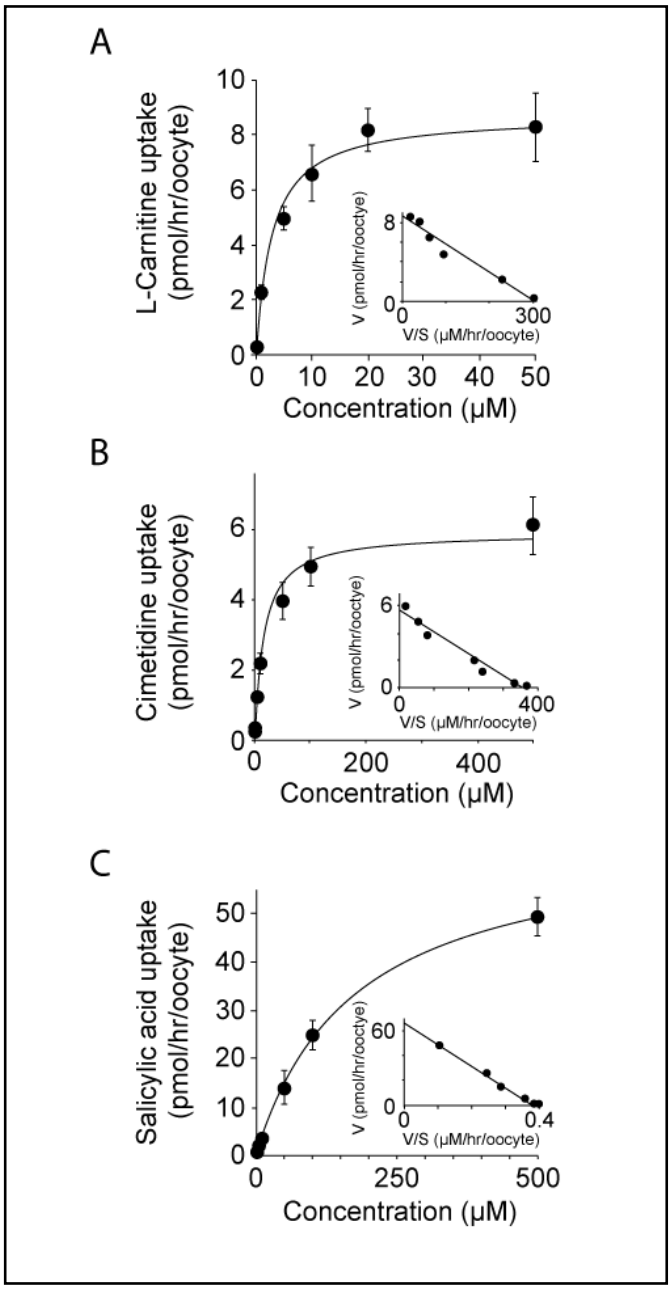

Fig. 5. Concentration-dependence of Oat9S-mediated uptake of [^{3}H]L-carnitine (A), [^{3}H]cimetidine (B), and [^{14}C]salicylic acid (C). Upate rates: [^{3}H]L-carnitine (50 nM), [^{3}H]cimetidine (200 nM), and [^{14}C]salicylic acid (20 μM) in control or Oat9S-expressing oocytes (1.0 h) measured at various concentrations (mean ± S.E.; n = 8 - 10). Oat9S-mediated transport was determined by subtracting the transport velocity in control oocytes from those via Oat9S-expressing oocytes. Insets: Eadie-Hofstee plots. V, transport velocity; V/S, velocity per concentration of substrate.

