Identification of a Novel Na\(^+\)-independent Acidic Amino Acid Transporter with Structural Similarity to the Member of a Heterodimeric Amino Acid Transporter Family Associated with Unknown Heavy Chains*

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We identified a novel Na\(^+\)-independent acidic amino acid transporter designated AGT1 (aspartate/glutamate transporter 1). AGT1 exhibits the highest sequence similarity (48% identity) to the Na\(^+\)-independent small neutral amino acid transporter Asc (aspartate/glutamate transporter)-2 a member of the heterodimeric amino acid transporter family presumed to be associated with unknown heavy chains (Chairoungdua, A., Kanai, Y., Matsuo, H., Inatomi, J., Kim, D. K., and Endou, H. (2001) J. Biol. Chem. 276, 49390–49399). The cysteine residue responsible for the disulfide bond formation between transporters (light chains) and heavy chain subunits of the heterodimeric amino acid transporter family is conserved for AGT1. Because AGT1 solely expressed or co-expressed with already known heavy chain 4F2hc (4F2 heavy chain) or rBAT (related to \(b_{0,+}\)-amino acid transporter) did not induce functional activity, we generated fusion proteins in which AGT1 was connected with 4F2hc or rBAT. The fusion proteins were sorted to the plasma membrane and expressed the Na\(^+\)-independent transport activity for acidic amino acids. Distinct from the Na\(^+\)-independent cystine/glutamate transporter \(xCT\) structurally related to AGT1, AGT1 did not accept cysteine, homocysteate, and \(\alpha\)-aminoacidopate and exhibited high affinity to aspartate as well as glutamate, suggesting that the negative charge recognition site in the side chain-binding site of AGT1 would be closer to the \(\alpha\)-carbon binding site compared with that of \(xCT\). The AGT1 message was predominantly expressed in kidney. In mouse kidney, AGT1 protein was present in the basolateral membrane of the proximal straight tubules and distal convoluted tubules. In the Western blot analysis, AGT1 was detected as a high molecular mass band in the nonreducing condition, whereas the band shifted to a 40-kDa band corresponding to the AGT1 monomer in the reducing condition, suggesting the association of AGT1 with other protein via a disulfide bond. The finding of AGT1 and Asc-2 has established a new subgroup of the heterodimeric amino acid transporter family whose members associate not with 4F2hc or rBAT but with other unknown heavy chains.

In the past, a large number of amino acid transport systems in mammals have been distinguished based on differences in substrate selectivity and ion dependence (1). For the last decade, molecular cloning approaches have revealed the molecular nature of amino acid transport systems (2). The amino acid transporters identified so far exhibit a variety of substrate selectivity and are composed of the members of several transporter families. Among them, the heterodimeric amino acid transporter family, a subfamily of SLC7, is unique in two aspects (3, 4). First, the members of this family are linked via a disulfide bond with single membrane spanning type II membrane glycoproteins such as 4F2hc (4F2 heavy chain) and rBAT (related to \(b_{0,+}\)-amino acid transporter) (3, 4). 4F2hc is the heavy chain of the cell surface antigen 4F2 (CD98) (5, 6). The 4F2 antigen is a heterodimeric protein composed of two subunits, an ~80-kDa glycosylated heavy chain and a ~40-kDa nonglycosylated light chain (5, 6). The 4F2 light chain has been revealed to be an amino acid transporter. Six proteins have so far been identified to be 4F2 light chains to form transporters subserving systems L, \(\gamma\)-L, \(\alpha\)-L, or asc (7–16). In addition, a member of the heterodimeric amino acid transporter family has been identified that couples with the other type II membrane glycoprotein rBAT to form a system \(b_{0,+}\)-amino acid transporter (17–19). The conserved cysteine residue in the predicted extracellular loop between transmembrane domains 3 and 4 is responsible for the disulfide bond formation between transporter proteins (light chains) and the heavy chains (20). Second, the heterodimeric amino acid transporter family is distinctive for its diversity in the substrate selectivity of its members. As already mentioned, they include transporters for neutral amino acids (systems L and asc), acidic amino acids as well as cysteine (system \(x\), \(\gamma\)-L, and \(b_{0,+}\)) (4).

