Cloning and Expression of Na^{+}-Dependent Neutral Amino Acid Transporter from Rat Kidney with Structural Similarity to the Glutamate Transporter Gene Family.

グルタミン酸トランスポーター遺伝子ファミリーと類似の構造をもつナトリウム依存性中性アミノ酸トランスポーター遺伝子のラット腎からのクローニングと機能発現

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SUMMARY

A cDNA encoding ASCT2B was isolated from a cDNA library of rat kidney superficial cortex. The predicted amino acid sequence was 39-43% identical to the amino acid sequences of mammalian glutamate transporters, 56% identical to that of the previously cloned neutral amino acid transporter ASCT1, and 80% identical to that of ASCT2, a Na^+-dependent neutral amino acid transporter recently cloned from mouse testis. Injection of RNA transcribed from ASCT2B cDNA into *Xenopus* oocytes resulted in the expression of transport activity characterized by low-affinity L-alanine uptake with K_m of 93 μM, whereas ASCT2 is a high-affinity neutral amino acid transporter, with a K_m of around 20 μM. The ASCT2B-mediated uptake of L-alanine was completely inhibited by L-serine, L-cysteine and L-threonine, which are typical substrates of the neutral amino acid transport system ASC. Northern blot analysis revealed ubiquitous expression of this gene in various organs except testis. ASCT2B is a novel system ASC-like neutral amino acid transporter cloned from rat kidney. The renal function of neutral amino acid transport system ASC will be clarified from the studies of this transporter.

INTRODUCTION

The total concentration of free amino acids in the plasma of the rat is about 2.7 mM. If the glomerular filtration rate is assumed to be 2.2 ml/min in a 0.3 kg rat, the filtered load of all free amino acids, except taurine, is 8.5 mmol/day. On the other hand, the urinary excretion of amino acids of the rat is only 42 μmol/day[1]. Therefore, the filtered load of all free amino acids is reabsorbed completely in the kidney and only 0.5% of the filtered load is excreted into the urine.

Among the amino acids, the concentrations of aspartate and glutamate were much higher in the tubular cells than in the extracellular fluid, using renal cortex slices[2] and a specific segment of a rat nephron[3]. This suggests that transcellular movement of glutamate occures via an active transporter mechanism. From the results of free-flow micropuncture studies, L-glutamate at endogenous plasma level is nearly completely reabsorbed in the first one-third of the proximal convoluted tubule of the superfical cortex of the rat kidney[4][5]. Therefore, there should be a glutamate transporter at the brush-border membrane of the rat proximal convoluted tubule. As a glutamate transporter molecule expressed in the kidney, Kanai and Hediger[6] isolated a cDNA encoding a high-affinity glutamate transporter, EAAC1 (K_m = 12.2 ± 1.2 μM), from rabbit jejunum. EAAC1 is present not only in the brain, but also in peripheral organs

inducing the kidney, small intestine, liver and heart. By in situ hybridization, rat EAAC1 mRNA is detected in medullary ray and outer stripe of the outer medulla of the kidney[7]. Thus, there may be a novel glutamate transporter other than EAAC1 at the superficial cortex of the rat kidney. The purpose of this study was to isolate a cDNA clone encoding this glutamate transporter expressed in the superfical cortex of the rat kidney. Two more mammalian Na^+-dependent glutamate transporters, GLAST[8] and GLT-1[9], have been isolated from rat brain cDNA libraries. The amino acid sequence identities among mammalian glutamate transporters (EAAC1, GLAST, and GLT-1) range from 51% to 55%. Two neutral amino acid transporters are also structural similar to the mammalian glutamate transporters. ASCT1 cDNA, isolated from human brain, encodes an amino acid sequence 34-39% identical to glutamate transporter sequences[10]. ASCT2[11], recently cloned from a mouse testis cDNA library, showed an amino acid sequence identity of 40-44% to the mammalian glutamate transporters and 57% to ASCT1. The transporter for the purpose of this study may belong to the same gene family, and this could be isolated by the similarity of cDNA structure to the cDNAs encoding glutamate transporters. With a cDNA library constructed from rat kidney superficial cortex, PCR cloning was undertaken using the conservative sequences of the gene family.

