Molecular cloning of mouse amino acid transport system $B^0$, a neutral amino acid transporter related to Hartnup disorder

Angelika Bröer*, Karin Klingel†, Sonja Kowalczuk*, John E.J. Rasko‡, Juleen Cavanaugh§ and Stefan Bröer*

*School of Biochemistry & Molecular Biology, Australian National University, Canberra, ACT 0200, Australia
† Department of Molecular Pathology, University of Tübingen, 72076 Tübingen, Germany
‡Gene Therapy Research, Centenary Institute of Cancer Medicine and Cell Biology, University of Sydney and Sydney Cancer Centre, Royal Prince Alfred Hospital
§ Medical Genetics Research Unit, The Canberra Hospital, Woden ACT 2607, Australia

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Corresponding author:
Dr. Stefan Bröer
Division of Biochemistry & Molecular Biology
Faculty of Science
Australian National University
Canberra, ACT 0200, Australia
Tel.: +61-2-6249-2540
Fax.: +61-2-6249-0313
e-mail.: stefan.broeer@anu.edu.au

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Summary

Resorption of amino acids in kidney and intestine is mediated by transporters, which prefer groups of amino acids with similar physico-chemical properties. It is generally assumed that most neutral amino acids are transported across the apical membrane of epithelial cells by system B\(^0\). Here we have characterised a novel member of the Na\(^+\)-dependent neurotransmitter transporter family (B\(^0\)AT1) isolated from mouse kidney, which shows all properties of system B\(^0\). Flux experiments showed that the transporter is Na\(^+\)-dependent, electrogenic and actively transports most neutral amino acids but not anionic or cationic amino acids. Superfusion of mB\(^0\)AT1 expressing oocytes with neutral amino acids generated inward currents, which were proportional to the fluxes observed with labelled amino acids. In situ hybridisation showed strong expression in intestinal microvilli and in the proximal tubule of the kidney. Expression of mouse B\(^0\)AT1 was restricted to kidney, intestine and skin. It is generally assumed that mutations of the system B\(^0\) transporter underlie autosomal recessive Hartnup disorder. In support of this notion mB\(^0\)AT1 is located on mouse chromosome 13 in a region syntenic to human chromosome 5p15, the locus of Hartnup disorder. Thus the human homologue of this transporter is an excellent functional and positional candidate for Hartnup disorder.
Introduction

Epithelial resorption of amino acids across the apical membrane in the kidney and intestine is thought to be carried out by four different transporters (1). Anionic amino acids are taken up by a Na⁺-dependent aspartate/glutamate transporter, which has been designated system XAG. Molecular cloning has identified this transporter as EAAT3 (2). Cationic amino acids are taken up by system b₀,⁺; the molecular correlate of this transporter being the heteromeric amino acid transporter rBAT/b₀,⁺AT (3). Proline and glycine are thought to be transported by the IMINO system (4) and it has recently been proposed that the molecular correlate of this transporter may be the proton-dependent amino acid transporter PAT1 (5). Most neutral amino acids are thought to be transported by system B₀, which has not yet been identified at the molecular level (6,7). System B₀ has been characterized in jejunal brush border vesicles (8-10), bovine epithelial cells (11) and Caco-2 cells (12). These studies suggest that system B₀ is a Na⁺-dependent, chloride independent transporter that accepts a wide variety of neutral amino acids. Failures to resorb amino acids in the kidney and intestine underlie a number of inherited transporter diseases, with mutations in either rBAT or b₀,⁺AT causing cystinuria (13), mutations of the IMINO system believed to cause iminoglycinuria (14) and mutations of system B₀ thought to cause Hartnup disorder (15).