Recently, we identified a transporter designated Asc-2 (asc-type amino acid transporter) (2) that exhibited relatively low but
significant sequence similarity to the members of the heterodimeric amino acid transporter family (21). Asc-2, however, does not associate with 4F2hc or rBAT and is presumed to link to unknown heavy chains. Although Asc-2 itself is not sorted to the plasma membrane when expressed in Xenopus oocytes, the fusion proteins in which Asc-2 is connected with rBAT or 4F2hc appeared on the plasma membrane and exhibits the functional properties corresponding to those of the transporter subserving Na+-independent small neutral amino acid transport system asc (21). In the present study, we have identified a novel transporter protein structurally related to Asc-2. The transporter is also proposed to associate with an additional protein, presumably through a conserved cysteine residue to form a functional complex. We have generated fusion proteins in which the transporter protein is connected with rBAT or 4F2hc and shown that they appear on the plasma membrane and exhibit the Na+-independent transport activity with distinct selectivity for acidic amino acids.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning of AGT1—The cDNA for a mouse expressed sequence tag (GenBankTM accession number AI314100) showing nucleotide sequence similarity to rat BAT1 (17) was obtained from the Integrated and Molecular Analysis of Genomes and their Expression (IMAGE). The 1.8-kb XhoI fragment was excised from the cDNA IMAGE cDNA clone number 1907807, labeled with [32P]dCTP (T7Quick prime; Amer sham Biosciences), and used as a probe for screening a mouse kidney cDNA library (22). The oligo(dT)-primed cDNA library was prepared from mouse kidney poly(A)+ RNA using the Superscript Choice System (Invitrogen) (23). The synthesized cDNA was ligated to ZipLox EcoRI arms (Invitrogen). Screening of the library and the isolation of positive plaques were performed as described elsewhere (22). The cDNAs in positive ZipLox phages were rescued into the plasmid pZL1 by in vitro excision in accordance with the manufacturer’s instructions (Invitrogen). The cDNA insert was subcloned into pcDNA3.1 (+) (Invitrogen) at a NotI site. The cDNA was sequenced in both directions by the dye terminator cycle sequencing method (PerkinElmer Life Sciences and Applied Biosystems). Transmembrane regions of proteins were predicted based on the SOSUI algorithm (24).

Construction of Fusion Proteins—Fusion proteins were constructed as described elsewhere with some modifications (21). To generate a BG1-rBAT fusion protein, AGT1 cDNA fragment was amplified by PCR using a sense primer corresponding to nucleotides 4–23 of AGT1 cDNA sequence extending at its 5' end by adding a HindIII restriction site and GCGCG (5'-GCGCGAAGCTTACCTATAGGCAGAAACATTC-3') and a reverse primer corresponding to the end of the coding sequence extended at its 3' end by adding a NotI restriction site and ATAAT (5'-ATATGCGGCCGCAATCTTTCTCTTGGATTGTTCTTG-3'). The PCR product was digested with HindIII and NotI and ligated to the HindIII and NotI sites of a mammalian expression vector pcDNA3.1 (+) (Invitrogen). Mouse rBAT cDNA was amplified using a sense primer corresponding to the coding sequence starting just after the start codon (ATG) extended at its 5' end by adding NotI restriction site and ATAT (5'-ATATGCGGCCGCGATAGGGAACAGACGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
The images were acquired using an Olympus Fluoview (FV 500) laser scanning confocal microscope (Olympus Optical, Tokyo, Japan). An Argon laser beam was used for excitation at 488 nm for Alexa Fluor™ 488 visualization. Emission from Alexa Fluor™ 488 was detected via BA505IF filter (30). For absorption experiments, the sections were treated with the primary antibodies in the presence of antigen peptides (200 μg/ml) (21).

Immunohistochemistry—Three-micrometer paraffin sections of mouse kidney were processed for light microscopic immunohistochemical analysis as described previously (17). For immunostaining, the sections were incubated with anti-AGT1 antisera (1:1,000) overnight at 4 °C. Thereafter, they were treated with Envision (+) rabbit peroxidase-diaminobenzidine (DAKO) for 30 min. To detect immunoreactivity, the sections were treated with diaminobenzidine (0.8 mM) (17). For absorption experiments, the tissue sections were treated with the primary antibodies in the presence of antigen peptides (50 μg/ml) (21). The sections were counterstained with hematoxylin.