EXPERIMENTAL PROCEDURES

cDNA cloning and sequencing

Degenerated sense primer (5′ GC(Inosine) GC(AGCT) (AG)TI TTC AT(ACT) GC(AGCT) CA 3′) and antisense primer (5′ TCC A(AGC)(AGCT) A(AGT)C CA(AG) TCI ACI GC 3′) were synthesized. The portions of these sequences are well conserved the genes encoding in the mammalian high-affinity glutamate transporters GLT-1[9], GLAST[8], and EAAC1[6], and the neutral amino acid transporter, ASCT1[10] (Figure 1). Total RNA was prepared from the superficial cortex of adult male Sprague-Dawley rat kidneys by the guanidinium thiocyanate method using cesium trifluoroacetic acid[12]. Poly(A)$^+$ RNA enriched by oligo(dT)-cellulose chromatography[13] was used to prepare the template for RT-PCR and the cDNA library. Mixed oligo(dT) and random-primed cDNA was synthesized using the Superscript Choice System (Life Technologies Inc., Gaithersburg, MD, USA) and ligated into λZIPLOX (Stratagene, La Jolla, CA, USA). Using a thermal cycler (GeneAmp PCR System 9600, Perkin-Elmer, USA), PCR amplification was conducted with degenerated primers and oligo(dT)-primed cDNA[14]. Amplification conditions were as follows: 5 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, followed by 30 cycles of 94°C for 20 sec, 55°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C of 5 min. PCR products (about 0.2 kb) were digested with Nhe I for degradation of the PCR product from rat EAAC1 cDNA. Uncut PCR products were isolated and ^{32}P-labeled for

screening the rat superficial cortex library. Conditions for hybridization and washing were as follows: the hybridization mixture contained 50 % formamide, pH 6.5, the hybridization was carried out at 37°C, and the washing was performed at 48°C in 0.1 x SSC/0.1% SDS (sodium dodecyl sulfate). The plasmid pBS(SK-)-ASCT2B, obtained by in vivo excision (Life Technologies Inc.) of the hybridizing λZIPLOX clone, contained a 2.6-kbp cDNA insert. This newly cloned cDNA insert was sequenced on both strands by the exonuclease III deletion method and dideoxy chain termination method using Sequenase 2.0 (U.S. Biochemical Corp., Cleveland, OH, USA). Synthetic oligonucleotide primers were used to complete the sequencing.

Expression of ASCT2B in Xenopus oocytes

Synthetic RNA was transcribed *in vitro* and injected into defolliculated stage V-VI *Xenopus* oocytes. Briefly, 30 μg/ml of ASCT2B cDNA was incubated with 1 U/μl of T7 polymerase and NTP mixture (0.4 mM each of ATP, UTP, and CTP and 0.04 mM GTP) at 37°C for 30 min; 0.1 mM GTP was added every 10 min. Two units per microliter of RNAsin, 30 mM DTT and 10 mU/μl of the cap analogue, $m^7G(5')ppp(5')G$, were mixed. After the transcription, template cDNA was digested by 20-min incubation with 0.4 U/μl of RNase-free DNase. Transcribed RNA was purified by phenol/chloroform extraction and twice ethanol precipitation. Denatured RNA was injected into the oocytes on the yellow side just above the meniscus.

After 3-day incubation, transport was assayed by measuring

uptake of [^{14}C]-alanine and [^{14}C]-glutamic acid (Du pont - New England Nuclear Research Products) in Na^+ uptake solution (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$, 10 mM HEPES, 5 mM Tris, pH 7.5) and by two-electrode voltage-clamp recording. Briefly, oocytes were voltage-clamped at -60 mV and continuously superfused with a buffer (ND-96) consisting of 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES pH 7.5. Uptake (V) as a function of substrate concentration (S) was fitted by a least squares method to $V=Vmax[S]/(Km+[S])$, where *Vmax* is the maximal uptake rate and *Km* is the transport constant. *Km* and *Vmax* were determined by fitting the results from individual oocytes in which five or more different concentrations were applied. Data were averaged from all oocytes tested and were expressed as mean ± S.E.

Northern blot analysis.

For northern blot analysis, total RNAs were prepared from various tissues of adult male Sprague-Dawley rats by the guanidinium isothiocyanate method using cesium trifluoroacetic acid. Three micrograms of poly(A)$^+$ RNAs were enriched from total RNA by oligo (dT)-cellulose chromatography, size-fractionated on denaturing formaldehyde gels and transferred to a nitrocellulose membrane. The 2.7-kbp sequence encoding ASCT2B was radiolabeled with α-[^{32}P]-dCTP (Du pont - New England Nuclear Research Products) by random priming method[15] (Pharmacia Biotech.). The filter was hybridized overnight

at 42°C with this cDNA probe in hybridization solution (5 x SSC, 50% formamide, 7.5 x Denhardt's solution, 2% SDS and 100 μg/ml denatured salmon sperm DNA). Autoradiography was performed after a 50-min wash at room temperature, a 10-min wash at 65°C in 5 x SSC/0.1% SDS/0.05% sarcosyl and a 10-min wash in 0.1 x SSC/0.1% SDS at 65°C.

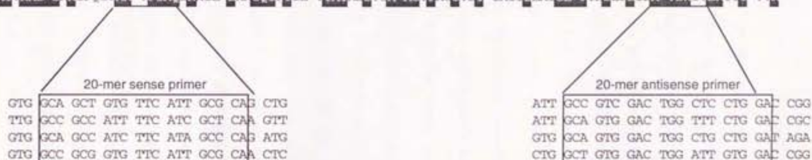

Figure 1. **Primers designed for the PCR cloning from the conserved region of the genes encoding the glutamate transporters, EAAC1, GLAST and GLT-1, and the neutral amino acid transporter ASCT1.** Frames enclose the nucleotide sequences corresponding to the degenerated primers.

