Cloning and Expression of a Mammalian Na⁺/Amino Acid Cotransporter with Sequence Similarity to Na⁺/Glucose Cotransporters*

(Received for publication, September 24, 1992)

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We describe the full-length sequence and functional expression of a cDNA cloned from LLC-PK₁ cells, which appears to encode a mammalian Na⁺-dependent neutral amino acid transporter with properties characteristic of system A. This sequence, designated SAAT₁, is 76% identical and 89% similar in amino acid sequence to the Na⁺-dependent glucose transporter SGLT₁ of the same species. A leucine zipper region was detected in both SAAT₁ and SGLT₁. The message for SAAT₁ was a single 2.4-kilobase species in kidney, but mRNA species of 2.4 and 3.7 kilobases were observed in LLC-PK₁ cells as well as in intestine. Transcripts were also found in spleen, liver, and muscle. Expression of SAAT₁ in COS-7 cells resulted in increased levels of Na⁺-dependent uptake of 2-(methylamino)isobutyric acid, a specific substrate for the system A amino acid transporter. Uptake due to cDNA expression was inhibited by a range of amino acids that are transported by system A and exhibited a kₘ of 0.8 ± 0.2 mM. These results suggest that the system A amino acid transporter is closely related to the Na⁺/glucose transporter SGLT₁.

A number of different nutrient transport systems in mammalian cells utilize a Na⁺-dependent cotransport mechanism (1). Na⁺-coupled transporters cloned thus far from both bacterial and mammalian sources share significant amino acid sequence homology, suggesting a fundamental similarity in the Na⁺-coupling mechanism for transport of these diverse substrates. Na⁺/glucose transporters (SGLT₁) cloned from rabbit intestine (2) and kidney (3), human intestine (4), and LLC-PK₁ cells (5) exhibit no sequence homology with Na⁺-independent facilitative glucose transporters (6) but do share a striking amino acid sequence homology with a rabbit renal Na⁺/nucleoside cotransporter (7) and the Madin-Darby canine kidney cell Na⁺/myo-inositol cotransporter (8). These Na⁺-coupled transporters have several conserved amino acid residues in common with the rabbit renal Na⁺/phosphate cotransporter (9), as well as bacterial Na⁺/cotransporters for proline (10), pantothenate (11), and glutamate (12).

Na⁺-coupled and Na⁺-independent transporters of distinct specificity mediate uptake of amino acids in mammalian cells (13). A Na⁺-independent transporter for cationic amino acids, system y⁺ (14, 15), as well as putative regulatory subunits of a long-chain and aromatic neutral amino acid transporter, system L (16), and of a transporter for cystine and dibasic and neutral amino acids, system b₀⁺ (17, 18), have been cloned and sequenced. These have no sequence homology with the SGLT₁-related family of Na⁺-coupled transporters. A Na⁺-dependent glycine transporter involved in glycine transport was cloned from rat brain (19).

In the present study we report the cloning and full-length sequence of a Na⁺/amino acid cotransporter cDNA obtained by low stringency screening of an LLC-PK₁ cDNA library for sequences with homology to the rabbit intestinal Na⁺/glucose cotransporter (SGLT₁). SAAT₁ exhibited 87% sequence similarity to rabbit intestinal SGLT₁. Expression of SAAT₁ in COS-7 cells indicated that it encoded a Na⁺-dependent neutral amino acid transporter with specificity characteristics of system A (13, 20).

