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Cloning and Expression of a Human Neutral Amino Acid Transporter with Structural Similarity to the Glutamate Transporter Gene Family*

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A cDNA was isolated from human brain that encodes an amino acid sequence 34–39% identical to previously published glutamate transporter sequences. Injection of RNA transcribed from this cDNA into *Xenopus* oocytes resulted in expression of a transport activity with the properties of the neutral amino acid uptake system ASC. Superfusion of alanine, serine, and cysteine evoked sodium-dependent inward currents in voltage-clamped oocytes expressing the transporter. These currents were dose-dependent, stereospecific, and saturable, with K_m values ranging from 29 to 88 μM. Northern blot analyses revealed ubiquitous expression of this gene, termed ASCT1, consistent with the general metabolic role ascribed to system ASC.

The cellular uptake of amino acids is mediated by multiple independent transport systems distinguished primarily by substrate preference and by ionic requirements. In mammalian cells, neutral amino acids are transported predominantly by two sodium-dependent systems termed ASC (for alanine, serine, and cysteine preferring) and A (for alanine preferring) (1–3). *N*-Methylated amino acids and the related compound MAIB[1] are not substrates for system ASC, a property that allows it to be operationally distinguished from system A (4). ASC-mediated transport is the major component of neutral amino acid uptake in a wide variety of cell types.

A number of the classic amino acid transport systems have now been cloned and demonstrated by structural homologies to be members of distinct gene families. These include system Gly (5–7), the neutral amino acid transport system A (8), the basic amino acid transport system y^+ (9, 10), and the acidic amino acid transport system X^- (referred to here as glutamate transporters). Glutamate transporter subtypes were recently isolated from rat brain (11, 12) and rabbit intestine (13), and demonstrated to be structurally related members of a transporter gene family that also exhibit regions of homology with glt-P (14) and dctA (15), two bacterial nutrient uptake systems. We report the cloning, expression, and functional characterization of a human gene product that is structurally related to the glutamate transporter gene family and whose properties correspond to the neutral amino acid uptake system ASC.

EXPERIMENTAL PROCEDURES

cDNA Cloning and Sequencing—The amino acid sequence of GLAST1 (11) was obtained from the Swiss Protein Database (accession no. P24942). A synthetic oligonucleotide complementary to the amino acid sequence ATINMDGTALYEALAAIFIAQ (residues 395–415 of GLAST1) was synthesized because the conservation of portions of this sequence in the *Escherichia coli* glutamate carrier protein glt-P (14) suggested an important and conserved structural motif. This oligonucleotide sequence (5′ CTG (A/G)GC (A/G)AT GAA (A/G)AT GGC AGC CAG GGC (C/T)TC ATA CAG GGC TGT GCC (A/G)TC CAT GTT (A/G)AT GGT (A/G)GC 3′) is 128-fold degenerate and utilizes human codon preferences. Human motor cortex RNA isolated by the method of Chomczynski and Sacchi (16) and poly(A^+) enriched by oligo(dT)-cellulose chromatography were used to prepare a cDNA library. Mixed oligo(dT) and random-primed cDNA was synthesized using the *Superscript* Choice System (Life Technologies Inc.) and ligated into λZAPII (Stratagene, La Jolla, CA). The oligonucleotide was ^{32}P-labeled for screening this human cortex library. Conditions for hybridization and wash were: 0.5 M Na_2HPO_4, pH 7.15, 7% SDS for hybridization at 50 °C, and wash at 60 °C in 2 × SSPE, 1% SDS. The plasmid pASCT1, obtained by *in vivo* excision (Stratagene) of a hybridizing λZAPII clone, contained a 2.7-kbp cDNA insert that was sequenced on both strands by the dideoxy chain termination method using Sequenase 2.0 (U. S. Biochemical Corp.) and synthetic oligonucleotide primers (Oligos Etc.).

