Amino acid transport System A resembles System N in sequence but differs in mechanism


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Three principal transport systems account for much of the amino acid uptake by mammalian cells (1, 2). System ASC preferentially transports alanine, serine, and cysteine but also recognizes other aliphatic amino acids (3). Although Na+-dependent, System ASC appears to mediate amino acid exchange rather than net uptake (4, 5). System L recognizes branched chain and aromatic amino acids (6). Like System ASC, System L catalyzes exchange rather than net uptake, but, unlike System ASC, System L does not depend on Na+. Thus, two of the three general amino acid transport systems mediate exchange rather than net flux. Their function as exchangers presumably relies on the active accumulation of amino acids through other mechanisms.

System A catalyzes the Na+-dependent net uptake of many neutral amino acids, in particular alanine, serine, and glutamine (7). System A and other more specialized transport systems may thus provide the concentrations of cytoplasmic amino acids necessary to drive the uptake of other amino acids by exchange through Systems ASC and L. System A also exhibits several unusual properties, including the recognition of N-methylated amino acids, tolerance of Li+ substitution for Na+, and sensitivity to inhibition by low extracellular pH (1, 2). In addition, amino acid transport System A has provided a paradigm for the short- and long-term regulation of transport activity (1, 8, 9). Multiple hormones increase System A function acutely through changes in the driving force for transport as well as through longer-term changes in gene expression. System A function also varies inversely with the concentration of cytoplasmic amino acids as part of adaptive regulation and up-regulates with cell proliferation and hypertonic shock, suggesting additional homeostatic roles. Association with the cytoskeleton (10) and complex genetic mechanisms (11, 12) may contribute to these forms of regulation. Although molecular cloning has recently identified the proteins that mediate Systems ASC and L (13–17), System A has eluded biochemical purification, genetic approaches, and expression cloning in Xenopus oocytes (10, 18–20).

We now report the isolation of a cDNA encoding System A that belongs to a family of mammalian proteins originally defined by a neurotransmitter transporter. The vesicular GABA transporter (VGAT) mediates uptake of the inhibitory neurotransmitters GABA and glycine into synaptic vesicles (21, 22). Like other transporters responsible for the storage of classical transmitters in secretory vesicles, VGAT relies on a proton electrochemical gradient generated by the vacuolar H+-ATPase and catalyzes the exchange of luminal protons for cytoplasmic transmitter (23, 24). VGAT also defined a novel family of mammalian proteins with 10–11 predicted transmembrane domains. We have recently identified another member of this family as responsible for classical amino acid transport System N. Unlike the virtually ubiquitous Systems A, ASC, and L, System N appears more tissue-specific and mediates the uptake specifically of glutamine, histidine, and asparagine (25). Transport by System N also depends on Na+ but shows a striking sensitivity to inhibition by low external pH. Consistent with this sensitivity, we found that the System N transporter (SN1) mediates proton exchange as well as Na+ cotransport (26). SN1 thus differs from VGAT by translocating Na+ as well as protons. Because System A also catalyzes Na+-dependent amino acid transport sensitive to low pH, we considered that the protein(s) responsible might belong to the same family as VGAT and, in particular, SN1. We now show that another member of this family catalyzes amino acid transport with all of the properties previously described for System A. Despite the sensitivity to low pH and the strong sequence similarity to SN1, however, the novel protein does not act as a proton exchanger.

Materials and Methods

CDNA Cloning. A fragment of the expressed sequence tag sequence AA314459 was amplified by PCR from a fetal human brain cDNA library and was used to screen a rat brain cDNA library (26). Sequence analysis identified one cDNA that contained the entire ORF with an additional 240-bp 5′ untranslated region and a 2,600-bp 3′ untranslated region.