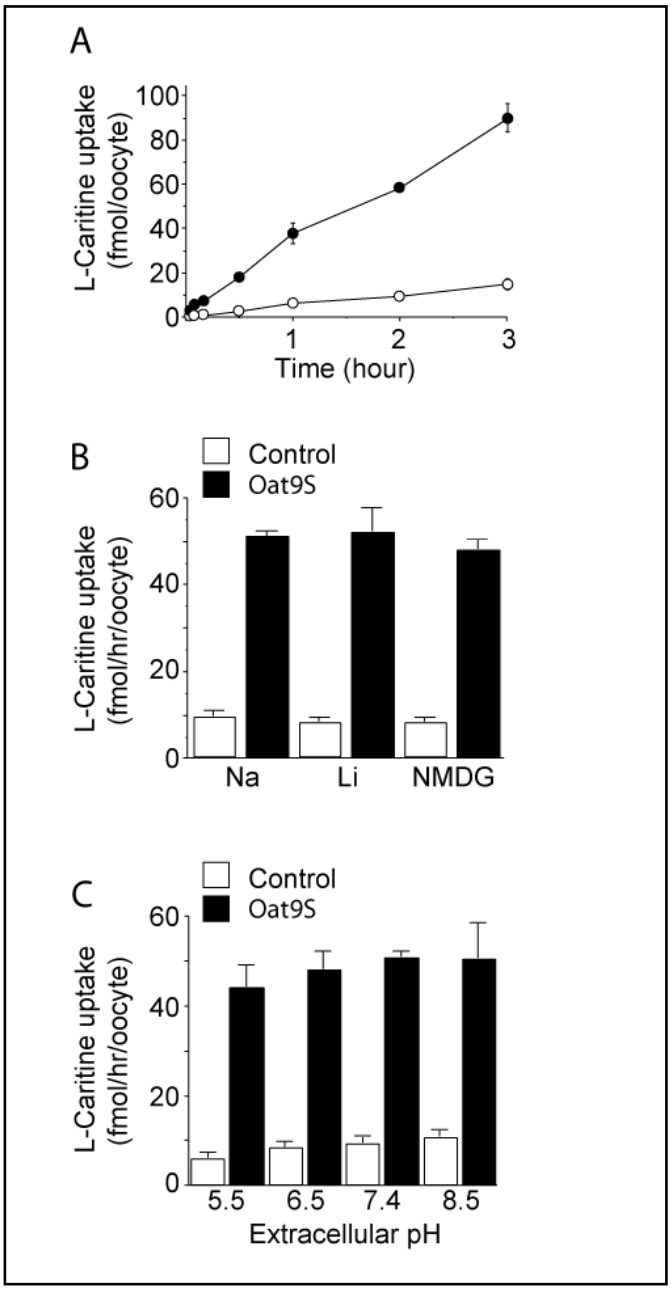

Fig. 6. Properties of L-carnitine transport via Oat9S. A, uptake of [^{3}H]L-carnitine (50 nM) in control oocytes (open circles) and Oat9S-expressing oocytes (closed circles) measured during a 3.0 h incubation (mean ± S.E.; n = 8 - 10). B, the effect of extracellular cation on [^{3}H]L-carnitine uptake in Oat9S-expressing oocytes. [^{3}H]L-carnitine (50 nM) uptake rate in control oocytes (open columns) or Oat9S-expressing oocytes (closed columns) for 1.0 h (mean ± S.E.; n = 8 - 10) in the presence or absence of extracellular Na^+. Extracellular Na^+ was replaced with equimolar lithium (Li^+) or *N*-methyl-D-glucamine (NMDG). C, extracellular pH dependence on [^{3}H]L-carnitine uptake in Oat9S-expressing oocytes. [^{3}H]L-Carnitine (50 nM) uptake rate by control oocytes (open columns) or Oat9S-expressing oocytes (closed columns) for 1.0 h was measured (mean ± S.E.; n = 8 - 10). Extracellular pH (5.5 - 8.5) was varied by adjusting the concentrations of MES, HEPES and Tris.

magnification, Oat9S immunoreactivity was observed at the apical (brush border) membrane of the proximal tubules (Fig. 3B). No immunoreactivity was observed in