Western Blot Analysis—The protein samples from mouse kidney were prepared as described elsewhere (21), with minor modifications. Briefly, the mouse kidney was homogenized in 9 volumes of 50 mM Tris-HCl (pH 7.5), 25 mM KCl, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 0.25% sucrose, with 15 strokes of a Dounce homogenizer. The homogenate was centrifuged for 10 min at 8,000 × g, and the supernatant was centrifuged further for 1 h at 100,000 × g. After centrifugation the membrane pellet was resuspended in 0.25 M sucrose, 100 mM KCl, 5 mM MgCl2, and 50 mM Tris (pH 7.4). The protein samples were heated at 100 °C for 5 min in the sample buffer either in the presence or absence of 5% 2-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred electrophoretically to a Hybond-P polyvinylidene difluoride transfer membrane (Amersham Biosciences). The membrane was treated with nonfat dried milk and diluted anti-AGT1 antiserum (1:10,000) and then with horseradish peroxidase-conjugated anti-rabbit IgG as a secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). The signals were detected with an ECL plus system (Amersham Biosciences). To verify the specificity of immunoreactions by absorption experiments, the membranes were treated with primary antibodies in the presence of antigen peptides (50 μg/ml) (17).

Northern Blot Analysis—RNA was prepared from the tissues of 4- to 5-week-old Jcl:ICR male mice and placenta of mice with late pregnancy by the guanidine isothiocyanate method using cesium trifluoroacetate acid (Amersham Biosciences) in accordance with the manufacturer’s instructions. Poly(A) RNA (3 μg/ml) selected by oligo(dT) cellulose chromatography (Amersham Biosciences) was separated on a 1% agarose gel in 0.5 x TBE buffer. The RNA was visualized on a Chemiluminescence Imaging System and the intensity of the bands was measured using a phosphorimager (Mirage Workstation System, Molecular Dynamics, Sunnyvale, CA) by NIH image software (13). The tissue RNA used for Northern blot analysis was digested with RNase A (10 μg/ml) for 30 min at 37 °C and then with RNase T1 (2 μg/ml) for 20 min.

RESULTS

Structural Features of AGT1—A mouse cDNA clone with a 2,141-bp insert was isolated from a mouse kidney cDNA library. It contained an open reading frame from nucleotides 59 to 1,495 encoding a putative 478-amino acid protein, designated as AGT1 (aspartate/glutamate transporter 1). The start of the coding sequence was defined by the first ATG and the surrounding sequences (CTCTCAATGG) corresponding to the Kozak consensus translation initiation sequence (32). The cDNA includes the poly(A) tail (16 As), which starts 23 nucleotide downstream from a typical polyadenylation signal AATAAA at the nucleotide 2,103. The amino acid sequence of AGT1 was identical to that of BCO14684, which was in a GenBank™ data base but not functionally characterized. The AGT1 amino acid sequence exhibited remarkable sequence identity to that of mouse system asc transporter Asc-2 (48% identity), which is presumed to be associated with unknown heavy chains (21). AGT1 also exhibits sequence identity to rat system L transporters, LAT1 (35% identity) (7) and LAT2 (37%) (12), the mouse system asc transporter, Asc-1 (37%) (16), the y-L transporters, rat y-LAT1 (37%) (33) and human KIAA0245/y-LAT2 (36%) (14, 34), the mouse system x_c transporter, xCT (37%) (15), and the rat system h_b+ transporter, BAT1 (36%) (17), all of which are associated with either 4F2hc or rBAT (Fig. 1). AGT1 also exhibited significant sequence identity to the system y+ transporters, CAT1-4 (30%) from mice and humans (35) and to the amino acid permeases from bacteria and yeast (e.g. 30% identity to Saccharomyces cerevisiae methionine permease MUP1 (36)).

As shown in Fig. 1, 12 transmembrane regions were predicted on the AGT1 amino acid sequence. There is a conserved cysteine residue (AGT1 amino acid residue 129) in the putative extracellular loop between predicted transmembrane domains 3 and 4, through which LAT1, LAT2, Asc-1, y-LAT1, y-LAT2, xCT, and BAT1/h_b+AT are proposed to link to 4F2hc or rBAT via a disulfide bond (20). Protein kinase C-dependent phosphorylation sites and a tyrosine phosphorylation site are predicted in the putative intracellular domains. A CaM-dependent phosphorylation site is predicted in the putative intracellular loops that is conserved between AGT1 and Asc-2 (see legend for Fig. 1).