Northern Blot Analysis and In Situ Hybridization. Poly(A)+ RNA (3 µg) from different rat tissues was separated by electrophoresis through formaldehyde-agarose, was transferred to nylon, and was hybridized in 50% formamide at 42°C with a 32P-labeled probe corresponding to base pairs 160–1,928 of the rat cDNA (21) before autoradiography for 36 h at −80°C with an enhancing screen. In situ hybridization with 35S-labeled probes corresponding to nucleotides 109–460 was performed in 5% dextran sulfate at 58°C as described (27). In situ hybridization with digoxigenin-labeled probes was carried out as recently reported (28).

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Abbreviations: VGAT, vesicular GABA transporter; MEAIB, methylaminoisobutyric acid.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF273024).

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Transfection. The SA1 cDNA (nucleotides 160–1,928) was expressed by using the vaccinia virus-T7 polymerase system (29). In brief, HeLa cells were plated at 1.5 × 10⁶ per well in a 24-well plate, were incubated for 12 h at 37°C, and were rinsed with serum-free media, and vaccinia virus was added to each well at 5 plaque-forming units per cell. After 30 min, 1 μg of DNA was added with 3 μl of Lipofectin (GIBCO/BRL), and the cells were incubated an additional 12–20 h at 37°C before the measurement of transport.

Transport Assay. Cells were rinsed extensively with Krebs Ringers Hepes (pH 7.4) and were incubated in Krebs Ringers Hepes (pH 8.0) containing 0.1 μCi of [14C]methylaminoisobutyric acid (MeAIB) (NEN), 50 μM unlabeled MeAIB, and other compounds noted in the text at 37°C for the indicated times before terminating the reaction with three washes of 2 ml of cold Krebs Ringers Hepes (pH 8.0) (30). The cells were lysed in 1% SDS, and the radioactivity was measured in Ecolume (ICN).

Electrophysiology. cRNA transcripts were synthesized from linearized cDNA templates by using T7 RNA polymerases (Ambion, Austin, TX). Defolliculated Xenopus laevis oocytes were injected with 15 ng of cRNA by using standard methods for oocyte preparation and maintenance (31). One to five days after injection, two-electrode voltage clamp recordings were performed at room temperature by using GeneClamp 500B (Axon Instruments, Foster City, CA). Except where noted, all voltage clamp experiments were performed by using ND96 solution [composition in mM: 96 NaCl, 2.0 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 5 Hepes (pH 8.2)]. Water-injected and uninjected oocytes were used as controls and were treated in the same way as oocytes injected with SA1 cRNA.

Results

The sensitivity of amino acid uptake mediated by both Systems A and N to inhibition by low extracellular pH suggested that the protein(s) responsible for System A transport might show sequence similarity to System N. We therefore screened the mammalian databases and identified an expressed sequence tag (EST) very similar to but distinct from SN1. The corresponding 4.3-kb cDNA isolated from a rat brain library predicts a protein with 65% similarity and 55% identity to rat SN1 and clear but weaker similarity to rat VGAT (Fig. 3a). Like SN1, the sequence also predicts 11 transmembrane domains, with the N terminus of the protein in the cytoplasm (Fig. 1b).