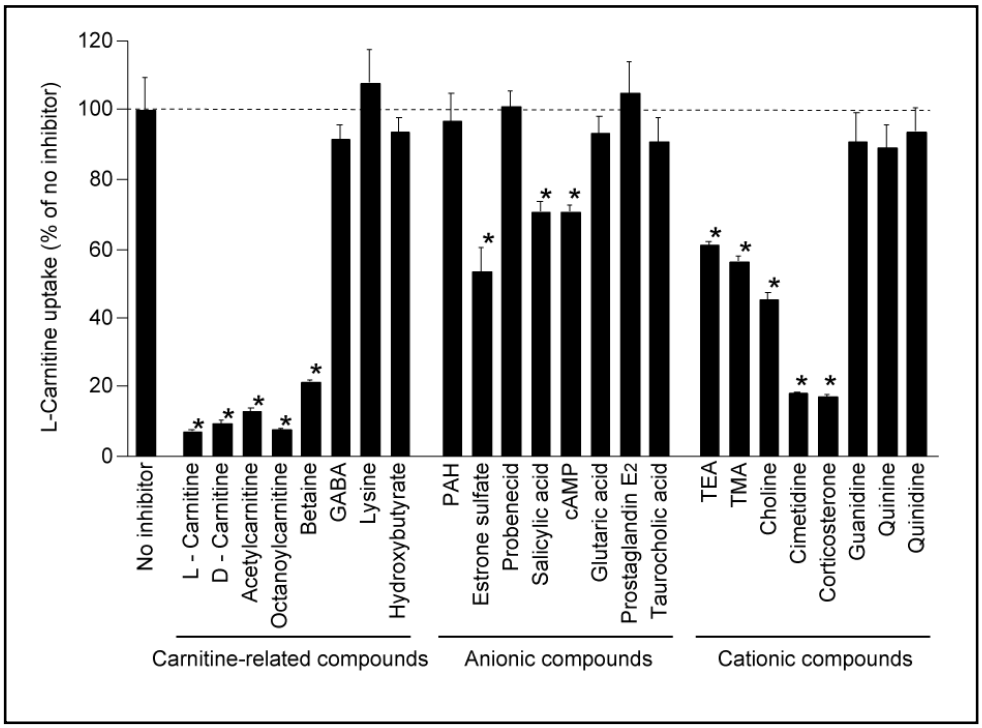

Fig. 7. Inhibition of Oat9S-mediated [^{3}H]L-carnitine uptake by various organic compounds. [^{3}H]L-Carnitine (50 nM) uptake rate in Oat9S-expressing oocytes was determined in the absence or presence of inhibitors (50 μM). Inhibitors were divided into three groups, compounds having structures related to carnitine (left), anionic compounds (middle), and cationic compounds (right). Inhibition was expressed as the percentage of uptake in Oat9S-expressing oocytes without inhibitors (mean ± S.E.; n = 8 - 10). GABA, γ-aminobutyric acid; PAH, *p*-aminohippurate; cAMP, adenosine 3', 5'-cyclic monophosphate; TEA, tetraethylammonium; TMA, tetramethylammonium. *P < 0.05 compared with no inhibitor.

the S_1 segment of proximal tubules, distal tubules, glomerulus, and arteries (data not shown). In mouse liver, Oat9S immunoreactivity was detected at the basolateral (sinusoidal) membrane but not at the canalicular membrane of hepatocytes surrounding the sinusoidal capillaries (Figs. 3E and F). Positive staining was not observed in the interlobular bile ducts nor interstitial tissues (Glisson's sheath data not shown). In the absorption experiments, where tissue sections were treated with primary antibodies in the presence of antigen peptides (50 μg/ml), immunostaining was not detected and illustrates immunoreaction specificity (Figs. 3C, D, G and H).

Functional expression in Xenopus oocytes

Using the *Xenopus* oocyte expression system, we investigated the transport function of Oat9S and Oat9L. Figs. 4B and 4C are immunofluorescence of both Oat9S and Oat9L stained with anti-mOat9 antibody. Because noninjected oocyte was not stained with the same antibody (Fig. 4A), protein expression of both Oat9S and Oat9L in the oocyte plasma membrane was confirmed. In the same system, using various radiolabeled test compounds, we successfully detected Oat9S's function; however, this was not the case with Oat9L. Compared to water injected control oocytes (Figs. 4D - F), Oat9S cRNA injected oocytes exhibited [^{3}H]L-carnitine, [^{3}H]cimetidine, and [^{14}C]salicylic acid transport. Consistent with carrier-mediated transport, Oat9S-mediated uptake by these organic ions was saturable. Next, we examined concentration dependent Oat9S-mediated [^{3}H]L-carnitine, [^{3}H]cimetidine and [^{14}C]salicylic acid uptake (Figs. 5A - C). Nonlinear regression analyses afforded Michaelis constants (K*m* values) of 2.8 ± 0.4, 16.1 ± 2.4 and 175.5 ± 14.1 μM (n = 3, means ± S.E.), respectively. Oat9S did not illustrate *p*-aminohippuric acid (PAH) (a prototypical OAT family member substrate) or tetraethylammonium (TEA) (a prototypical OCT family member substratemembers) uptake (data not shown); hence, Oat9S has unique substrate selectivity.