Functional Expression of AGT1—The expression of AGT1 did not induce functional activity in Xenopus oocytes (Fig. 2a) or COS-7 cells (Fig. 2b). Therefore, AGT1 was coexpressed with 4F2hc or rBAT, because AGT1 exhibited structural similarity to the members of the heterodimeric amino acid transporter family. The coexpression of AGT1 and 4F2hc, however, did not induce amino acid transport activity in Xenopus oocytes (Fig. 2a). This result was confirmed in COS-7 cells in which the coexpression of AGT1 with 4F2hc or rBAT did not induce amino acid transport activity (Fig. 2b). Then, following the functional characterization of Asc-2 (21), we generated fusion proteins in which the C terminus of AGT1 was connected with the N terminus of 4F2hc or rBAT. When expressed in Xenopus oocytes, AGT1–4F2hc and AGT1-rBAT fusion proteins exhibited [14C]-l-aspartate uptake (Fig. 2a).

To examine whether the fusion proteins were expressed in the oocyte plasma membrane, we performed confocal immunofluorescence microscopic analysis using specific antibodies raised against C-terminal parts of AGT1 and 4F2hc. As shown in Fig. 3 (a and b), these antibodies did not exhibit specific staining in the control oocytes injected with water instead of cRNAs. When AGT1 was solely expressed, AGT1 protein did not appear on the plasma membrane (Fig. 3d). In contrast, when the AGT1–4F2hc fusion protein was expressed in Xenopus oocytes, both anti-4F2hc antibody and anti-AGT1 antibody recognized the immunoreactivity on the plasma membrane (Fig. 3, e and f), indicating that the AGT1–4F2hc fusion protein is expressed in the plasma membrane. In the absorption experiments in which the tissue sections were treated with the primary antibodies in the presence of their antigen peptides, the immunostainings were not detected, confirming the specificity of the immunoreactions (data not shown).

Transport Properties—When expressed in Xenopus oocytes, AGT1–4F2hc and AGT1-rBAT fusion proteins mediated the Na+ - and Cl–-independent transport (Fig. 4). The uptake of [14C]-l-aspartate mediated by the fusion proteins was saturable and followed Michaelis-Menten kinetics (Fig. 5). The K_m values for l-aspartate were calculated to be 25.5 ± 5.9 μM in AGT1–4F2hc fusion protein and 20.1 ± 6.1 μM in AGT1-rBAT fusion protein.

Substrate Selectivity—The substrate selectivity of the AGT1–4F2hc fusion protein and the AGT1-rBAT fusion protein was investigated by inhibition experiments in which the uptake of 20 μM L-aspartate was measured in the presence of 2 mM of nonlabeled amino acids. As shown in Fig. 6, the...
L-aspartate uptake by the oocytes expressing AGT1–4F2hc fusion protein or AGT1–rBAT fusion protein was markedly inhibited by acidic amino acids such as L-aspartate and L-glutamate. L-Cysteine exhibited weaker but significant inhibitory effect on the [14C]L-aspartate uptake. Other L-/H9251-amino acids including neutral amino acids and basic amino acids/H9252- alanine and H9253- aminobutyric acid did not inhibit [14C]L-aspartate uptake mediated by AGT1–4F2hc fusion protein (Fig. 6). D-Amino acids such as D-aspartate, D-glutamate, D-asparagine, D-glutamine, D-alanine, D-serine, and D-valine did not exhibit significant inhibitory effects on the [14C]L-aspartate uptake. The [14C]L-aspartate uptake was not affected by the system L-specific inhibitor 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid or the system A-specific inhibitor (aminomethyl)isobutyric acid (Fig. 6).

Consistent with the results from the inhibition experiments, high uptake levels of 14C-labeled L-aspartate and L-glutamate were observed for the AGT1–4F2hc fusion protein (Fig. 7). The uptake of neutral amino acids including L-cysteine and L-cystine and basic amino acids such as L-arginine, L-glutamine, L-lysine, and L-histidine was not inhibited by 20 μM [14C]L-aspartate (Fig. 7). The [14C]L-aspartate uptake was not affected by the system L-specific inhibitor 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid or the system A-specific inhibitor α-(aminomethyl)isobutyric acid (Fig. 7).