Tissue Distribution. To characterize the expression of the novel sequence, we examined the distribution of the mRNA. Northern blot analysis detects expression of an ~4.3-kb mRNA transcript by a wide range of different tissues (Fig. 1c). Although consistent with the wide distribution of System A, the lung and pancreas lack detectable mRNA transcripts, indicating that expression is not ubiquitous. In situ hybridization further indicates expression of the novel sequence by multiple brain regions as well as the kidney and liver (Fig. 2). In the brain, expression appears strong in the cortex, hippocampus, thalamus, cerebellum, and brainstem but less intense in the caudate-putamen (Fig. 2a and c). Within the cerebellum, the granule cell layer labels more strongly than the molecular layer or white matter (Fig. 2d). In situ hybridization at higher resolution with a digoxigenin-labeled probe indicates expression by dentate gyrus granule cells and pyramidal cells in the hippocampus (Fig. 3a). Many cortical neurons also express the novel sequence (Fig. 3c). However, glial cells in the posterior commissure express the sequence as well (Fig. 3e), suggesting that many of the cells labeled in the cortex, hippocampus, and cerebellum are also glia. Further, the choroid plexus expresses the SN1-related sequence at high levels (Fig. 3f), indicating that it may contribute to the blood–brain barrier and the composition of the cerebrospinal fluid. In the kidney, expression is widespread, although more intense in the medulla than the cortex (Fig. 2e). In contrast, expression in the liver appears uniform (Fig. 2f).
Transport of MeAIB. To determine the function of the novel sequence, we used heterologous expression in HeLa cells. The similarity to VGAT as well as SN1 raised the possibility of a role in transport across intracellular membranes as well as at the plasma membrane. However, the putative transporter resembles SN1 much more strongly than VGAT (Fig. 1), and the introduction of an epitope tag at the N terminus of the protein allowed us to identify expression at the plasma membrane (data not shown). We therefore measured the uptake of radiolabeled amino acids across the plasma membrane of transfected cells. In light of the similarity to SN1, we first used [3H]glutamine and [3H]alanine. However, the considerable background uptake of these amino acids by untransfected cells complicated the analysis. Other transport systems for these amino acids and endogenous expression of the novel sequence itself may contribute to the uptake in untransfected cells. Because System A has the unusual property of recognizing N-methyl amino acid derivatives that are not recognized by most other plasma membrane amino acid transport activities, we then used [14C]MeAIB, a classical substrate for System A. Fig. 4a shows a clear time-dependent increase in the uptake of [14C]MeAIB by transfected cells that greatly exceeds that observed in untransfected cells, particularly at early times, suggesting a role for the novel protein as the System A transporter.

The analysis of flux mediated by the putative System A transporter (SA1) agrees with the results of classical studies in native tissue (7). The uptake of [14C]MeAIB by SA1 is saturable, with a $K_m$ of $\approx 140$ $\mu$M (Fig. 4b), very similar to the high micromolar values previously reported. In addition, 10 mM alanine, proline, methionine, serine, and glutamine dramatically inhibit the uptake of [14C]MeAIB, whereas histidine inhibits less potently, and the charged glutamate and arginine do not substantially inhibit flux mediated by SA1 (Fig. 4c). The IC$_{50}$ values for alanine, proline, glutamine, and serine also cluster in the high micromolar range, very close to the $K_m$ for MeAIB (Fig. 4d). The inhibition of SA1 by these amino acids parallels very closely the observations previously made by using hepatocytes (32). The System L substrate BCH does not inhibit MeAIB uptake by SA1, and SA1 recognizes D-serine poorly relative to the standard L-amino acid (Fig. 4e).

Coupling of Transport to $Na^+$ but not $H^+$. Because VGAT mediates the exchange of protons for neurotransmitter and SN1 the symport of $Na^+$ as well as proton exchange for amino acid substrates, we determined the ionic dependence of the closely related SA1. The replacement of $Na^+$ by choline essentially eliminates transport mediated by SA1 (Fig. 5a), as anticipated from the known $Na^+$ dependence of System A. Like SN1 but unlike many other $Na^+$-dependent transporters, SA1 tolerates the substitution of Li$^+$ for $Na^+$. In contrast to SN1, however, Li$^+$ appears much less effective than $Na^+$ in supporting amino acid flux (26). Further, replacement of Cl$^-$ in the Krebs Ringer buffer by gluconate substantially diminishes but does not eliminate the uptake of MeAIB. Thus, SA1 does not require Cl$^-$ for transport, but Cl$^-$ influences SA1 activity.

Low extracellular pH inhibits the function of SA1 (Fig. 5b), as predicted by previous work on System A. Low external pH also inhibits System N, but we have found that this apparently derives from a role for SN1 in proton exchange (26). The mechanism of proton exchange indeed predicts that an inwardly directed pH gradient should promote amino acid efflux and inhibit uptake. Thus, the sensitivity of SA1 to low external pH may similarly reflect a role for proton exchange. In this case, dissipation of the pH gradient across the plasma membrane should reduce the
inwardly directed pH gradient and hence promote amino acid uptake. However, the proton ionophores nigericin (Fig. 4c) and CCCP (data not shown) both reduce rather than increase MeAIB uptake at both pH 7.4 and pH 6.5, suggesting that SN1, unlike SA1, does not mediate proton exchange. The reduction of transport by CCCP may result from dissipation of the membrane potential, but the electroneutrality of exchange catalyzed by nigericin (Na\(^+\) or K\(^+\) for H\(^+\)) suggests that other mechanisms may account for the reduction of transport by proton ionophores.