Hartnup disorder [MIM 234500] is an autosomal recessive disorder resulting from impaired neutral amino acid transport, largely limited to the kidneys and the small intestine. Its diagnostic hallmark is a striking neutral hyperaminoaciduria (15,16). A number of additional symptoms have been reported in Hartnup disorder patients, including photosensitive skin rash, ataxia and psychotic behaviour (15,16). Nevertheless, many of those affected remain asymptomatic despite the hyperaminoaciduria. This variation in manifestations of Hartnup disorder may be a consequence of compensation for the reduced
amino acid transport by increased peptide transport in the intestine or the presence of genetic or environmental modifiers, such as dietary variations. Despite its apparently mild phenotype, Hartnup disorder has been a model disease, because it illustrates the principles of amino acid resorption in epithelial cells. Homozygosity mapping in consanguineous families has recently localized the disorder to Chromosome 5p15 (17).

Here we describe the cloning and characterization of a neutral amino acid transporter from mouse kidney, which belongs to the family of Na\(^+\) and Cl\(^-\) dependent neurotransmitter transporters, has all the functional hallmarks of system B\(^0\), and is expressed mainly in kidney and intestine. Its human homologue is found on chromosome 5p15. It is suggested that the human homologue of this gene is an excellent candidate for Hartnup disorder.
Materials and Methods

cDNA cloning and plasmids

Total RNA was isolated from mouse kidney by the acid-guanidinium-thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (18). For cloning of mouse \( \text{B}^0\text{AT1} \), 0.5 µg oligo(dT)\(_{15} \) was added to 1.5 µg kidney RNA in a total volume of 4.5 µl. The mixture was incubated for 10 min at 65°C and then chilled on ice. Reverse transcription was carried out in a buffer designed to improve the temperature stability of reverse transcriptase (19). The reaction was assembled by adding the following components to the RNA mixture: 2 µl 0.1 M DTT, 1 µl dNTPs (each 10 mM), 0.5 µl 40 x reverse transcriptase buffer, 30 U RNaseout (Invitrogen, Mulgrave, VIC, Australia), 10 µl 1.8 M trehalose, 2 µl 100% glycerol. The mixture (20 µl) was then incubated at 42°C for 2 min. For cDNA synthesis 200 U of Superscript II-RT (Invitrogen, Mulgrave, VIC, Australia) was added followed by incubation at 55°C for one hour. The cDNA was purified by using a PCR-product purification kit (Qiagen, Clifton Hill, VIC, Australia). For PCR a proofreading polymerase was used (Pfu polymerase, Promega, Madison, Wisconsin, USA). The coding sequence was amplified during 30 PCR cycles with the temperature profile 94°C 30 sec, 55°C 60 sec and 72°C 12 min using the sense primer 5' GAC ACA ACC ACT TGC CCT TT and the antisense primer 5' GTC CTG CAT CTT GCT TCC TC. The final 11 base pairs of the sense primer correspond to bases 1-11 of the mouse cDNA clone XM_127449 (Riken cDNA 4632401C08). The antisense primer corresponds to bases 2005-2024 of the same cDNA clone. The amplified PCR product was purified by agarose gel electrophoresis and the 2033 bp fragment isolated using a gel elution kit (Qiagen, Clifton Hill, VIC, Australia). The isolated PCR fragment was initially cloned using the Zero Blunt TOPO PCR cloning kit (Invitrogen, Mulgrave, VIC, Australia). The mouse \( \text{B}^0\text{AT1} \) sequence was determined by BigDye Terminator v3.1 Cycle Sequencing using conditions recommended by the manufacturer (Applied Biosystems Foster...
City, CA, USA) and a ABI 3730 capillary genetic analyser (Biomolecular Resource Facility, Australian National University). Its sequence is deposited as AJ633679 in the EMBL database. For expression studies, mouse B0AT1 was excised with HindIII-XbaI and inserted into the same sites of the oocyte expression vector pGEM-He-Juel. (20).

**Oocytes and injections**

Oocyte isolation and management have been described in detail elsewhere (21). For expression in oocytes mouse B0AT1 in pGem-He-Juel was linearised with SalI and transcribed *in vitro* using the T7 mMessage mMachine Kit (Ambion, Austin Texas, USA). Oocytes were injected with 20 ng of cRNA encoding mouse B0AT. Transport measurements were carried out after 3-6 days of expression.