EXPERIMENTAL PROCEDURES
cDNA Cloning and Sequencing—An oligo(dT)/random-primed cDNA library in λ ZAPII (Stratagene, La Jolla, CA) was prepared from poly(A⁺) RNA isolated from LLC-PK₁ cells and screened using the rabbit intestinal SGLT₁ clone pMJC424 (2) obtained through the generosity of E. Wright (UCLA, Los Angeles, CA) under low-stringency conditions as described previously (21) with the omission of dextran sulfate. In order to obtain the 5' region, a second LLC-PK₁, λ ZAPII library was prepared using both an internal primer based on the SAAT₁ sequence 5'-CGCTTGTGAGATGAGG-3' and an oligo(dT) primer. This library was screened as described above except that 528-base pair PCR product corresponding to bases 673-1201 of the SAAT₁ sequence and labeled by random priming was used as probe, pBluescript SK(-) was excised from positive plaques and purified phage using an in vitro excision protocol (Stratagene). DNA was purified by mini-prep alkaline lysis (22) with polyethylene glycol-purified phage using an in vivo excision protocol (Stratagene). DNA was purified by mini-prep alkaline lysis (22) with polyethylene glycol precipitation (23). Double-stranded DNA sequencing was carried out by the dideoxy chain termination method using Sequenase version 2.0 (United States Biochemical Corp.) and synthetic oligonucleotide primers (Genosys). Compressions were resolved using dexta nucleotides. The complete sequence was determined on both strands and also confirmed by automated DNA sequencing.

Northern Blot Analysis—Poly(A⁺) RNA was isolated from cell cultures or tissues (24) and resolved and transferred by Northern blot (25) to a Duralon-UV membrane (Stratagene). The filters were washed with U.S.A.

The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase(s); sMGF, α-methyl-D-glucopyranoside; MeAIB, 2-(methylamino)isobutyric acid.
in a 20-μl reaction mixture containing 1 unit/μl RNasin (Promega), 1 mM dNTPs (Boehringer Mannheim), 7.5 μg/ml oligo(dT) (17-mer, Promega), 10 units/μl Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) in 1 X RT buffer. The mixture was held at room temperature for 5 min, then incubated at 37°C for 1 h. Termination was carried out by heating at 90°C for 10 min and then chilling on ice. Primers (20-mers) specific for porcine SAAT1, were as follows: sense, 5'-ATACTGGTCGTCCTGGCAAT-3'; antisense, 5'-GATGTTCACTATAGTCTTCC-3'. The reverse-transcribed sample was added to 80 μl of a mixture containing 0.03 unit/μl Hot Tub polymerase (Amersham Corp.), 25 ng of each specific primer, and 1 X Hot Tub reaction buffer. The predominant PCR cDNA amplification product was predicted to be 318 base pairs, and the position was confirmed by amino acid residues 538 and 643. The product was ligated to the pcR-1000<sup>TM</sup> vector using a TA cloning kit (Invitrogen), and possible clones were isolated and confirmed by double-strand sequencing, pCR318, the LLC-PK<sub>1</sub> SAAT1-specific vector, was obtained using this procedure.

Expression of SAAT1 in COS Cells—Two partial SAAT1 clones spanning the entire coding region were constructed into a full-length clone, pCTK, by ligation at their internal ApaLI site. An XhoI/XbaI restriction fragment containing either the full-length SAAT1 cDNA from pMJC424 (2 kb) as positive control) was ligated into the NhEl/XhoI site of the inducible expression vector pMAMneo (Clontech). Exponentially growing COS-7 cells grown in the same medium previously described for LLC-PK<sub>1</sub> cells (25) were washed and resuspended at 7.5 x 10<sup>5</sup> cells/ml in 0.8 ml aliquots containing 21 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.3 mM Na<sub>2</sub>HP<sub>4</sub>, 1 mM dNTPs (Boehringer Mannheim), 10 units/ml RNasin (Promega), 1 mM dNTPs (Boehringer Mannheim), 10 units/ml Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) in 1 X RT buffer. The mixture was held at room temperature for 5 min, then incubated at 37°C for 1 h. Termination was carried out by heating at 90°C for 10 min and then chilling on ice. Primers (20-mers) specific for porcine SAAT1, were as follows: sense, 5'-ATACTGGTCGTCCTGGCAAT-3'; antisense, 5'-GATGTTCACTATAGTCTTCC-3'. The reverse-transcribed sample was added to 80 μl of a mixture containing 0.03 unit/μl Hot Tub polymerase (Amersham Corp.), 25 ng of each specific primer, and 1 X Hot Tub reaction buffer. The predominant PCR cDNA amplification product was predicted to be 318 base pairs, and the position was confirmed by amino acid residues 538 and 643. The product was ligated to the pcR-1000<sup>TM</sup> vector using a TA cloning kit (Invitrogen), and possible clones were isolated and confirmed by double-strand sequencing, pCR318, the LLC-PK<sub>1</sub> SAAT1-specific vector, was obtained using this procedure.