**Electrogenic Transport.** Because SA1 mediates the cotransport of Na\(^+\) with neutral amino acids but without proton exchange, it might be expected to generate a current coupled to the flux of substrate. The reductions of transport by Cl\(^-\) substitution support this possibility. We have therefore expressed SA1 in *Xenopus* oocytes and measured currents by two-electrode voltage clamp in the presence of added substrate. Fig. 6a shows that increasing concentrations of alanine activate inwardly directed currents, and the concentration that elicits a half-maximal response falls in the high micromolar range, very similar to the \(K_m\) measured for flux. Using this assay, we have determined whether amino acids simply inhibit the transport of [\(^1^4\)C]MeAIB or act as substrates that undergo net accumulation and hence generate currents. Very similar to their potency as inhibitors of flux, serine, alanine, methionine, proline, and glutamine generate currents whereas bulky aliphatic, aromatic, and charged amino acids produce very small, if any, currents (Fig. 6b).

Asparagine and serine also appear much better substrates than the closely related glutamine and threonine, consistent with previous observations of flux mediated by System A (32). The correlation between amino acid potency as inhibitors of flux and potency in producing currents further suggests that the currents generated by SA1 are closely coupled to transport, at least under these conditions. The currents produced by alanine also depend
on $\text{Na}^+$, as anticipated if they reflect electrogenic transport (Fig. 6c). In addition, the parabolic rather than sigmoidal shape of the curve suggests the translocation of 1 $\text{Na}^+$ per cycle, as suggested by previous studies (33, 34), and low external pH inhibits the alanine-induced currents (Fig. 6d) as well as the flux of MeAIB. Replacement of extracellular chloride by gluconate reduces the amplitude of the currents by $29 \pm 9\%$ ($n = 6$). Because the electrophysiologic analysis is performed under voltage clamp conditions, the results support a role for the anion independent of membrane potential.

**Discussion**

The results indicate that SA1 exhibits all of the properties previously described in native tissues for System A. SA1 transports N-methyl amino acids such as MeAIB and recognizes standard amino acids as substrates with the same order of affinity as previously reported for System A. In addition, the affinities for preferred substrates appear very similar to those previously described for System A. Further, transport by SA1 depends on $\text{Na}^+$ but tolerates substitution by $\text{Li}^+$ and shows sensitivity to inhibition by low extracellular pH. As predicted from previous work, transport by SA1 is also electrogenic. Thus, SA1 mediates System A transport activity. However, the tissue distribution and sequence similarity to SN1 make several unanticipated predictions.

Despite the presumed ubiquity of System A in mammalian cells, SA1 does not occur uniformly in all tissues and cells. Northern blot analysis shows no detectable expression in the lung and pancreas, suggesting either that these tissues do not express any System A activity or, more likely, that they express a distinct isoform. Indeed, the recent identification of a System A-like transporter (GlnT) expressed relatively specifically by neurons indicates the potential for tissue-specific isoforms of SA1 (35). Further, the neuronal transporter appears to recognize glutamate with slightly higher affinity than other System A substrates, suggesting a possible role in the glutamine-glutamate cycle that sustains glutamate release at excitatory synapses. Interestingly, the closely related SN1 also appears to contribute to this cycle (26).