RESULTS

Cloning and structural properties of ASCT2B

One novel cDNA clone was isolated by screening 600,000 plaques from a rat kidney superficial cortex cDNA library. Nucleotide sequence analysis of this 2564-bp clone revealed an apparent open reading frame of 557 amino acids and corresponding to a protein of ~59kDa, flanked by 414 bp of 5'-untranslated sequence and 476 bp of 3'-untranslated sequence (Figure 2). Although it does not match the consensus initiation sequence [GCC(A/G)CCAUGG][16], the first ATG present in the cDNA was assigned as the initiation codon on the basis of it being located 54 base pairs downstream of an in-frame stop codon. The 3'-untranslated region of this clone ends in a poly(A)$^+$ tail (12 A's). A classical polyadenylation signal, the canonical AATAAA is located 20 bp upstream of the start of the poly(A)$^+$ tail. I termed this sequence ASCT2B (Alanine, Serine and Cysteine Transporter 2B) because of the protein's functional properties of neutral amino acid transport (see below).

The ASCT2B amino acid sequence shown in Figure 3 exhibits remarkable similarity to amino acid sequences reported for various glutamate transporter subtypes and neutral amino acid transporters. The amino acid residues indicated by white letters on a black background are identical to those of ASCT2. ASCT2B has an amino acid sequence identity of 43% to rat EAAC1, 41% to rat GLAST, 39% to rat GLT-1, 57% to human ASCT1[10] and 80% to mouse ASCT2[11]. All sequences exhibit unrelated NH_2 and COOH termini, and diversity in a putative

extracellular domain containing conserved potential N-linked glycosylation sites. Figure 4 shows results of Kyte-Doolittle hydropathy analysis[17] of the putative proteins when the value of the window for calculation was 15 amino acid residues. The transmembrane topology of this transporter family is currently unknown; many hydrophobic regions have been predicted based on result of sequence hydropathy analysis: six membrane-spanning domains in the NH_2-terminal portion of ASCT2B, consistent with domains proposed for the glutamate transporters, and four in the COOH-terminal portion. Prediction of the secondary structure of ASCT2B by the procedure of Chou and Fasman[18] indicated that distinct hydrophobic domains 1-6 contain α-helical and/or β-sheet structures, whereas random structures are more predominant in the hydrophobic domains 7-10 except an α-helical structure predicted at the end of the hydrophobic stretch (data not shown). The sites for N-linked glycosylation are present not only on a presumably extracellular 33-amino acid hydrophilic loop between putative transmembrane domains 3 and 4 (ASCT2B residues Asn^{164} and Asn^{215}), but also on the COOH-terminus (ASCT2B residue Asn^{536}). A number of consensus sequences for protein kinase C mediated phosphorylation are also present (ASCT2B residues Ser^{41}, Thr^{327}, and Ser^{504}).

Figure 2. **Nucleotide sequence of cDNA and deduced amino acid sequence of the Na⁺-dependent neutral amino acid transporter ASCT2B.** Putative transmembrane domains, potential N-linked glycosylation sites predicted by N-(X)-T/S, and polyadenylation sequences (AATAAA) are underlined. See text for details.

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rASCT2B   1 MAVDPPKADP KGVVAVD--- --PTANCGSG LRSREDQSAK AGGCCGSRDQ VRRCLRANLL VLLTVAAVVA GVALGLGVSA AGGADALGPA RFTRFAFPGE
mASCT2    1 MAVDPPKADP KG-VAVDSSR RCPAL-GS-- -R--EDQSAK AGGCCTSRDR VRRCIRANLL VLLTVAAVVA GVVLGLGVSA AGGADALGPA RLTRFAFPGE
hASCT1    1 ---------- ------MEKS NETNGYLDSA QAGPAAGPGA PGTAAGRARR CARFLRRQAL VLLTVSGVLA GAGLGAALR- ---GLSLSRT QVTYLAFPGE
rEAAC1    1 ---------- ---------- ---------- ---------- MGKPTSSGCD WRRFLRNHWL LLSTVAAVVL GIVVGVLVRG ---HSELSNL DKFYFAFPGE
rGLT1     1 ---------- ----MASTEG ANNMPKQVEV RMHDSHLSSE EPKHRNLGMR MCDKLGKNLL LSLTVFGVIL GAVCGGLLRL MA---PIHPD VVMLIAFPGD
rGLAST    1 -------MT KSNGEEPRMG SRMERFQQGV RKRTLLAKKK VQNITKEDVK SYLFRNAFVL L--TVSAVIV GTILGFALRP Y----KMSYR EVKYFSFPGE