RESULTS AND DISCUSSION

Low stringency screening with the rabbit intestinal SGLT1 cDNA of two different LLC-PK<sub>1</sub> cDNA libraries was utilized to isolate nine overlapping SAAT1 cDNA clones, from which a composite full-length cDNA nucleotide sequence was obtained by sequencing both strands. The composite cDNA and deduced amino acid sequence of SAAT1 are shown in Fig. 1. The full sequence encodes a protein of 610 amino acids. The 3'-untranslated region contains a consensus polyadenylation sequence, AAUAAA (underlined), but does not include a poly(A<sup>T</sup>) tail. Fig. 2 demonstrates the significant amino acid sequence similarity between SAAT1 and several SGLT1 sequences. The LLC-PK<sub>1</sub> SAAT1 sequence shows 74% DNA sequence homology, 89% amino acid similarity, and 76% amino acid identity over their region of overlap to the partial LLC-PK<sub>1</sub>-SGLT1 sequence reported by Ohta et al. (5). Two residues, 576 and 613, were deleted in the porcine SAAT1 sequence compared with porcine SGLT1. LLC-PK<sub>1</sub> SAAT1 shows 88% amino acid similarity and 75% identity with the human (4) intestinal SGLT1 sequence and 87% similarity and 74% identity with rabbit intestinal SGLT1 (2). Furthermore, hydropathy plots of both SAAT1 and SGLT1 were nearly identical (not shown). Our alignment indicates that the porcine SAAT1 sequence lacks 4 residues found in human intestinal SGLT1 and 2 residues are missing compared with the rabbit intestinal SGLT1.

We observed only one potential N-linked glycosylation site indicated by the asterisk (*) in the SAAT1 sequence (Fig. 2), corresponding to Asn<sup>248</sup>. By contrast, two potential N-linked glycosylation sites, at Asn<sup>246</sup> and Asn<sup>298</sup>, are conserved in the three SGLT1 sequences, although it appears that only Asn<sup>246</sup> is glycosylated in SGLT1 (27).

One apparent leucine zipper region, located at the loop between transmembrane domains 7 and 8, was noted in all of the SGLT1 sequences and in SAAT1, indicated by the vertical bars in Fig. 2. A leucine zipper has not previously been reported for SGLT1 sequences but has been found in the family of facilitative glucose transporters (28) and may be involved in transporter subunit oligomerization (29).

Amino acid residues Gly<sup>380</sup>, Ala<sup>417</sup>, Gly<sup>426</sup>, and Arg<sup>427</sup> in the SAAT1 sequence (Fig. 2), are conserved in all of the Na<sup>+</sup> symporters, as well as the Escherichia coli Na<sup>+</sup>/proline (10), glutamate (12) and pantothenate symporters (11), the rabbit Na<sup>+</sup>/phosphate (9), rabbit Na<sup>+</sup>/nucleoside (7), and Madin-Darby canine kidney cell Na<sup>+</sup>/myo-inositol transporter (8). Leu<sup>281</sup> was conserved in all except the proline cotransporter. This conserved region, designated the SOB motif (12), located next to the Na<sup>+</sup> binding and energetic coupling to the Na<sup>+</sup> gradient (30). Asp<sup>275</sup>, the residue that is mutated in SGLT1 of patients with glucose-galactose malabsorption (31), is present in SAAT1 and all of the Na<sup>+</sup> symporters, as well as the E. coli Na<sup>+</sup>/proline cotransporter shown by mutation to be implicated in Na<sup>+</sup> binding and energetic coupling to the Na<sup>+</sup> gradient (30). Asp<sup>278</sup> is conserved in SGLT1 sequences and corresponds to Arg<sup>232</sup> in the E. coli Na<sup>+</sup>/proline cotransporter shown by mutation to be implicated in Na<sup>+</sup> binding and energetic coupling to the Na<sup>+</sup> gradient (30). Asp<sup>278</sup>, the residue that is mutated in SGLT1 of patients with glucose-galactose malabsorption (31), is present in SAAT1 and corresponds to Asp<sup>278</sup> in the sodium/ nucleoside cotransporter SNT1 (7).