The effects of acidic amino acid analogues on AGT1-mediated transport were also investigated by inhibition experiments in which the uptake of 20 μM [14C]L-aspartate was measured in the presence of 2 mM nonlabeled acidic amino acid analogues. As shown in Fig. 8, the [14C]L-aspartate uptake by the oocytes expressing the AGT1–4F2hc fusion protein was markedly inhibited by threo-H9252-hydroxyaspartate, L-serine-O-sulfate, L-cysteine sulfinate, and L-cysteate as well as L-aspartate and L-glutamate, whereas it was not inhibited by L-aminoadipate, L-homocysteate, L-trans-pyrrolidine-2,4-dicarboxylate, and dihydrokainate.

Tissue Distribution of Expression—The expression of AGT1 mRNA was analyzed by Northern blotting of poly(A)+ RNAs from various mouse tissues. A strong 2.2-kb band was detected only in the kidney (Fig. 9).

Protein Characterization under Nonreducing and Reducing Conditions—Western blot analyses were performed on the membrane fraction prepared from mouse kidney in the presence of 2 mM nonlabeled acidic amino acid analogues. As shown in Fig. 8, the [14C]L-aspartate uptake by the oocytes expressing the AGT1–4F2hc fusion protein was markedly inhibited by three-β-hydroxyaspartate, L-serine-O-sulfate, L-cysteine sulfinate, and L-cysteate as well as L-aspartate and L-glutamate, whereas it was not inhibited by L-α-aminoadipate, L-homocysteate, L-trans-pyrrolidine-2,4-dicarboxylate, and dihydrokainate.

**Fig. 1.** Sequence alignment of AGT1 and the structurally related transporters. The deduced amino acid sequence of AGT1 (mouse) is shown aligned with those of system asc transporter Asc-2 (mouse) (12), system L transporter LAT1 (rat) (7), system γ-L transporter γ-LAT1 (rat) (33), system xC transporter xCT (mouse) (15), and system b0,–transporter BAT1 (rat) (17). Identical residues in at least two sequences are shaded. Predicted transmembrane regions of AGT1, numbered 1–12, are shown by bold lines above the sequences. The conserved cysteine residue (AGT1 amino acid residue 129) in the predicted extracellular loop, through which LAT1, γ-LAT1, xCT, and BAT1 are proposed to link to 4F2hc or rBAT is indicated by an asterisk. Protein kinase C-dependent phosphorylation sites are predicted on the AGT1 sequence at residues 5, 36, 282, 312, and 419, among which the ones at 5 and 312 are predicted intracellularly (labeled with +). A potential cAMP-dependent phosphorylation site is located at residue 237, which is predicted to be intracellular (labeled with #). A tyrosine phosphorylation site is predicted at residue 17 (labeled with &). Although a potential N-glycosylation site is located at residue 259, it is predicted to be in the membrane-spanning region. The residue numbers indicated above the aligned sequences are in reference to those in the amino acid sequence of AGT1.

| AGT1 | Asc-2 | LAT1 | γ-LAT1 | xCT | BAT1 |
|------|-------|------|--------|-----|------|
| H     | H     | H     | H       | H   | H    |
| 1     | 1     | 1     | 1       | 1   | 1    |
| 2     | 2     | 2     | 2       | 2   | 2    |
| 3     | 3     | 3     | 3       | 3   | 3    |
| 4     | 4     | 4     | 4       | 4   | 4    |
| 5     | 5     | 5     | 5       | 5   | 5    |
| 6     | 6     | 6     | 6       | 6   | 6    |
| 7     | 7     | 7     | 7       | 7   | 7    |
| 8     | 8     | 8     | 8       | 8   | 8    |
| 9     | 9     | 9     | 9       | 9   | 9    |
| 10    | 10    | 10    | 10      | 10  | 10   |
| 11    | 11    | 11    | 11      | 11  | 11   |

**[14C]L-aspartate uptake by the oocytes expressing AGT1–4F2hc fusion protein or AGT1–rBAT fusion protein was markedly inhibited by acidic amino acids such as L-aspartate and L-glutamate.**
bands disappeared in the presence of antigen peptides in the absorption experiment, confirming the specificity of immunoreactions (data not shown).

**Immunolocalization of AGT1 in the Mouse Kidney**—Immunohistochemical analysis on mouse kidney revealed the strong immunoreactivity for AGT1 in the proximal tubules in the outer medulla and the distal tubules in the cortex (Fig. 11a). AGT1 immunoreactivity appeared to be localized on the basolateral membrane of the proximal tubule S3 segments (Fig. 11c) and the distal convoluted tubules (Fig. 11d). In the absorption experiments in which the tissue sections were treated with the primary antibodies in the presence of antigen peptides, the immunostaining was not detected, confirming the specificity of the immunoreactions (Fig. 11b).

