January 1993

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Recommended Citation
Shafqat, S., Tamarappoo, B., Kilberg, M., Puranam, R., McNamara, J., -Ferraz, A., Fremeau, R. (1993). Cloning and expression of a novel Na(+)-dependent neutral amino acid transporter structurally related to mammalian Na+/glutamate cotransporters. Journal of Biological Chemistry, 268(21), 15351-15355.
Available at: https://ecommons.aku.edu/pakistan_fhs_mc_med_neurol/113
Cloning and Expression of a Novel Na+-dependent Neutral Amino Acid Transporter Structurally Related to Mammalian Na+/Glutamate Cotransporters

(Received for publication, April 26, 1993)

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A cDNA has been isolated from human hippocampus that appears to encode a novel Na+-dependent, Cl−-independent, neutral amino acid transporter. The putative protein, designated SATT, is 529 amino acids long and exhibits significant amino acid sequence identity (39–44%) with mammalian l-glutamate transporters. Expression of SATT cDNA in HeLa cells induced stereospecific uptake of l-serine, l-alanine, and l-threonine that was not inhibited by excess (3 mM) 2-(methylamino)-isobutyric acid, a specific substrate for the System A amino acid transporter. SATT expression in HeLa cells did not induce the transport of radiolabeled l-cysteine, l-glutamate, or related dicarboxylates. Northern blot hybridization revealed high levels of SATT mRNA in human skeletal muscle, pancreas, and brain, intermediate levels in heart, and low levels in liver, placenta, lung, and kidney. SATT transport characteristics are similar to the Na+-dependent neutral amino acid transport activity designated System ASC, but important differences are noted. These include: 1) SATT's apparent low expression in ASC-containing tissues such as liver or placenta; 2) the lack of mutual inhibition between serine and cysteine; and 3) the lack of trans-stimulation. SATT may represent one of multiple activities that exhibit System ASC-like transport characteristics in diverse tissues and cell lines.

Carrier-mediated amino acid transport has evolved to maintain transmembrane fluxes of amino acids for cellular nutrition and metabolism. In eukaryotic cells, a large number of distinct amino acid transport systems have been distinguished based on differences in substrate specificity, kinetic properties, and ionic dependence (1, 2). Our knowledge of the structural properties of amino acid transporters is limited. However, recent advances in the cloning of amino acid transporters have begun to provide insights into the molecular basis of carrier-mediated amino acid transport. A cDNA clone, SATT1, has recently been described that exhibits structural and sequence similarity with the Na+/glucose cotransporter family (3) and codes for a Na+-dependent neutral amino acid transporter with properties similar to System A (4). A murine ectropic retroviral receptor, which exhibits sequence homology with yeast permeases for histidine and arginine, has recently been found to induce Na+-independent transport of cationic amino acids with properties similar to System y* (5, 6). cDNAs have also been reported for putative regulatory subunits of a transporter for cystine, di- and neutral amino acids with properties similar to System b0,+ which appear to contain a single transmembrane domain and exhibit sequence similarity to glucosidases (7–9). Furthermore, cDNAs have been isolated for plasma membrane transporters for the neurotransmitter/modulator amino acids, including γ-aminobutyric acid (10–13), glycine (14, 15), and proline (16). Recently three distinct, but related, Na+/glutamate cotransporters have been cloned and functionally characterized (17–19), which appear to comprise a distinct gene family of membrane transport proteins. An intriguing result to emerge from the molecular cloning studies is the extent of structural diversity within the group of amino acid transporter proteins.

To explore the molecular diversity within the family of glutamate transporters, we performed a polymerase chain reaction on human hippocampal poly(A)+ RNA using primers corresponding to a human expressed sequence tag that exhibits distant homology to an Escherichia coli glutamate/aspartate carrier (20, 21). We identified a novel PCR product that was used to isolate a full-length cDNA clone from human hippocampus that exhibited 39–44% homology with mammalian glutamate transporters. Surprisingly, despite the sequence relationship with mammalian glutamate transporters, expression of this cDNA clone in HeLa cells did not induce l-glutamate transport but did induce Na+-dependent neutral amino acid uptake with several properties similar to System ASC. However, important differences were noted indicating that SATT may represent only one of several molecular entities that collectively account for System ASC-like transport.