In order to prevent cross-hybridization between the closely related sequences SAAT1 and SGLT1, an SAAT1-specific probe pCR318, which contained a 318-base pair insert corresponding to amino acid residues 538–643, was utilized for Northern blot analysis. This region exhibited the most sequence diversity between the various Na<sup>+</sup>-coupled transporters.
Cloning of a Na⁺/Amino Acid Cotransporter

Fig. 2. Alignment of deduced amino acid sequences for LLC-PK₁ pig renal SAAT₁ with SGLT₁ sequences from LLC-PK₁ cells and rabbit and human intestine. The sequences are aligned against the top sequence shown, using the Genetics Computer group program GAP (38). Porcine SGLT₁ indicates the LLC-PK₁ sequence (5); rabbit (2) and human (4) intestine SGLT₁ sequences are shown. Vertical bars indicate leucine zipper regions. The predicted SAAT₁ N-linked glycosylation site is indicated by the asterisk (*). Gaps and identical amino acid residues are indicated by dots and dashes, respectively.

Fig. 3. Expression of SAAT₁ mRNA in LLC-PK₁ cells and porcine tissues. Samples (1 μg) of poly(A⁺) RNA from each of the indicated sources were analyzed by Northern blot. The filter was hybridized with antisense RNA transcribed from the SAAT₁-specific probe pCR-318. Lane 1, LLC-PK₁ cells, confluent; lane 2, kidney; lane 3, intestine; lane 4, liver; lane 5, spleen; lane 6, muscle.

ers. Control experiments established that the SAAT₁ antisense probe transcribed from pCR318 did not hybridize with sense RNA transcribed in vitro from an LLC-PK₁ SGLT₁ template but did specifically hybridize with sense RNA transcribed from an SAAT₁ template under the same stringency conditions used in the Northern blot. The antisense RNA probe transcribed from pCR318 detected both 2.4- and 3.7-kb bands by Northern blot analysis of LLC-PK₁ poly(A⁺) RNA (Fig. 3). This cell population was recloned twice from a single cell in order to avoid complications arising from the considerable cellular heterogeneity of the parental LLC-PK₁ cell line (32, 33). By contrast, an SGLT₁ probe detected transcripts of 2.2 and 2.9 kb in LLC-PK₁ cells (5). A single 2.4-kb SAAT₁ transcript was observed in kidney, while transcripts of 2.4, 3.7, and 4.9 kb were observed in poly(A⁺) RNA from small intestine (Fig. 3). PCR analysis of poly(A⁺) RNA from pig kidney and small intestine indicated that in these tissues, the region corresponding to amino acid residues 538–643 was identical in sequence to that shown for porcine LLC-PK₁ SAAT₁ in Fig. 1. A single 4.9-kb transcript was detected in liver, whereas skeletal muscle exhibited a predominant 1.4-kb band with faintly visible bands of 2.4 and 4.9 kb. In spleen, predominant bands of 2.4 and 4.9 kb were evident with a faint band of 3.7 kb (Fig. 3). The transcript sizes and tissue distribution of SAAT₁ differ from those observed for SNST₁ and six other distinct partially characterized cDNAs isolated from a rabbit kidney library by high stringency hybridization with SGLT₁ (7). Southern blot analysis of LLC-PK₁ cell DNA using a SAAT₁-specific probe revealed a different pattern of hybridization from that obtained using a probe specific for SGLT₁ (not shown).

In order to determine the function of SAAT₁, the full-length cDNA including the start codon was subcloned into the dexamethasone-inducible mammalian expression vector pMAMneo, which provided the missing poly(A⁺) tail. The resulting construct, pCTK-MAMneo, was used to transiently transfet COS-7 cells by electroporation. Initial studies indicated that pCTK-MAMneo was not able to express Na⁺-dependent α-methyl-D-glucopyranoside (αMGP) uptake in COS cells (<0.57 nmol/h/mg protein), whereas the positive control, a similarly constructed plasmid, pMJC-MAMneo,