**Flux measurements**

For each determination, groups of 7-10 cRNA or non-injected oocytes were washed twice with 4 ml ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES); titrated with NaOH to pH 7.4 unless indicated otherwise) before incubation at room temperature in a 5 ml polypropylene tube containing 70 µl of the same buffer supplemented with [14C] labelled amino acids and different amounts of unlabeled substrate. Transport was stopped after different time intervals by washing oocytes three times with 4 ml ice-cold ND96 buffer. Single oocytes were placed into scintillation vials and lysed by addition of 200 µl 10% SDS. After lysis, 3 ml scintillation fluid was added, and the radioactivity determined by liquid scintillation counting. In Na+-free incubation buffer NaCl was replaced by NMDG-Cl or LiCl. To determine the dependence of transport activity on the Na+ concentration NaCl-ND96 (pH 7.4) was mixed with NMDG-Cl-ND96 (pH 7.4, NaCl replaced by NMDG-Cl) in different proportions. Different pH values were adjusted by mixing MES-buffered ND96 (5 mM HEPES replaced by 5 mM
Morpholinoethanesulfonic acid) with Tris-buffered ND96 (5 mM HEPES replaced by 5 mM Tris-base). Uptake of $^{14}$Cphenylalanine or $^{14}$Cleucine increased linearly with time for up to 20 min. As a result, uptake was determined using an incubation period of 15 min. To determine substrate specificity the following compounds were used (all Amersham Pharmacia Biotech, Castle Hill, Australia): $[{U-14}^{14}$C]phenylalanine, $[{U-14}$C]glutamine, $[{U-14}$C]alanine, $[{U-14}$C]leucine, $[{U-14}$C]glycine, $[{U-14}$C]arginine, $[{U-14}$C]glutamate, $[{U-14}$C]histidine, $[{U-14}$C]proline and $[{U-14}$C]isoleucine. For competition experiments uptake of 100 µM labeled amino acid was challenged with an excess of 20 mM unlabelled amino acid.

**Electrophysiological recordings**

Amino acid induced currents were analysed by two-electrode voltage clamp recording. The recordings were performed with 1 x LU and 10 x MGU headstages connected to a Geneclamp 500B electronic amplifier (Axon Instruments, Union City, CA, USA). The output signal was amplified ten times and filtered at 50 Hz. The analog signal was converted into digital figures by a Digidata 1322A (Axon Instruments, Union City, CA, USA) and data were sampled at 3Hz using pCLAMP software (Axon Instruments, Union City, CA, USA). Oocytes were chosen that had a membrane potential of more negative than -30 mV. Once a stable membrane potential was reached under current clamp conditions, the amplifier was switched to voltage clamp mode holding the oocytes at -50 mV. Oocytes were superfused with ND96 solution with or without saturating amounts of amino acids (5 mM). A complete change of the bath to a new solution was accomplished in about 10 sec.

**RT-PCR**

Total RNA was isolated from male adult NRMI mouse tissues by the acid-guanidinium-thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (18). For reverse transcription 0.5 µg oligo(dT)$_{15}$ was added to 2 µg total RNA in a total volume of 12
µl. The mixture was incubated for 10 min at 65°C and then chilled on ice. DTT, dNTPs, 5 x reverse transcriptase buffer and 30 U RNasin (Promega, Madison, Wi; USA) were added and the whole mixture (20µl) was incubated at 42°C for 2 min. For cDNA synthesis 200 U of Superscript II-RT (Invitrogen, Mulgrave, VIC, Australia) was added followed by incubation at 42°C for one hour. A standard PCR protocol with 100 pmol of each primer and a 2µl aliquot of the purified cDNA was used for amplification of the fragments during 40 cycles (95°C 30 sec; 55°C 1 min; 72°C, 1 min) in a Thermocycler using Taq-polymerase (Qiagen, Clifton Hill, VIC, Australia). After amplification the samples were analysed by agarose gel electrophoresis. The mB0AT1 specific fragment was amplified with sense primer: 5’ CTTCATGGTGGCCTGTACT (corresponding to positions 428-447 of XM_127449) and antisense primer: 5’ GTCCGTCCATCTGGCTCTCTC (corresponding to positions 2005-2025 of XM_127449). To determine the identity of the amplified fragment it was cloned into the pCR-XL-TOPO vector and its sequence was determined (Biomolecular Resource Facility, Australian National University). From both kidney and intestine, six clones were isolated and all sequences were found to be identical to that of XM_127449. A 1kb actin cDNA fragment was amplified during 30 cycles as a control using the sense primer 5’ GCT CAC CAT GGA TGA TGA TAT CGC 3’ and the antisense primer 5’ GGA GGA GCA ATG ATC TTG ATC TTC 3’.