EXPERIMENTAL PROCEDURES

PCR and Library Screening—Poly(A)+ mRNA (1 μg) purified from human hippocampus was converted to single-stranded cDNA (SuperScript, Life Technologies Inc.) using random primers (Pharmacia LKB Biotechnology Inc.) and subjected to PCR (22) with Taq polymerase as described by the manufacturer (Perkin-Elmer). Amplification was conducted with primers 5′-CCATTTCGACAGATTIT3′ and 5′-GGCCTGCACAGACACGACAA′ corresponding to a previously described human hippocampal expressed sequence tag (21). Amplification conditions were: 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final extension step of 72°C for 7 min. The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L18444.

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* The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase(s); KRP, Krebs-Ringer-phosphate; MeAIB, 2-(methylamino)-isobutyric acid; bp, base pair(s).
were 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 1 min, with a final extension of 10 min. The resulting PCR product (~0.3 kb) was cloned into the TA cloning vector (Invitrogen, San Diego, CA). The cloned insert was used as a template in a PCR containing 0.25 mCi of [32P]dCTP to generate a probe that was used to screen the human hippocampal cDNA library in λ-zap (Stratagene, La Jolla, CA). The inserts, as defined by a restriction enzyme digest, were recloned into the TA cloning vector. Overlapping clones were aligned by restriction map. Several mRNAs were found to be present on the basis that they were 6 base pairs downstream from a single in-frame stop codon. The 3'-untranslated region of clone 5 ends in a poly(A) tail but lacks a classical polyadenylation signal; however, the stretch of six As (27 bases upstream from the start of the poly(A) tail may substitute for the canonical AAUAAA. Kyte-Doolittle hydropathy analysis of the putative protein reveals the presence of multiple regions (6-10) of significantly extended hydrophilic domains (data not shown). Based on the subjective nature of hydropathy analysis, it is not possible to reliably predict the number of transmembrane domains. The amino terminus does not contain a readily identifiable signal sequence (25), suggesting that this domain resides on the cytoplasmic face of the membrane. Two canonical sites for N-linked glycosylation are present on a presumably extracellular 72-amino acid hydrophilic loop between putative transmembrane domains 3 and 4. Interestingly, a large, glycosylated hydrophilic extracellular loop is also present between transmembrane domains 3 and 4 in the Na+ and Cl- dependent neurotransmitter transporter family (26). As shown in Fig. 1, a number of consensus sequences for protein kinase-mediated phosphorylation are present in the putative cytoplasmic domains of the SATT protein. A leucine heptad repeat motif extends from the fourth transmembrane domain through the second cytosolic loop into the fifth transmembrane domain (amino acid residues 223-258). It remains to be determined whether this motif plays a role in transporter subunit oligomerization. A search of protein sequence data bases (April, 1993) with the putative SATT protein sequence revealed significant amino acid sequence similarity (39-44% amino acid sequence identity) with a recently described family of mammalian Na+/glutamate cotransporters including GLUTP (17), EAAC1 (19), and GLT1 (18) (Fig. 2). As shown in Fig. 2, the amino acid sequence conservation is approximately 3-fold higher in the carboxyl-terminal half of these proteins. Furthermore, SATT and the cloned mammalian glutamate transporters exhibit very similar hydropathy profiles (data not shown) and share a conserved putative glycosylation site (SATT residue Asn203) and several putative phosphorylation sites (SATT residues Ser250, Thr258, and Ser364) (see Fig. 2). Of particular interest, the sequence motif AA[I/V, L]FiFaQ that is conserved throughout the evolutionary diversity of glutamate transporters from prokaryotes to mammals is also conserved in SATT. In contrast, SATT does not share significant structural or amino acid sequence homology with other known ion-coupled cotransporters.