         96 LLLRLLKMII LPLVVCSLIG GAASLDPSAL GRLGAWALLF FLVTTLLSSA LGVALALALK PGAAFAAI-- -NSSVVDSSV HR-APTKEVL DSFLDLARNM
         94 LLLRLLKMII LPLVVCSLIG GAASLDPSAL GRVGAWALLF FLVTTLLASA LGVGLALALK PGAAVTAITS INDSVVDPCA -RSAPTKEAL DSFLDLARNI
         81 MLLRMLRMII LPLVVCSLVS GAASLDASCL GRLGGIRVAY FGLTTLSASA LAVALAFIIK PGSGAQTLQS SDLGLEDSGP PP-VP-KETV DSFLDLARNL
         58 ILMRMLKLVI LPLIISSMIT GVAALDSNVS GKIGLRAVVY YFSTTVIAVI LGIVLVVSIK PGVTQKVNEI NRTGK-TP-- -----EVSTV DAMLDLIRNM
         84 ILMRMLKMLI LPLIISSLIT GLSGLDAKAS GRLGTRAMVY YMSTTIIAAV LGVILVLAIH PGNPKLKKQL -GPGKKND-- -----EVSSL DAFLDLIRNL
         87 LLMRMLQMLV LPLIISSLVT GMAALDSKAS GKMGMRAVVY YMTTTIIAVV IGIIIVIIIH PGKGTKENMY -REGK----- ---IVQVTAA DAFLDLIRNM

        192 FPSNLVSASA AFRI------ PCGA-C--PQ ----RS-NAT ---------M DQPHCEMK-- ---MNILGLV VFAIVFGVAL RKLGPEGELL IRFFNSFNDA
        193 FPSNLVSA-- AFRSFATSYE PKDNSCKIPQ SCIQREINST ---------M VQLLCEVE-- --GMNILGLV VFAIVFGVAL RKLGPEGELL IRFFNSFNDA
        179 FPSNLVVA-- AFRTYATDYK VV-------- TQNSSSGNVT --------HE KIPIGTEIE- --GMNILGLV LFALVLGVAL KKLGSEGEDL IRFFNSLNEA
        150 LGENLVQACF QQYKTKREEV KPASDPGGNQ TEVSVTTAMT TMSENKTKEY KIVGLYSD-- --GINVLGLI IFCLVFGLVI GKMGEKGQIL VDFFNALSDA
        176 FPENLVQACF QQIQTVTKKV LVAPPSEEAN TTKAVISLLN ETMNEAPEET KIVIKKGLEF KDGMNVLGLI GFFIAFGIAM GKMGVAGQAD GGVLQHSERD
        178 FPPNLVEACF KQFKTSYEKR SPKVPIQANE TLLGAVINNV SEAMETLTRI REEMVPVPGS VNGVNALGLV VFSMCFGFVI GNMKEQGQAL REFFDSLNEA

        264 TMVLVSWIMW YAPVGILFLV AGKIVEMKDI RQLFISLGKY IVCCLLGHAI HGLLVLPLIY FLFTRKNPYR FLWGIVTPLA TAFGTSSSSA TLPLMMKCVE
        278 TMVLVSWIMW YAPVGILFLV ASKIVEMKDV RQLFISLGKY ILCCLLGHAI HGLLVLPLIY FLFTRKNPYR FLWGIVTPLA TAFGTSSSSA TLPLMMKCVE
        258 TMVLVSWIMW YAPVGIMFLV GSKIVEMKDI IVLVTSLGKY IFASILGHVI HGGIVLPLIY FVFTRKNPFR FLLGLLAPFA TAFATCSSSA TLPSMMKCIE
        246 TMKIVQIIMC YMPIGILFLI AGKIIEVEDW EIF-RKLGLY MATVLSGLAI HSLIVLPLIY FIVVRKNPFR FALGMAQALL TALMISSSSA TLPVTFRCAE
        276 CHEVSDHDHV VFPAGIHCLI CGKIIAIKDL EVVARQLGMY MITVIVGLII HGGIFLPLIY FVVTRKNPFS FFAGIFQAWI TALGTASSAG TLPVTFRCLE
        278 IMRLVAVIMW YAPLGILFLI AGKILEMEDM GVIGGQLAMY TVTVIVGLLI HAVIVLPLLY FLVTRKNPWV FIGGLLQALI TALGTSSSSA TLPITFKCLE

        364 EKNGVAKHIS RFILPIGATV NMDGAALFQC VAAVFIAQLN GMSLDFVKII TILVTATASS VGAAGIPAGG VLTLAIILEA ISLPVKDISL ILAVDWLVDR
        378 EKNGVAKHIS RFILPIGATV NMDGAALFQC VAAVFIAQLN GMSLDFVKII TILVTATASS VGAAGIPAGG VLTLAIILEA VSLPVKDISL ILAVDWLVDR
        358 ENNGVDKRIS RFILPIGATV NMDGAALFQC VAAVFIAQLN NIELNAGQIF TILVTATASS VGAAGVPAGG VLTIAIILEA IGLPTHDLPL ILAVDWIVDR
        345 EKNRVDKRIT RFVLPVGATI NMDGTALYEA VAAVFIAQLN GMDLSIGQII TISITATAAS IGAAGVPQAG LVTMVIVLSA VGLPAEDVTL IIAVDWLLDR
        376 DNLGIDKRVT RFVLPVGATI NMDGTALYEA VAAIFIAQMN GVILDGGQIV TVSLTATLAS IGAASIPSAG LVTMLLILTA VGLPTEDISL LVAVDWLLDR
        378 ENNGVDKRIT RFVLPVGATI NMDGTALYEA LAAIFIAQVN NFDLNFGQII TISITATAAS IGAAGIPQAG LVTMVIVLTS VGLPTDDITL IIAVDWFLDR