Table I

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Inserted cDNA</th>
<th>Na⁺-stimulated MeAIB uptake</th>
<th>Na⁺-independent MeAIB uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCTK-MAMneo</td>
<td>SAAT₁</td>
<td>4.0 ± 0.02</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>pMJC-MAMneo</td>
<td>SGLT₁</td>
<td>1.8 ± 0.06</td>
<td>0.2 ± 0.005</td>
</tr>
<tr>
<td>pMAMneo</td>
<td>None</td>
<td>2.0 ± 0.3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>1.9 ± 0.1</td>
<td>0.1 ± 0.008</td>
</tr>
</tbody>
</table>

*COS-7 cells subjected to the electroporation procedure without addition of vector DNA.
containing the full-length rabbit intestine SGLT1 CDNA did express αMGP uptake (1.59 nmol/h/mg protein). Furthermore, the SAAT1 construct pCTK-MAMneo was not able to induce expression of Na+-dependent [3H]uridine uptake (not shown); SAAT1 shares 80% amino acid sequence similarity with SNST1, the Na+/nucleoside cotransporter (7).

Table I demonstrates that COS-7 cells transfected with SAAT1 cDNA via pCTK-MAMneo exhibited a 2-fold increase in Na+-dependent αMGP uptake compared with controls. A similar transport activity was observed at either 24 or 48 h after inducing the murine mammary tumor virus promoter activity of the vector (Table I). Negative controls included COS-7 cells subjected to the electroporation procedure without the addition of vector DNA, and COS-7 cells transfected with either the SGLT1-containing plasmid pMJC-MAMneo or the empty plasmid pMAMneo, under parallel conditions (Table I). The lack of induction of MeAIB cotransport activity by transfection with the closely related SGLT1 cDNA argues against the possibility of nonspecific regulatory effects on endogenous amino acid transport after expression of a foreign recombinant protein in the membrane. However, the possibility that up-regulation of endogenous system A transport is an indirect result of expression of the protein encoded by SAAT1 cannot be ruled out by this approach. As a positive control for the efficiency of the electroporation procedure, cells transfected with the SGLT1 plasmid pMJC-MAMneo were also assayed for Na+-dependent αMGP uptake in each experiment. Na+-dependent MeAIB uptake exhibited a ko of 0.8 ± 0.2 mM in both mock-transfected and SAAT1-transfected COS-7 cells.

MeAIB is a specific substrate for the system A Na+-dependent neutral amino acid transporter (13, 20). Competitive interactions among amino acids for inhibition of MeAIB uptake expressed in the SAAT1 transfectants are consistent with properties described for system A activity (13). Addition of a 20-fold excess of unlabeled alanine resulted in 70% inhibition of the component of MeAIB uptake activity due to cDNA expression, followed in potency by serine (45%), cysteine, proline (34%) > glycine (25%). By contrast leucine, glutamic acid or histidine did not inhibit uptake. The less specific nonmetabolizable analog 2-aminoisobutyric acid, which is transported by systems A, ASC, and L (13), exhibited a 50% increase in transport after expression of SAAT1 in COS-7 cells compared with mock-transfected cells.

In summary, SAAT1, which appears to encode the system A Na+-/amino acid cotransporter, is closely related in sequence to other members of the SGLT1-related gene family, which includes transporters for various substrates including glucose and nucleosides. SAAT1 does not share any sequence homology with any of the Na+-independent amino acid transporters cloned thus far (14–19). The broad tissue distribution of SAAT1 transcripts is consistent with the known ubiquity of system A, although the possibility of tissue-specific isoforms remains to be established. The protein components involved in the system A transport process have not yet been unequivocally identified, although a candidate polypeptide of apparent molecular mass 120–130 kDa copurified with system A transport activity after solubilization and partial purification (34, 35). The identification of SAAT1 will be useful in analysis of the specific gene defect in human inherited disorders of amino acid transport such as Hartnup disease, which affects Na+-dependent neutral amino acid transport in kidney and intestine (36). The system A amino acid transporter is of particular interest due to cellular regulation of its expression by growth state, hormones, and oncogenic transformation (37).

Acknowledgments—We thank Anjali Varde for cell culture and plasmid preparation, Lily Li for mRNA purification, and Beto Zumga for graphics and photography.

REFERENCES