Synaptic transmission requires mechanisms to recapture the massive amounts of neurotransmitter released by exocytosis. For several classical transmitters, plasma membrane reuptake systems located at the nerve terminal serve this function (36–38). In the case of the major excitatory transmitter glutamate, however, the known plasma membrane transporters generally reside either postsynaptically or on glia (39, 40). Indeed, glutamate has been proposed to recycle through astrocytes (41–43), undergoing conversion to glutamine by the enzyme glutamine synthetase after uptake from the synapse (44). Glutamine is then released from the glia and taken up by neurons before conversion back to glutamate. Although the transporters responsible for glutamine efflux from astrocytes have remained unknown, the demonstration that SN1 resides on astrocytic processes surrounding synapses and predominantly mediates efflux rather than uptake under physiological conditions strongly suggests that SN1 contributes to the glutamate-glutamine cycle by catalyzing glutamine efflux from glia (26). The distribution and functional characteristics of GlnT further suggest a role in the uptake of glutamine by neurons (35). SN1 and GlnT may therefore act sequentially in the glutamine-glutamate cycle. However, we have also observed the expression of SA1 by neurons, suggesting that SA1 as well as GlnT contributes to the uptake of glutamate by neurons. Glial cells appear to express SA1 as well as SN1, but the ionic coupling of SA1 suggests that it may not mediate efflux under physiological conditions.

Differences in ionic coupling contribute to the divergent functions proposed for SN1 and SA1. SN1 mediates the cotransport of $\text{Na}^+$ with a neutral amino acid in exchange for a proton. The mechanism of proton exchange predicts that the inwardly directed proton electrochemical gradient (due mostly to the negative membrane potential because the cell is usually slightly more acidic than the extracellular medium) opposes the inwardly directed $\text{Na}^+$ electrochemical gradient and hence makes the SN1 more likely to mediate efflux if intracellular amino acid concentrations rise sufficiently high or extracellular concentrations fall sufficiently low, conditions that occur physiologically. In contrast, SA1 catalyzes only $\text{Na}^+$ cotransport and does not appear to mediate proton exchange. Low extracellular pH inhibits uptake by SA1 as well as SN1, but nigericin does not relieve the inhibition as anticipated if an inwardly directed pH gradient (rather than simply an acidic environment) opposed amino acid uptake. We have also found that amino acid substrates increase intracellular pH in cells expressing SN1 (26) but not SA1 (data not shown). The apparent failure of SA1 to translocate a proton in exchange for $\text{Na}^+$ and a neutral amino acid predicts that flux involves net charge movement across the membrane and we have directly demonstrated the electrogenic nature of SA1 transport. However, we have recently observed inward currents caused by uptake by SN1 as well (F.A.C., R.J.R., and R.H.E., unpublished work). Although the stoichiometry of $\text{Na}^+$ coupling may differ between SA1 and SN1, the lack of $\text{H}^+$ translocation by SA1 indicates that the inwardly directed $\text{H}^+$ electrochemical gradient will not oppose the $\text{Na}^+$ electrochemical gradient that drives uptake by SA1. As a result, the net driving force for amino acid uptake by SA1 is much more...
strongly inward directed than that for SN1, enabling SA1 to accumulate much higher intracellular amino acid concentrations than SN1 without net efflux. Thus, the ionic coupling may contribute to a primarily unidirectional role for SA1 in amino acid uptake and a bi-directional role for SN1 in efflux as well as uptake.

Functions for SA1 in amino acid uptake and for SN1 in efflux as well as uptake suggest the potential for antagonistic roles in vivo. In tissues such as the brain and liver that widely express both proteins, many of the amino acids taken up by SA1 will not be transported inside the cell. In the case of glutamine, however, SN1 may well contribute to a primarily unidirectional role for SA1 in amino acid uptake and a bi-directional role for SN1 in efflux as well as uptake.

Although SA1 and SN1 differ in proton translocation, suggesting very different physiological roles, SA1 remains exquisitely sensitive to inhibition by low external pH, similar to SN1-mediated amino acid uptake. The extensive sequence similarity between SA1 and SN1 presumably contributes to this shared pH sensitivity. Conversely, the difference between SA1 and SN1 in proton translocation must reflect a subtle but important difference in their structure. The intact sensitivity to low pH indeed suggests that SA1 retains at least part of the proton binding site present in SN1 but lacks the pathway for proton translocation.

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