**DISCUSSION**

In the present study, we identified a novel transporter AGT1 that exhibits structural similarity to Asc-2, a member of the heterodimeric amino acid transporter family presumed to be associated with unknown heavy chains. By generating fusion proteins with 4F2hc or rBAT, we were able to express AGT1 on Xenopus oocyte plasma membrane. AGT1 transported acidic amino acids at high affinity in a Na$^+$-dependent manner.

In the family of heterodimeric amino acid transporters, the transporter proteins are linked via a disulfide bond to the single membrane spanning heavy chains 4F2hc or rBAT (7–19). Among the members of this family, a cysteine residue is conserved in the extracellular loop between predicted transmembrane domains 3 and 4 (Fig. 1). Through this cysteine residue, the transporter proteins of this family are proposed to form a disulfide bond with heavy chains (20). The cysteine residue is also conserved in AGT1 as well as Asc-2 (Fig. 1). It is therefore predicted that AGT1 is linked to the other protein(s) by forming a disulfide bond through the conserved cysteine residue.

For the members of the heterodimeric amino acid transporter family, the association with the heavy chain type II membrane glycoproteins is required for the light chain transporter proteins to be sorted to the plasma membrane (8, 18, 37). In the previous investigation, we showed that the fusion proteins in which Asc-2 was connected with 4F2hc or rBAT were sorted to the plasma membrane and exhibited their functions, although Asc-2 was not functional when solely expressed or coexpressed with 4F2hc or rBAT (21). This indicates that 4F2hc and rBAT are capable of supporting the membrane sorting of the light chain subunit transporter proteins when their fusion proteins are constructed even though not between the right partners. AGT1 is also proposed to require additional associating proteins similar to 4F2hc or rBAT but not 4F2hc and rBAT themselves, because AGT1 was not functional when solely expressed or coexpressed with 4F2hc or rBAT (Fig. 2) and also because AGT1 is not colocalized with 4F2hc or rBAT in kidney in vivo; AGT1 is expressed in the basolateral membrane of proximal straight tubules and distal convoluted tubules (Fig. 11), whereas rBAT is present in the apical membrane of proximal tubules, and 4F2hc is most densely expressed in the basolateral membrane of proximal convoluted tubules (17, 19, 31, 38). We thus generated fusion proteins in which the C terminus of AGT1 is connected with the N terminus of 4F2hc or rBAT to examine the functional properties of AGT1. The fusion proteins in fact appeared on the plasma membrane and exhibited the transport activity when expressed in Xenopus oocytes (Figs. 2 and 3, e and f).

The fusion proteins of heavy chain and light chain subunits of heterodimeric amino acid transporters were first generated by Pfeiffer et al. (19) for the characterization of system b$^{0,+}$ transporter b$^{0,+}$AT/BAT1 in Xenopus oocytes. They showed that the fusion protein in which the C terminus of the light chain b$^{0,+}$AT/BAT1 was connected with the N terminus of its associating heavy chain rBAT was functional and exhibited the identical properties to those obtained by the coexpression of b$^{0,+}$AT/BAT1 and rBAT (19). In addition, the mutant fusion protein whose light chain portion was mutated was not functional, confirming that the detected transport activity was due to the fusion protein itself and not to the associated oocyte endogenous light chain (19). For the characterization of Asc-2,
we generated fusion proteins in which the C terminus of Asc-2 is connected with the N terminus of 4F2hc or rBAT (21). We showed that two Asc-2 fusion proteins connected with rBAT or 4F2hc exhibited basically identical properties in their ion dependence, affinity, and substrate selectivity, suggesting that the rBAT or 4F2hc portion of the fusion proteins does not affect the transport properties of light chain transporter subunits (21). Consistent with this, LAT1 fusion proteins connected with 4F2hc or rBAT exhibited substrate selectivity and affinity basically identical to those obtained by the coexpression of LAT1 and its partner heavy chain 4F2hc (21). Therefore, it is suggested that to generate fusion proteins with 4F2hc or rBAT exhibited substrate selectivity and affinity basically identical to those obtained by the coexpression of LAT1 and its partner heavy chain 4F2hc (21). In the present study, the fusion proteins AGT1–4F2hc and AGT1-rBAT were also shown to exhibit basically identical transport properties. Therefore, it is suggested that to generate fusion proteins with 4F2hc or rBAT is a useful strategy to examine the functional properties at least for the heterodimeric amino acid transporters and their related proteins.