When a human multiple tissue Northern blot was screened at high stringency with the SATT probe (Fig. 3), four prominent transcripts were observed, at 4.8, 3.5, 2.8, and 2.2 kb. Hybridization signals were most prominent in skeletal muscle, pancreas, and brain; a weaker signal was observed in heart. Only low levels of SATT mRNA were detected in liver, kidney, lung, and placenta. The cDNA whose sequence and expression characteristics are described here most likely corresponds to the 2.2-kilobase species seen in Fig. 3.

To examine whether the multiple transcripts observed by Northern blot analysis represent the products of related genes or differentially processed species arising from a single primary transcript, a probe identical to the one employed in Northern blot analysis was used to locate the SATT gene in the human genome by hybridization of a DNA panel from somatic hybrid cell lines containing each of the human chromosomes. The SATT gene was localized to human chromosome 2 in two separate experiments that each employed a different

RESULTS AND DISCUSSION

Screening of a human hippocampal cDNA library with the PCR-generated probe described under "Experimental Procedures" yielded 10 overlapping cDNA clones. One full-length cDNA, clone 5, was isolated and sequenced to identify an open reading frame that codes for 529 amino acids (Fig. 1). The first ATG present in the cDNA was assigned as the initiation codon on the basis that it was 6 base pairs downstream from a single in-frame stop codon. The 3'-untranslated region of clone 5 ends in a poly(A) tail but lacks a classical polyadenylation signal; however, the stretch of six As (27 bases upstream from the start of the poly(A) tail may substitute for the canonical AAUAAA.
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restriction enzyme (EcoRI or HindIII) for cell line DNA digestion. EcoRI digestion of control human DNA resulted in hybridization to a single band; hybridization with HindIII-digested DNA revealed three bands (data not shown). In each case, the corresponding bands were seen only in the human chromosome 2 somatic hybrid cell line. These results are consistent with the hypothesis that the multiple SATT transcripts detected by Northern blotting arise from a single gene (although additional genetic loci on chromosome 2 cannot be excluded). The biological significance of SATT transcripts of different sizes, including the possible generation of functionally distinct isoforms by differential RNA processing within the coding sequence, remains to be determined.

To determine the function of SATT, clone 5 was expressed in HeLa cells to induce the transport of radiolabeled α-ketoglutarate, pyruvate, t-glutamate, L-glutamine, @alanine, taurine, L-histidine, L-ornithine, L-phenylalanine, or L-tyrosine. HeLa cells transfected with SATT cDNA consistently exhibited a significant increase in 3 μM l-[3H]serine and l-[3H]halaline uptake over basal levels (Table I). Preliminary studies using the System A-specific substrate MeAIB established that −10% of the basal transport of alanine and serine in our HeLa cell population was mediated by System A. Subsequently, all experiments were performed in the presence of 3 mM MeAIB to minimize System A-mediated transport of neutral amino acids. The presence of 3 mM MeAIB did not inhibit SATT-induced l-[3H]serine uptake. Transection-induced transport was stereospecific; 3 mM l-serine did not significantly inhibit transport of 3 μM l-[3H]serine in transfected cells (Table I). The Kₘ for l-[3H]serine uptake by SATT-transfected cells was estimated to be 56.5 μM by Lineweaver-Burk analysis of the dependence of initial velocity data on substrate concentration (data not shown). Removal of Na⁺ from the uptake buffer reduced basal l-[3H]serine or l-[3H]halaline transport by 90% in untransfected HeLa cells and eliminated the SATT-mediated induction in transport (data not shown). However, SATT-in-
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**Fig. 2.** Multiple sequence alignment depicting relationships of primary structure between the mammalian glutamate transporters and SATT. Shaded residues are conserved across all four proteins.

**Fig. 3.** Northern hybridization analysis using an SATT probe (see “Experimental Procedures”) against mRNA from multiple human tissues. The main species are the bands at 4.8, 3.5, 2.8, and 2.2 kb in skeletal muscle, pancreas, and brain.