        464 SCTVLNVEGD AFGAGLLQSY VDRTKMPSSE PELIQVKNEV SLNPLPLATE EGNPLLKQCR EPSGTPVPHA KRNLSCECLE GFYALVTSLW KLGS...... 557
        478 SCTVLNVEGD AFGAGLLQSY VDRTKMPSSE PELIQVKNEV SLNPLPLATE EGNPLLKQYQ GPTGDSSATF -E-KESVM.. .......... .......... 553
        458 TTTVVNVEGD ALGAGILHHL NQKATKKG-E QELAEVKVEA I--PNCKSEE ETSPLVTHQN PAGPVASAPE LESKESVL.. .......... .......... 532
        445 FRTMVNVLGD AFGTGIVEKL --------SK KELEQVDVSS EVNIVNPFAL EPTILDNEDS DTKKSYVNGG FSVDKSDTIS FTQTSQF... .......... 523
        476 MRTSVNVVGD SFGAGIVYHL --------SK SELDTIDSQH RMHEDIEMTK TQSVYDDTKN HRESNSNQCV YAAHNSVVID ECKVTLAANG KSADCSVEEE PWKREK 573
        479 LRTTTNVLGD SLGAGIVEHL --------SR HELKNRDVEM GNSVIEENEM KKPYQLIAQD NEPEKPVADS ETKM...... .......... .......... 543
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Figure 3. **Multiple sequence alignment depicting relationships among the primary structure of glutamate transporters and neutral amino acid transporters.** The amino acid residues indicated by white letters on a black background are conserved between rat ASCT2B and mouse ASCT2.

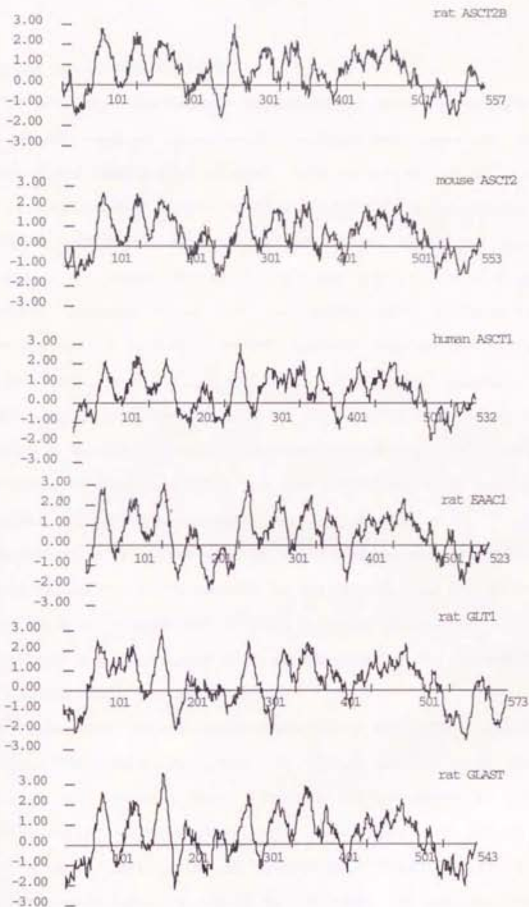


Figure 4. **Kyte-Doolittle hydropathy plots for glutamate transporters, neutral amino acid transporters and ASCT2B.**

Transport properties of ASCT2B

The sequence similarity of ASCT2B to known glutamate transporters and neutral amino acid transporters suggested that this protein might transport L-glutamic acid or neutral amino acids. The rate of radiolabeled L-alanine uptake into ASCT2B-expressing oocytes was 121.8 ± 18.8 $pmol \cdot hr^{-1} \cdot oocyte^{-1}$ at 100 μM, while the corresponding value for water-injected controls was 20.2 ± 2.0 $pmol \cdot hr^{-1} \cdot oocyte^{-1}$. Removal of Na^{+} from the uptake buffer reduced the basal L-alanine uptake by 90% in water-injected oocytes and ASCT2B mRNA-injected ones, to 3.1 ± 1.9 and 4.9 ± 2.8 $pmol \cdot hr^{-1} \cdot oocyte^{-1}$, respectively. No significant uptake of ^{14}C-labeled L-glutamic acid was observed in ASCT2B-expressing oocytes (Figure 5). The concentration dependence of the L-alanine uptakes indicated that transport was saturable. The *Km* for L-alanine was 93.3 μM (Figure 6).