The AGT1–4F2hc and AGT1-rBAT fusion proteins exhibited Na\(^+\) and Cl\(^-\)-independent transport of acidic amino acids (Fig. 4). Furthermore, the AGT1–4F2hc fusion protein showed high affinity to L-aspartate and L-glutamate. For mammalian acidic amino acid transport systems, four transport systems have been characterized so far: Na\(^+\)-dependent X\(^-\)\(_{AG}\) and X\(^-\)\(_{A}\) and Na\(^+\)-independent x\(^-\)\(_{G}\) and x\(^-\)\(_{C}\) (1). X\(^-\)\(_{AG}\) transports both glutamate and aspartate, whereas X\(^-\)\(_{A}\) largely excludes glutamate

![Figure 4](image-url) | ![Figure 5](image-url)
and longer analogues. \( x^-_G \) transports glutamate and its analogues, largely excluding aspartate and short analogues. \( x^-_C \) is similar to \( x^-_G \) except that it transports cystine as well as glutamate (1). The functional properties of AGT1 is, however, not able to be assigned to any of these classically characterized amino acid transport systems. The reason for this is probably in its restricted localization in the basolateral membrane of renal tubules (Figs. 9 and 11). In addition, \( \text{Na}^+ \)-dependent transport systems for acidic amino acids were present not only in the apical membrane but also in the basolateral membrane of the tubular epithelial cells (39), which may mask the contribution of \( \text{Na}^+ \)-independent transport system in the basolateral membrane. The transporters subserving systems \( X^-_{A,G} \) and \( x^-_C \) have been identified so far by molecular cloning approaches (15, 40). Five isoforms of \( \text{Na}^+ \)-dependent high affinity glutamate transporters, which belong to SLC1 family, and \( \text{Na}^+ \)-independent cystine/glutamate transporter \( xCT \), which belongs to SLC7 family, have been identified for systems \( X^-_{A,G} \) and \( x^-_C \), respectively (15, 40, 41).

\( xCT \) is a heterodimeric amino acid transporter that is associated with 4F2hc (15). \( xCT \) accepts \( \text{l}-\text{glutamate}, \text{l}-\text{homocysteate}, \text{and l-cysteine. l-Aspartate is not transported at high rate by xCT (15). It is proposed that the substrate-binding site of xCT possesses a negative charge recognition site in the side chain-binding site so that xCT recognizes amino acids as anions. Thus, the length of the side chain of substrate amino acids plays a crucial role in substrate recognition.}

Fig. 6. Inhibition of the [\(^{14}\text{C}]\text{l-aspartate uptake by various amino acids. The [\(^{14}\text{C}]\text{l-aspartate uptake (20 \muM) mediated by AGT1–4F2hc fusion protein (a) or AGT1–rBAT fusion protein (b) was measured in the presence of 2 mM nonradioiodinated indicated amino acids. The uptake was measured in the Na\(^+\)-free uptake solution, and the values are expressed as percentages of the control l-aspartate uptake in the absence of inhibitors (–). The l-aspartate uptake was inhibited by l-aspartate, l-glutamate, and l-cysteine. The asterisks indicate statistical significance. **, \( p < 0.01 \), Student's unpaired t test.}
acids, namely the distance between the α-carbon and the negative charge on the side chain, would be an important determinant for the amino acids to be accepted by the substrate binding site of xCT (4). It is therefore understandable that glutamate and homocysteate are well accepted by xCT, whereas aspartate, which has a shorter side chain, is not. The distance between two carboxyl groups of L-cystine is probably well suited to meet the requirement, so that cystine is proposed to be recognized as an anionic amino acid to be accepted by the binding site (4).

Although AGT1 is structurally related to xCT, AGT1 exhibits a remarkable difference in the selectivity for acidic amino acid substrates. In contrast to xCT, AGT1 well accepts acidic amino acids with shorter side chains such as L-aspartate, threo-hydroxyaspartate, L-serine-O-sulfate, L-cysteine sulfinate, and L-cysteate (Figs. 7 and 8). AGT1 does not accept acidic amino acids with longer side chains such as L-homocysteate, L-trans-pyrrolidine-2,4-dicarboxylate (PDC), and dihydrokainate (DHK). The asterisks indicate statistical significance. **, p < 0.01, Student’s unpaired t test.