Expression of ASCT1 in Oocytes—The coding sequence of pASCT1 was isolated with unique flanking restriction sites by the polymerase chain reaction using synthetic oligonucleotide primers. Plasmid pOTV-ASCT1 contains this DNA sequence subcloned into the polylinker of an oocyte transcription vector that utilizes *Xenopus* β-globin 5′- and 3′-untranslated regions derived from pSP64T (17) to enhance expression levels in oocytes. Synthetic RNA was transcribed *in vitro* (18) and injected into defolliculated stage V–VI *Xenopus* oocytes. Transport was assayed as described previously by measuring uptake of [^{3}H]alanine, [^{3}H]serine, or [^{35}S]cysteine (all radiolabeled compounds from Du Pont-New England Nuclear) and by two-electrode voltage-clamp recording (18). 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. For transport measurements, this solution was changed to the indicated concentration of amino acid in ND-96. Current (I) as a function of substrate concentration ([S]) was fitted by least squares to $I = I_{max}\cdot[S]/(K_m + [S])$, where I_{max} is the maximal current and K_m is the transport constant. Values of K_m and I_{max} 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 are expressed as mean ± S.E.

Northern Blot Analysis—The human tissue Northern blot was obtained from Clontech Laboratories, and human brain region RNAs were prepared (16) from tissue provided by the Oregon Brain Repository. RNAs were size-fractionated on denaturing formaldehyde gels and transferred to a nylon membrane. The 1.7-kbp coding sequence of ASCT1 was radiolabeled with α-[^{32}P]dCTP (Du Pont-New England Nuclear) by the random priming method (Boehringer Mannheim). Filters were hybridized overnight at 42 °C with this cDNA probe (10^6 cpm/ml) in 5 × SSPE (20 × SSPE = 3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M Na_2EDTA, pH 7.4), 50% formamide, 7.5 × Denhardt's solution, 2% SDS, and 100 μg/ml denatured salmon sperm DNA. Autoradiography was performed

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L14595.

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[1] The abbreviations used are: MAIB, α-(methylamino)isobutyric acid; kb, kilobase(s); kbp, kilobase pair(s).

after two 30-min room temperature washes in 2 × SSPE, 0.1% SDS and two 20-min washes at 50 °C in 0.1 × SSPE, 0.1% SDS. Filters were subsequently reprobed with a radiolabeled human β-actin probe (Clontech Laboratories, Palo Alto).

RESULTS

A novel cDNA clone was isolated during the course of screening a human motor cortex cDNA library for sequences related to rat (11) and *E. coli* (14) glutamate transporters. Nucleotide sequence analysis of this clone predicted an open reading frame of 532 amino acids and corresponding to a protein of ~56 kDa, flanked by ~180 base pairs of 5′-untranslated sequence and ~0.9 kbp of 3′-untranslated sequence. The start of the coding sequence was defined by the first ATG downstream of an in-frame stop codon, and the surrounding sequences (GCCATGG) conform to a consensus translation initiation site (19). A fragment of this cDNA sequence (~nucleotides 1156–1492) was among the randomly selected clones sequenced by Adams *et al.* (20) and referred to as EST02446. We term this sequence ASCT1 because of the functional properties of the encoded transport activity.

The ASCT1 amino acid sequence shown in Fig. 1 exhibits remarkable similarity to those reported for glutamate transporter subtypes. Amino acid identities among these sequences have been highlighted to illustrate the conservation of glutamate transporter structural motifs within the ASCT1 sequence. ASCT1 has 39, 34, and 39% sequence identity with rat GLAST1 (11), rat GLT1 (12), and rabbit EAAC1 (13), respectively. This sequence identity is comparable with the identity seen between the subtypes themselves. All sequences exhibit unrelated NH_2 and COOH termini, and diversity in a putative extracellular transporter domain containing conserved potential *N*-linked glycosylation sites (Fig. 1). Another notable difference resides in a region highly conserved in the glutamate transporters (see the ASCT1 sequence IFQC, residues 384–387) and may be related to the determination of substrate specificity. The absence of a signal peptide sequence suggests the amino terminus is intracellular, but other aspects of the transmembrane topology of this transporter family are currently controversial because many of the hydrophobic regions predicted by sequence hydropathy analyses are more consistent with β-sheets than with α-helices. Six membrane-spanning regions are predicted in the NH_2-terminal portion of ASCT1, consistent with domains proposed for the glutamate transporters (11–13), and as many as four transmembrane regions may be present in the COOH-terminal portion.