Superfusion of ASCT2B mRNA-injected oocytes voltage-clamped at -60 mV failed to induce current in the presence of 100 μM L-alanin. In the case of uninjected oocytes voltage-clamped at -60 mV, the current was also unchanged upon superfusion in the presence of 100 μM L-alanine (data not shown, n=3).

To determine the substrate specificity of ASCT2B, inhibition of radiolabeled L-alanine uptake by 10 mM amino acids and their analogues was investigated. Uptake of L-alanine at 100μM was slightly inhibited by α-methylamino isobutyric acid (66.4% of control), N-methylalanine (82.3% of control), L-valine (47.7% of control) or L-phenylalanine (51.0% of control). In contrast, uptake of

L-alanine at 10 μM was almost completely inhibited by 1 mM L-serine (0.7% of control), L-cysteine (-2.3% of control) or L-threonine (2.4% of control).

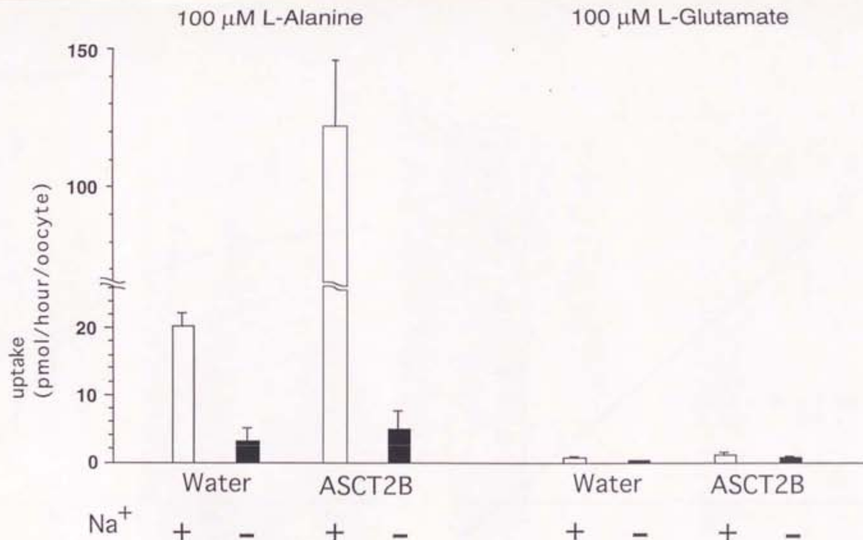
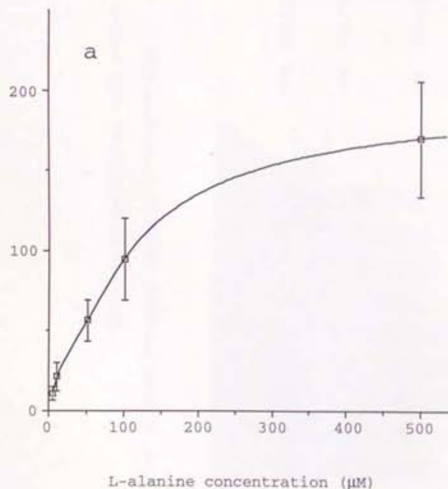


Figure 5. **Uptake of ^{14}C-labeled L-alanine and L-glutamate into *Xenopus* oocytes.** Oocytes were injected with water or in vitro-transcribed ASCT2B mRNA. Each column represents the mean ± SEM (n=6-8 oocytes). Vertical bars give the standard error. Open columns indicate rates of uptake in the presence of Na^+ (100mM); closed columns show rates of uptake in Na^+-free medium, in which Na^+ was replaced by choline.



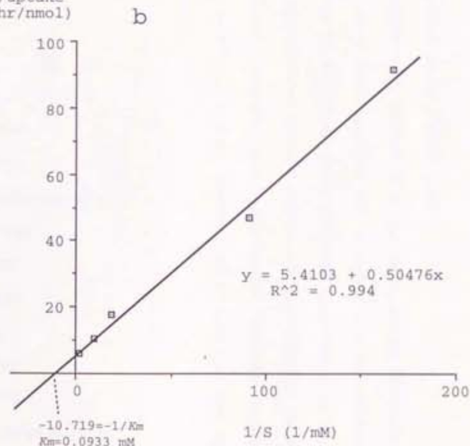


Figure 6. **a, Representative plot showing concentration dependence of ASCT2B-mediated L-alanine transport**
b, Lineweaver-Burk plot. The calculated K_m was 93.3 μM.

Tissue distribution of ASCT2B mRNA

The expression of ASCT2B mRNA was analyzed by Northern blotting of poly(A)$^+$ RNAs from various rat tissues. A hybridization band corresponding to a 2.7 kb mRNA fragment was detected in blots of samples of all tissues tested except testis. ASCT2B mRNA was most abundant in lung, skeletal muscle and colon, whereas it was detected at moderate levels in kidney, heart, small intestine and eye (Figure 7).