Hydroxyaspartate, L-serine-O-sulfate, L-cysteine sulfinate, L-cysteate, and L-glutamate (Figs. 7 and 8). AGT1 does not accept acidic amino acids with longer side chains such as L-homocysteate and L-α-amino adipate (Fig. 8). L-Cystine is not transported by AGT1. Again, the length of the side chain of acidic amino acids is an important determinant for substrates of AGT1. It is predicted that the negative charge recognition site in the side...
chain-binding site of AGT1 is closer to the α-carbon binding site than that of xCT. It is interesting that Asc-2 structurally related to AGT1 also accepts amino acids with short side chains, although Asc-2 prefers neutral amino acids (21). It is proposed that, although the substrate-binding site of AGT1 possesses similar spatial configuration to that of Asc-2, AGT1 has acquired the additional mechanisms for negative charge recognition in the course of evolution.

Na⁺-dependent high affinity glutamate transporters transport not only L-glutamate and l-aspartate but also d-aspartate (40), whereas AGT1 is quite stereoselective for both aspartate and glutamate (Figs. 6 and 7). Although five isoforms of Na⁺-dependent high affinity glutamate transporters exhibit remarkable differences in the inhibitor selectivity, l-trans-pyrrolidine-2,4-dicarboxylate is accepted by all of the isoforms and in general is regarded as a selective inhibitor for Na⁺-dependent high affinity glutamate transporters (40, 41). Dihydrokainate and L-α-aminoadipate are selective for the isoforms GLT-1/EAAT2 and EAAT4, respectively (40, 42–44). AGT1-mediated l-aspartate uptake is not affected by these inhibitors for Na⁺-dependent high affinity glutamate transporters. AGT1 is thus supposed to possess quite distinct mechanisms of substrate recognition compared with Na⁺-dependent high affinity glutamate transporters.

AGT1 is only expressed in kidney. In the immunohistochemical analysis, AGT1 immunoreactivity was detected in the basolateral membrane of the proximal straight tubules, particularly S3 segments in the outer medulla, and of the distal tubules in the cortex (Fig. 11, a, c, and d). In the S2 and S3 segments of the proximal tubules, Na⁺-dependent high affinity glutamate transporter EAAC1 is present in the apical membrane (45). EAAC1 plays a critical role in the reabsorption of acidic amino acids from the luminal fluid because EAAC1 knockout mice exhibit acidic amino aciduria (46). Although the level of expression is less than that in the proximal tubules, EAAC1 is also present in the apical membrane of the distal convoluted tubules in agreement with previous physiological studies showing significant glutamate reabsorption distal to the proximal tubules, including the distal convoluted tubules (45, 47, 48). Although the functional role of AGT1 is not clear at this moment, considering the distribution of AGT1 along nephron segments apparently corresponding to that of EAAC1, it is speculated that AGT1 might function as an exit path for the acidic amino acids at the basolateral membrane of tubular epithelial cells in the reabsorption of acidic amino acids from the luminal fluid. It is also possible that AGT1 might contribute to provide tubular epithelial cells with metabolically important acidic amino acids from basolateral side, although Na⁺-dependent glutamate transport systems with higher concentrating capability have been reported to be present in the basolateral membrane (39).

In the Western blot that we performed on mouse kidney, a high molecular mass band detected in the nonreducing condition shifted to the lower molecular mass band, which seems to correspond to the AGT1 monomer in the reducing condition (Fig. 10). This observation is interesting because AGT1 is proposed to be linked to the other protein by a disulfide bond through the conserved cysteine residue, although it is still unclear at this stage whether the high molecular mass band in the nonreducing condition is because AGT1 forms a heteromeric complex or because AGT1 oligomerizes with other cysteine residues.

In summary, we identified and characterized a novel amino acid transporter AGT1 with structural similarity to the members of heterodimeric amino acid transporter family particularly Asc-2 that is proposed to be associated with unknown heavy chains (21). AGT1 exhibits distinct Na⁺-independent transport activity with substrate selectivity for acidic amino acids. Similar to Asc-2, AGT1 appears to be associated with unknown protein(s) other than 4F2hc or rBAT. The finding of AGT1 has established a subgroup of the heterodimeric amino acid transporter family, which includes transporters such as Asc-2 and AGT1 associated not with 4F2hc or rBAT but with other unidentified proteins.

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