The sequence similarity of ASCT1 with known glutamate transporters suggested that this protein might function to transport glutamate or other amino acids. We have previously utilized expression in oocytes and voltage clamp recording to characterize system y^+ (10), and others have utilized similar assays to characterize the activity of glutamate transporters (13). Superfusion of ASCT1 RNA-injected oocytes voltage-clamped at −60 mV with the amino acids alanine, serine, and cysteine produced inward currents that were absent in uninjected controls (Fig. 2*A*). These uptake currents were dose-dependent and saturable (Fig. 2, *B* and *C*, and Table I). Threonine and valine also elicited currents, but with lower affinities. Lysine or glutamate did not elicit detectable responses (Fig. 2*A*), and the system A-specific substrate MAIB neither evoked a detectable current nor inhibited the current elicited by alanine. *N*-Methylalanine at 1 mM did not produce a detectable current nor did proline, glycine, glutamine, asparagine, methionine, arginine, or leucine (data not shown). The rate of radiolabeled amino acid uptake (in pmol/min·oocyte, determined at 100 μM) in ASCT1-expressing oocytes was 18.0 ± 2.0 for alanine, 20.1 ± 5.1 for serine, and 19.2 ± 5.9 for cysteine, while the comparable values in uninjected controls were 0.6 ± 0.1, 0.4 ± 0.1, and 1.0 ± 0.3, respectively (n = 5). Uptake of 100 μM [^{3}H]alanine was unaffected by the addition of 10 mM MAIB (107% of control; n = 2).

ASCT1-mediated transport was also examined for stereospecificity and ion sensitivity. The current induced by D-alanine at 1 mM was only 12 ± 3% of the current obtained with 1 mM L-alanine (n = 3). The inward current elicited by 1 mM L-alanine superfusion was not seen when Tris was substituted for Na^+ (Fig. 2*A*). The current induced by 1 mM alanine was also reduced 61 ± 4% (n = 4) when extracellular K^+ was increased from 2 to 20 mM.

The expression of ASCT1 mRNA was analyzed by Northern blotting of RNAs from various human tissues. Two prominent RNA species 5.1 and 4.0 kb in length, and a smaller hybridizing band corresponding to 2.4 kb, were detectable in all lanes (Fig. 3*A*). The nature of these multiple RNA species is unclear but may reflect differential RNA processing. ASCT1 RNA appears to be most abundantly expressed in brain, muscle, and pancreas but was detectable in all tissues examined. The expression of ASCT1 in various regions of human brain was also examined and found to be uniformly distributed (Fig. 3*B*).