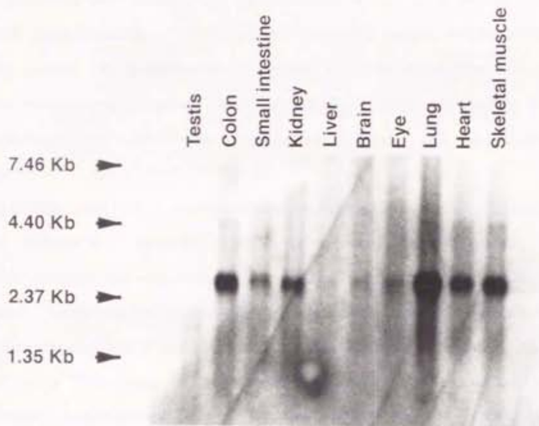


Figure 7. **High-stringency Northern blot analysis of mRNA from rat tissues probed with ^{32}P-labeled ASCT2B cDNA.**

DISCUSSION

Before amino acid transporters were cloned, the transport of amino acids was studied using epithelial membrane vesicles. On the basis of their transport properties, Na^+-dependent neutral amino acid transporters has been categorized in three systems: system A, ASC and $B^{0,+}$. System A contains Na^+-dependent neutral amino acid transporters which transports most dipolar amino acids and N-methyl amino acids. Particularly α-methylamino isobutyric acid is used as a model substrate for system A. System ASC contains the transporter that prefers to transport 3 to 5 carbon atoms in a chain except N-methyl amino acids. They accepts anionic amino acids of similar chain length on protonation. System $B^{0,+}$ contains the transporter which transports neutral amino acids including the bulky 3-amino-endo-bicyclo[1,2,3]octane-3-carboxylic acid, and cationic amino acids[19].

ASCT2B exhibited Na^+-dependent neutral amino acid transport. Thus, ASCT2B must be a transpoter of system A or $B^{0,+}$ or ASC. The system A-specific substrate α-methylamino isobutyric acid (αMAIB) slightly inhibited ASCT2B-mediated L-alanine uptake. The rate of L-alanine uptake was slightly inhibited by N-methylalanine. L-Phenyl-alanine, a substrate of system $B^{0,+}$, also slightly inhibit ASCT2B-mediated L-alanine uptake. Serine, cysteine and threonine were suggested to be the most effective substrates by the inhibition of ASCT2B-mediated L-alanine uptake. These findings indicate that ASCT2B is a neutral amino acid transport similar to system ASC.

The first system ASC-like transporter to be identified, ASCT1, was cloned from human brain and is structurally similar to mammalian glutamate transporters[10]. Recently, ASCT2, which also shows similarity to high-affinity glutamate transporters, was cloned from mouse testis[11]. Despite the structural similarity of ASCT2B with glutamate transporters, L-glutamate was not an effective substrate at pH 7.5. Similar to the reports that system ASC could transport glutamate with low affinity when the pH was reduced[20][21], mouse ASCT2 also transported glutamate at low pH[11]. The functional properties of ASCT2B at low pH, therefore, should be further studied. System ASC has been reported to be electroneutral[22] and to be insensitive to the extracellular K^+ concentration. I detected no current during ASCT2B-mediated L-alanine transport: this is in agreement with mouse ASCT2[11]. This suggests ASCT2B-mediated transport to be electroneutral. Further studies are needed to clarify effects of changes in extracellular ion concentrations on ASCT2B activity, because L-alanine uptake via ASCT1 and ASCT2 was affected by changes in extracellular K^+ concentrations. The *Km* values of mouse ASCT2-mediated L-alanine uptake were less than 20 μM[11]. The corresponding values of rat ASCT2B were 90 μM, implying that ASCT2B has a lower affinity than ASCT2 for L-alanine uptake. In this study, ASCT2B was cloned from the superficial cortex of the rat kidney by an RT-PCR method. On the basis of amino acid sequence homology, ASCT2B is considered to be a member of the family of proteins encoded by genes defined by the cloning of mammalian glutamate transporter subtypes[6][8][9] and

ASCT1[10]. Although ASCT2B cloned from rat kidney shows 80% amino acid sequence similarity to ASCT2[11] from mouse testis, the following reasons suggest that ASCT2B be different from ASCT2. 1) In the amino acid sequence of ASCT2B, there can be seen defects of two parts in consecutive amino acid sequence that corresponds to the ones from Ser^{17} to Cys^{21} and from Phe^{205} to Gln^{224} of ASCT2. 2) Different from ASCT2, ASCT2B is not expressed in testis. 3) The *Km* of L-alanine transport in ASCT2B was higher than that in ASCT2.