Historically, zwitterions (*i.e.* L-carnitine) are not considered as OAT family member substrates. However, as presented in Fig. 6A, [^{3}H]L-carnitine uptake via Oat9S increased linearly (3 h) and indicates that L-carnitine was translocated across the plasma membrane; L-carnitine was not merely non-specifically bound to the cell surface. We then compared Oat9S carnitine transport to that via carnitine transporter 2 (OCTN2) [12-14]; we also examined extracellular sodium and pH effects. Oat9S-mediated L-carnitine uptake rate was not affected by replacing extracellular sodium with lithium or *N*-methyl-D-glucamine (NMDG); results which demonstrate that Oat9S-mediated L-carnitine uptake does not require a direct sodium-carnitine cotransport and that sodium does not supply the driving force (Fig. 6B). We then analyzed [^{3}H]L-carnitine transport and its pH dependence in oocytes expressing Oat9S (Fig. 6C). Carnitine uptake rate

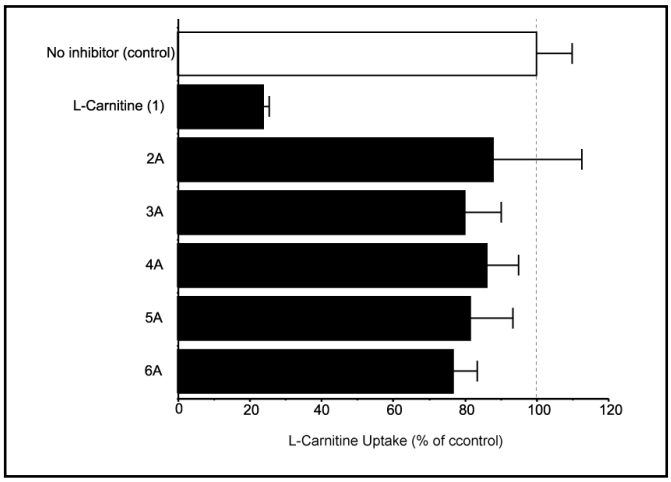

Fig. 8. Inhibition of Oat9S-mediated [^{3}H]L-carnitine uptake by various carnitine-related compounds. An Oat9S-mediated [^{3}H]L-carnitine uptake inhibition study was performed using synthesized carnitine-related compounds. The rate of [^{3}H]L-carnitine (50 nM) uptake in Oat9S-expressing oocytes was determined in the absence or presence of carnitine (1) and each synthetic compound 2A-6A (50 μM). Inhibition was expressed as the percentage of [^{3}H]L-carnitine uptake in Oat9S-expressing oocytes in the absence of inhibitor (control) (mean ± S.E.; n = 8 - 10).

via Oat9S was not altered when extracellular medium pH was changed; data which suggest that H^+ or OH^- are not involved. These features for Oat9S are clearly distinct from OCTN2/CT1 [12-14] and hCT2 [19].

Substrate selectivity of the transport

Oat9S substrate selectivity was examined; competition assays in the presence of various organic anionic, cationic, and zwitterionic compounds were performed (Fig. 7). The assay probed Oat9S-mediated [^{3}H]L-carnitine (50 nM) uptake rate and *cis*-inhibitory effects via nonradiolabeled compounds (50 μM). Carnitine-related compounds (*i.e.* D-carnitine, acetylcarnitine, octanoylcarnitine, and betaine) significantly inhibited Oat9S-mediated L-carnitine uptake. Conversely, γ-aminobutyric acid (GABA), hydroxybutyrate, and lysine did not display inhibition. Various organic anionic compounds were tested (Fig. 7) to reveal that estrone sulfate, salicylic acid, and cAMP moderately inhibited Oat9S-mediated L-carnitine uptake. However, PAH, glutaric acid, prostaglandin E_2 and taurocholic acid showed no inhibitory effects. These results are interesting because probenecid, a known organic anion inhibitor [33, 34], did not alter Oat9s-mediate L-carnitine uptake. Among the tested organic cationic compounds, cimetidine and corticosterone showed strong inhibitory effects, while TEA, tetramethylammonium (TMA) and choline showed moderate inhibitory effects. In contrast, guanidine, quinine and quinidine did not significantly inhibit Oat9S-mediated L-carnitine uptake. These results are consistent with Oat9S being classified as a unique organic ion transporter; an organic ion transporter with selectivity for specific organic anions with overlap for certain organic cations and zwitterions.