Induced transport was independent of extracellular Cl⁻ in the assay buffer. Substitution of extracellular Cl⁻ with thiocyanate or acetate anions did not inhibit the SATT-mediated induction of L-[³H]serine transport (Table I). Excess unlabeled L-threonine (3 mM) markedly reduced both the basal and SATT-induced uptake of 3 mM L-[³H]serine (Table I).

These transport properties of SATT are similar to System ASC, a Na⁺-dependent neutral amino acid transport activity that does not transport N-methylated substrates such as MeAIB (1, 2, 27). Unlike System A, which can be identified by MeAIB uptake, no diagnostic ligands are available to distinguish System ASC. Typically, Na⁺-dependent, MeAIB-insensitive uptake of alanine, serine, threonine, or cysteine has been assigned to System ASC. However, subtle differences in System ASC transport properties have been described in different tissues. It is known, for example, that L-cysteine is a better ASC substrate than threonine in rat liver but that the converse is true in the hepatoma cell line HTC (28-31). Indeed, System ASC enhance the activity of the system, a process called trans-stimulation (see Ref. 27 for review). However, preloading of HeLa cells with L-alanine or L-threonine did not stimulate SATT-mediated transport (data not shown). Finally, only low levels of SATT mRNA were observed in tissues such as liver and placenta that exhibit prominent ASC transport activity.

The magnitude of SATT-induced transport in our expression system (1.7-2.0-fold, Table I) is comparable with that seen with the System A cDNA, SAAT-1, which produced a 2-fold increase in MeAIB uptake in transiently transfected COS cells (4). The relatively small magnitude of SATT-induced transport is most likely related to high background transport of ASC substrates in HeLa cells. Indeed, a significant proportion of such basal transport is probably occurring through the SATT carrier protein itself, because we observed substantial levels of SATT mRNA (corresponding to the three most abundant transcripts of 2.2, 3.5, and 4.8 kb seen in Fig. 3) in Northern blots of transfected HeLa cell mRNA (data not shown).

In summary, we have isolated and characterized a cDNA clone, SATT, which induces Na⁺-dependent, MeAIB-insensitive, transport of serine, threonine, and alanine in transfected HeLa cells. These SATT transport characteristics are similar to the Na⁺-dependent neutral amino acid transport activity designated System ASC. However, several properties of the cloned SATT transporter, as expressed in the HeLa cell, distinguish it from the generically described System ASC. These include: 1) the lack of L-cysteine transport; 2) the lack of trans-stimulation; and 3) the low levels of expression of SATT in ASC-rich tissues such as liver and placenta. Thus, SATT is likely to represent one of multiple activities that collectively account for System ASC-mediated amino acid uptake in diverse tissues and cell lines. Interestingly, SATT does not share significant structural or amino acid sequence homology with SAAT-1, the Na⁺-dependent, MeAIB-sensitive “System A” neutral amino acid transporter (4), even though both transporters share several
common substrates and Na\(^+\) dependence (1, 2, 27). Previously, a putative "Na\(^+\)-binding domain" has been proposed for a bacterial Na\(^+\)/glutamate cotransporter based on a short, loosely defined region of amino acid sequence similarity present in several Na\(^+\)-dependent symporters (26, 34), including SAAT1 (4); this sequence motif is not present in SATT. Although SATT does not transport l-glutamate or related dicarboxylates under our assay conditions (sodium KRP, pH 7.4), it does exhibit significant structural and amino acid sequence homology with a recently described family of mammalian Na\(^+\)/glutamate cotransporters (17–19). Interestingly, Vadgama and Christensen (30) demonstrated that System ASC does transport anionic amino acids such as cysteate and cysteine sulfinate at pH <6. It will therefore be of interest to characterize the pH dependence of the transport properties of SATT to investigate the functional basis for the structural and sequence similarity between SATT and the mammalian Na\(^+\)/glutamate cotransporters.

Acknowledgments—We thank Drs. Randy Blakely, David Pickup, and Bernard Moss for providing recombinant vaccinia virus, VTF, for expression studies. We are grateful to Dr. Jeff Arriza for providing crucial insights into potential substrates for this transporter and for sharing unpublished information.

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