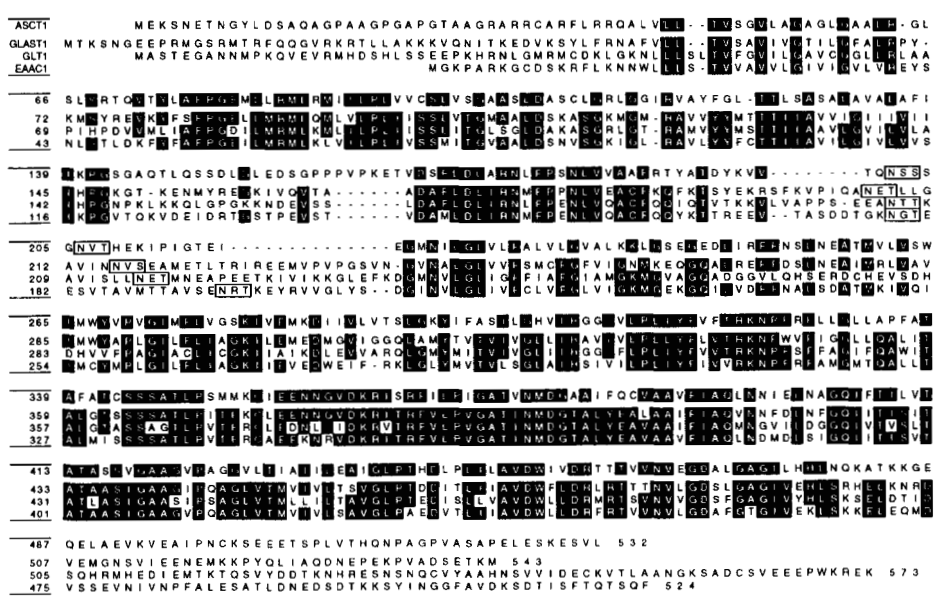

FIG. 1. **ASCT1 is structurally related to mammalian glutamate transporters.** The deduced amino acid sequence of human ASCT1 is shown aligned with the sequences reported for the glutamate transporter subtypes GLAST1 (11), GLT1 (12), and EAAC1 (13). Gaps (indicated by *dashes*) were introduced into the protein sequences to facilitate their alignment. Positions where at least three of the four protein sequences have identical amino acid residues are indicated by *white* on *black lettering*. Potential *N*-linked glycosylation sites (N-*X*-S/T) are outlined within *boxes*.

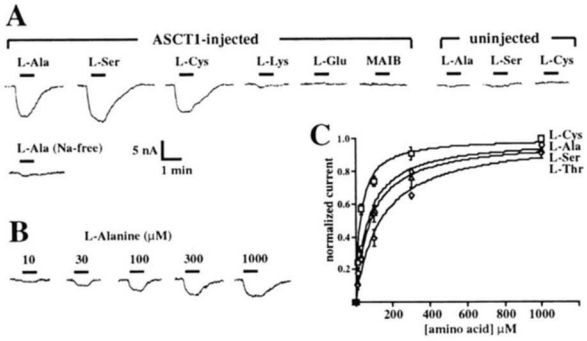

FIG. 2. **Inward currents evoked by amino acids in voltage-clamped ASCT1-expressing oocytes.** *A*, amino acids (1 mM) were superfused for the duration indicated by the *bar*. Sodium-dependent currents were induced by L-alanine, L-serine, and L-cysteine in ASCT1-expressing oocytes but not in uninjected controls. *B*, currents elicited were dose-dependent and saturable. *C*, concentration response data were fitted to the Michaelis-Menten equation to obtain the kinetic parameters listed in Table I.

TABLE I
Kinetic parameters of amino acid substrates

Amino acid	K_m	I_{max}[a]
	μM	
L-Alanine	71 ± 14	(1.0)
L-Serine	88 ± 11	1.2 ± 0.08
L-Cysteine	29 ± 6	1.0 ± 0.04
L-Threonine	137 ± 19	1.4 ± 0.03
L-Valine	390 ± 8	0.6 ± 0.11

[a] Maximum currents obtained with each substrate were normalized to the maximal current induced by alanine in the same oocyte (n = 3–5).