To further probe Oat9S substrate recognition, we tested modified L-carnitine compounds; compounds previously reported [19]. We examined five carnitine-related compounds (2A - 6A; 50 μM) and their ability to compete with [^{3}H]L-carnitine (50 nM) Oat9S mediated transport. As shown in Fig. 8, these five compounds, which demonstrated a strong inhibitory effect on both CT1 and CT2, did not readily inhibit Oat9S mediated L-carnitine transport. These results help to illustrate that Oat9S has distinct substrate recognition and clearly different from OCTN2/CT1 [12-14] or hCT2 [19].

Discussion

In the present study, we identify a novel OAT family member which we term 'Oat9S' (short Oat9 isoform). Expressed in liver and kidneys, Oat9S has been found to mediate the transport of several organic drugs as well as L-carnitine; the L-carnitine result suggests that Oat9S may contribute to L-carnitine homeostasis in the body.

Mitochondrial lipid metabolism requires carnitine [16]; during fasting or stressful conditions, carnitine deficiency may significant impair the body's ability to utilize fatty acids as a fuel source. In particular, cardiac and skeletal muscles utilize fatty acids as a key energy source; hence, carnitine deficiency in these tissues can lead to cardiomyopathy, muscle weakness, and/or Reye syndrome [35]. Plasma carnitine levels are regulated by the diet via extraction across the intestinal epithelial cell membrane [36, 37], via liver synthesis, and kidney excretion/reabsorption via renal tubular epithelial cells [38-40]. In our attempts to probe Oat9's physiological function, we investigated its localization by immunohistochemistry techniques; the results show Oat9 to be predominantly expressed in mouse kidney at the proximal tubules, specifically the apical membrane in the S_2 and S_3 segments (Fig. 3). The kidney localization, and overall L-carnitine transport activity by Oat9, suggest that Oat9 may participate in carnitine reabsorption via the tubular lumen. In mouse liver, Oat9 protein was located at the hepatocyte basolateral membrane along the

sinusoidal capillaries; this observation suggests that Oat9 may transport carnitine from hepatocytes into the blood and thereby help to maintain carnitine plasma levels. A speculation further supported by a physiological study which demonstrated that carnitine may be released into the medium by hepatocytes when incubated in a carnitine-free medium [41]. The importance of L-carnitine synthesis in hepatocytes has also been illustrated by another study; a study which showed that plasma carnitine levels in fasting rats initially decreased but subsequently increased above the initial levels after 3 - 4 d of fasting [42]. Also expressed in liver and kidneys, OCTN2 plays a critical role in carnitine homeostasis; carnitine deficiency patients show genetic abnormality, a result also observed in its mouse model, juvenile visceral steatosis (*jvs*) mice [18, 43, 44]. However, dietary carnitine supplementation to carnitine deficient patients may correct metabolic abnormalities and reverse skeletal and heart muscle abnormalities; an observation suggests that other carnitine transporter(s) exist in various organs, such as the liver and/or kidneys [11]. Tamai et al. reported OCTN2 apical membrane localization in mouse and rat renal tubular epithelial cells [45]. Although its precise intranephron localization was not mentioned, Oat9 may work as a supportive pathway in OCTN2-deficient animals; both transporters are expressed in the renal apical membrane. To clarify the functional difference between OCTN2 and Oat9 in liver and kidneys, establishing knockout mice with a disruption in the *Oat9* gene should be considered.

Kyte-Doolittle hydropathy plot analysis (Fig. 1) suggests that Oat9S contain eight transmembrane domains. To our knowledge, a unique feature among OAT family members. The amino acid sequence predicted for Oat9S lacks four putative membrane-spanning regions as compared to Oat9L (corresponds to third to sixth transmembrane domains of Oat9L) (Figs. 1a and b). Urakami et al. reported the splice variant of human OCT2, named hOCT2-A. It has nine transmembrane domains (lacking the three final transmembrane domains of OCT2) and shows different tissue distributions and characteristics [46]. Another report has described mammalian transporter spliced forms, such as rat kidney-specific organic anion transporter (OAT-K1 and K2) [47]. OAT-K1 and K2 were cloned from rat kidney as Oatp (organic anion transporting polypeptide) family homologues. OAT-K2, lacking the predictive four transmembrane region in OAT-K1, showed substrate selectivity similar to OAT-K1. Although the mechanisms underlying specific Oat9S substrate recognition, compared to other OAT family members, remains to be clarified, it appears that transmembrane domains 3-6 common to OAT family members are critical for organic anion selectivity.