Hydropathy analysis revealed the structural similarity between ASCT2B and the mammalian glutamate transporters and the neutral amino acid transporters (Figure 4). Many structural features are highly conserved between the predicted ASCT2B amino acid sequence and the neutral amino acid transporters as depicted in Figure 3. In addition, ASCT2B contains the heptapeptide well-conserved motif AAVFIAQ (A^{395}-Q^{401} of ASCT2B) that is found in the bacterial proteins for a glutamate/aspartate carrier[23] and a dicarboxylate carrier[24], as well as in the mammalian glutamate transporters and the neutral amino acid transporters. From these viewpoints, ASCT2B may belong to the glutamate transporter gene family.

The mammalian glutamate transporters and the neutral amino acid transporters have potential N-glycosylation sites (N-X-S/T), which are predicted to face the extracellular side. Based on the result of deglycosylation studies, at least one or two sites are N-glycosylated[6][8]. Two of the predicted N-glycosylation sites of ASCT2B

are located at similar positions in the extracellular loop between the 3rd and 4th membrane-spanning regions. The other predicted site is located in the carboxyl-terminal domain that was assumed to be intracellular in ten membrane-spanning domain models. Thus, further study is required to determine the number of membrane-spanning domains of ASCT2B.

ASCT2B mRNA was found in all rat tissues examined except testis, with the highest levels detected in lung, skeletal muscle and colon. These distributional patterns of ASCT2B are different from those of ASCT1 and ASCT2. The former is expressed predominantly in the brain and skeletal muscle, and the latter is in the lung, colon, skeletal muscle, kidney and testis. Considering the strong structural similarity between ASCT2B and ASCT2, it would be interesting to determine the exact tissue distributions of the mRNAs of these proteins in the same species.

Based on the results of the studies on epitherial membrane vesicles of intestine, some system ASC-like neutral amino acid transporters transports L-glutamate in low-affinity manner at the brush border membrane of the intestine. On the other hand, some system ASC-like neutral amino acid transporters transport L-alanine, L-serine, L-cystein and L-threonine at the basolateral membrane of the intestine. To date, only ASCT2B is the cloned neutral amino acid transporter that belongs to system ASC and is expressed in the small intestine. Thus, for clarifying the role of ASCT2B in small intestine, it is required to identify the sites of intracellular

ASCT2B expression by immunohistochemistry.

Since there is no information about the function and localizations of system ASC-like transporters in the kidney, studies of in situ hybridization or immunohistochemistry may be able to answer whether ASCT2B is expressed at the superficial cortex of rat kidney. Furthermore, it is required to identify the sites of intracellular ASCT2B expression by immunohistochemistry and to clarify if ASCT2B can transport glutamate at low pH. Thus, several studies remain to be further investigated on ASCT2B in the kidney.

ABBREVIATIONS

ASC; alanine, serine, cystein

ASCT1; alanine, serine, cystein, transpoter 1

ASCT2; alanine, serine, cystein, transpoter 2

ASCT2B; alanine, serine, cystein, transpoter 2B

bp; base pair

cDNA; complementary DNA

EAAC1; excitatory amino acid carrier 1

GLAST; glutamate and aspartate transporter

GLT-1; glutamate transporter 1

kDa; kilodalton

Km; the transport constant or the affinity constant

PCR; polymerase chain reaction

mRNA; messenger RNA

SDS;sodium dodecyl sulphate

SSC; standard sodium citrate solution

Vmax; the maximal uptake rate

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謝 辞

本論文の研究にあたり、以下の皆様に対し厚く感謝の念を述べさせていただきます。

指導教官	遠藤 仁	杏林大学医学部薬理学教授
同	野々村禎昭	前東京大学医学部第一薬理学教授
同	三品昌美	現東京大学医学部第二薬理学教授
共同研究者	金井好克	杏林大学医学部薬理学講師
	関根孝司	同助手
技術補助	大羽尚子	同実験助手
	小林麻美	同実験助手
杏林大学	松田博青理事長	
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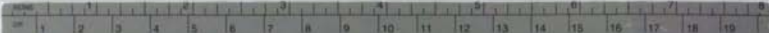
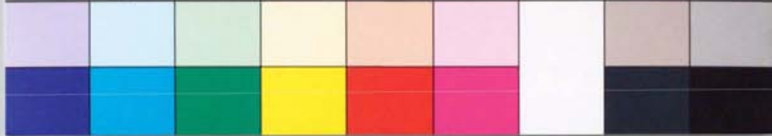
杏林大学	長澤俊彦医学部長	

博樹会西クリニック　西　忠博院長およびコメディカルの方々
東京慈恵会医科大学第二内科　川口　良人教授および透析室スタッフの方々

この研究には以下の研究助成の一部が用いられました。
文部省科学研究費補助金　試験研究(B)(2)（課題番号04557122）
　　　同　　　　　　　　　　　　　　（課題番号07557317）
厚生省厚生科学研究費補助金
ソルトサイエンス研究財団助成金（9337
財団法人上原記念生命科学財団研究助成金

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