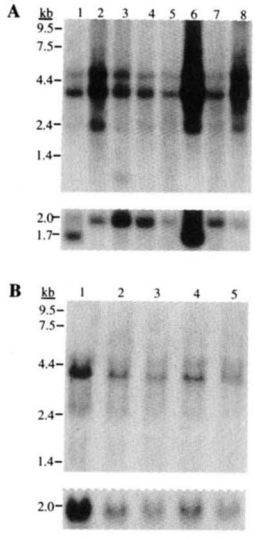

FIG. 3. **Expression of ASCT1 mRNA in human tissues.** *A*, Northern blot analysis using the radiolabeled coding sequence of ASCT1 as a hybridization probe. Two μg of poly(A$^+$) RNA was loaded per lane. *Lane 1*, heart; *lane 2*, brain; *lane 3*, placenta; *lane 4*, lung; *lane 5*, liver; *lane 6*, skeletal muscle; *lane 7*, kidney; *lane 8*, pancreas. In the *lower panel*, the same filter was reprobed with a human β-actin cDNA. β-Actin mRNA is approximately 2.0 kb in length, but in heart and skeletal muscle an abundant 1.7-kb actin mRNA species also hybridizes. *B*, 20 μg of total RNA prepared from human brain regions was analyzed as in *A*. *Lane 1*, motor cortex; *lane 2*, frontal cortex; *lane 3*, hippocampus; *lane 4*, basal ganglia; *lane 5*, cerebellum. Hybridization of this filter with the β-actin probe is shown in the *bottom panel*.

DISCUSSION

On the basis of amino acid sequence homology, ASCT1 is a member of the gene family recently defined by the cloning of mammalian glutamate transporter subtypes (11–13). The predicted ASCT1 amino acid sequence contains many of the highly conserved structural features of the GLAST1, GLT1, and EAAC1 transporter subtypes, as depicted in Fig. 1. Moreover, ASCT1 also contains the heptapeptide sequence AA(I/V)FIAQ that is found in the bacterial genes for a glutamate/aspartate carrier protein (14) and a dicarboxylate carrier protein (15), as well as in the mammalian sequences. These structural parallels with acidic amino acid carrier proteins are striking in view of the marked difference ASCT1 has in substrate specificity. These findings demonstrate that the functional diversity of this gene family is broader than previously suspected.

In agreement with previous studies of system ASC transport, ASCT1 demonstrated a broad specificity for neutral amino acids. Alanine, serine, cysteine, and threonine were the most effective substrates, and cysteine had the highest measured transport affinity (K_m = 29 μM). ASCT1 exhibited sodium dependence and stereospecificity for the L-enantiomer of alanine and did not transport *N*-methylalanine. The system A-specific substrate MAIB was not a substrate for ASCT1, and it did not inhibit alanine uptake. These are defining characteristics of system ASC (2, 3). Despite the structural homology of ASCT1 with glutamate transporters but consistent with system ASC, glutamate and aspartate were not effective substrates. As there have been reports that system ASC does transport glutamate with low affinity when the pH is reduced (21, 22), further studies will be required to analyze this phenomenon.

Although the substrates of ASCT1 and the glutamate transporters differ, the structural similarities of these proteins suggest that they may function by similar mechanisms. Uptake of glutamate is an electrogenic process that appears to be coupled to the cotransport of Na^+ and the countertransport of intracellular K^+ and of an additional pH-changing anion (23–25). Consistent with the notion of a conserved ionic mechanism, ASCT1-mediated transport was found to be electrogenic and sensitive to elevated extracellular K^+ as well as reduced extracellular Na^+. System ASC has been suggested to be electroneutral (26), and K^+ sensitivity has not been reported. Previous studies of system ASC have necessarily been conducted in cells with multiple partially overlapping transport activities. However, the overexpression of the cloned ASCT1 gene product in *Xenopus* oocytes and the utilization of voltage-clamp control allow direct demonstration of these properties. The substrate specificity of ASCT1-mediated transport argues that this cDNA clone encodes the biochemically defined cellular uptake system ASC.

ASCT1 mRNA was found to be expressed in all human tissues examined, with the highest levels detected in brain, skeletal muscle, and pancreas. The abundant expression in brain is particularly intriguing because cysteine, the ASCT1 substrate with the highest affinity, has been reported to have neurotoxic effects in the central nervous system (27). Thus, ASCT1 and the glutamate transporters may have similar roles in preventing the accumulation of endogenous neurotoxic amino acids. The molecular cloning of ASCT1 will provide an avenue to explore further the role of this neutral amino acid transport system in neuronal and cellular physiology. Moreover, the structural similarity of ASCT1 with glutamate transporter subtypes may aid in the elucidation of structure/function relationships in this transporter gene family.

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