Oat9S cRNA injected oocyte experiments demonstrate that Oat9S may interact with different organic compound types, such as L-carnitine (zwitterionic), salicylic acid (anionic), and cimetidine (cationic) (Figs. 4 and 5). Hence, regardless of the fact that it has high OAT homology, Oat9S can mediate zwitterionic, organic anionic, and even organic cationic translocation across the plasma membrane. Oat9S-mediated L-carnitine uptake was inhibited by D-carnitine, acetylcarnitine, octanoylcarnitine, and betaine (a precursor of carnitine synthesis). These compounds contain two or three carbon atoms between the carbonyl moiety and the positively charged quaternary ammonium nitrogen. GABA and lysine possess structures similar to carnitine; but these compounds do not inhibit Oat9S-mediated L-carnitine transport. Lysine has a negatively charged carboxyl group and positively charged quaternary nitrogens separated by four methylene units; at physiological pH, lysine has a formal +1 charge (cationic). GABA does not contain a permanent quaternary ammonium cation. These results suggest that Oat9S preferentially accepts zwitterions with a quaternary amine and a carboxylic acid moiety, similar to OCTN2/CT1 [12-14] and CT2 [19]. However, as shown in Fig. 7, Oat9S-mediated L-carnitine transport was inhibited by organic anions such as estrone sulfate (a steroid hormone conjugate and a prototypical substrate of OAT3, OAT4 and Oat5), salicylic acid and cAMP, or by organic cations such as TEA (a mainstream OCT substrate), TMA, choline, cimetidine and corticosterone. The fact that certain organic ions may afford moderate inhibition suggests that Oat9S's carnitine binding site has retained some organic anion transport properties. In contrast, TEA, TMA, and choline afford moderate inhibition and illustrate the cationic similarity to carnitine; that is, they are permanent quaternary ammonium cations. On the other hand, cimetidine and corticosterone, which do not possess quaternary nitrogens or carnitine-like structures, inhibit Oat9S-mediated L-carnitine uptake more so than TEA, TMA or choline. Cimetidine, a bisubstrate known to interact with both organic anion and cation transporters [48], may be recognized by Oat9S as a zwitterion. On the other hand, corticosterone does not possess a positive charge, and shown to interact with organic cation transporters [49]. Furthermore, cortisone has been reported to possess a strong inhibitory effect on hOCTN2-mediated carnitine transport [50].

These Oat9S substrate selectivity studies suggest that researchers may need to reevaluate our view

that OATs predominantly interact with anionic compounds. OCTN2 and OCTN3, members of the OCT/OCTN family, show L-carnitine transport activity [11, 12]. As Ullrich has indicated, an appropriately sized hydrophobic part and an ionic charge are necessary for substrate binding to both PAH (organic anion) and TEA (organic cation) transporters [51]. OCT's binding sites are proposed to contain negatively charged amino acid residues which accept the cationic compound positive charge; vice versa, OAT's contain positively charged amino acid residues that accept anionic compound negative charge. OCTN2 interacts with L-carnitine in the presence of extracellular Na^+, whereas Oat9S-mediated L-carnitine transport was not dependent on extracellular Na^+ (Fig. 6). It has been speculated that Na^+ helps to neutralize the negative charge within the carnitine molecule, and thereby helps the complex to become accepted by the OCTN2 binding site [15]. Distinct from OCTN2, one may propose that Oat9S recognizes carnitine as a zwitterion; Na^+ was not essential for carnitine transport. The anionic binding sites common to OAT family members are proposed to contribute to carnitine binding via Oat9S.

In summary, we have identified a new organic ion transporter expressed in liver and kidneys. The first identified OAT family member able to transport carnitine, Oat9S may also interact with organic drugs, such as salicylic acid and cimetidine. These results suggest its potential function in the handling of drugs in these organs.

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