In situ hybridization

Tissue specimens of kidney, liver, lung, pancreas, small intestine, skeletal muscle, lymph nodes and brain from C57BL/6, SWR/J and DBA1/J mice were fixed in 4% paraformaldehyde /0.1 M sodium phosphate buffer (pH 7.2) for 4 hours and embedded in paraffin. Five µm tissue sections were dewaxed and hybridized as previously described (22). Hybridization probes were generated by in vitro transcription of mB0AT1 cDNA cloned into pCR-blunt II-TOPO using SP6 polymerase (antisense) and T7 polymerase (sense). The hybridization mixture (10 mM Tris HCl,
pH 7.4, 50% (vol/vol) deionized formamide, 600 mM NaCl, 1 mM EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.05% bovine serum albumin, 10% dextrane sulfate, 10 mM dithiothreitol, 200 µg/ml denatured sonicated salmon sperm DNA, 100 µg/ml rabbit liver tRNA) contained either the 35S-labeled RNA antisense or sense control mB0AT1 probe at a concentration of 500 ng/ml. Hybridization with RNA probes proceeded at 42ºC for 18 hr. Following washing steps, the slide preparations were dipped in NTB2 emulsion (Kodak, Rochester, NY) and exposed at 4°C for 3 weeks. After development the slides were stained with hematoxylin/eosin and photographed with a Sony DSC digital camera.

Calculations, statistics and computer analysis
Each datapoint or bar in figures and tables represents the mean ± SD activity of m = 7-10 mB0AT1 expressing oocytes minus the mean ± SD activity of m = 7-10 non-injected oocytes. Kinetic constants were derived by non-linear curve fitting of the means to the corresponding equation using Origin7.0 software (OriginLab corporation, Northampton, MA, USA). To determine $K_m$ and $V_{max}$ the Michaelis-Menten equation $v = (V_{max} * S/(K_m + S))$ was used. The cotransport stochiometry was determined by using a linear form of the Hill equation:

$$\log\left(\frac{v}{(V_{max} - v)}\right) = n * \log [S] + \log K_m.$$  

The number “n” of independent experiments is indicated in the figure legends. Throughout the manuscript we show representative figures from one of these experiments.

Sequence alignments and the tree were calculated using programs of GCG and PHYLIP packages supplied by the Australian National Genomic Information Service (ANGIS). Sequence alignment was performed using ClustalW (23). Subsequently, protein distance was calculated by using the Dayhoff PAM matrix (24) and converted into a tree diagram using an additive tree model. The peptide sequence of mB0AT1 was analysed by hydropathy plotting using the TMHMM 2.0 program (http://www.cbs.dtu.dk/services/TMHMM-2.0) and the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html). The structural plot was
Results

Cloning of mouse $B^\theta AT1$

Hydrophobicity analysis of predicted open reading frames on human chromosome region 5p15 in the NCBI human genome database and the ENSEMBL database revealed the presence of only a limited number of proteins with more than 5 hydrophobic putatively membrane spanning domains (data not shown). Some of these had already been annotated as transporter genes, such as the dopamine transporter (SLC6A3), the Na$^+/H^+$ exchanger NHE-3 (SLC9A3) and the potassium-chloride cotransporter KCC4 (SLC12A7). These are well characterised transporters and were unlikely to mediate or affect neutral amino acid transport and were not considered valid candidates. Two additional proteins with multiple hydrophobic domains were identified in this region. One of these is XT2, an orphan member of the SLC6 family, which does not appear to have a transport activity (25,26). The second lies distal to XT2 on chromosome 5p15, is a close homologue of XT2 and has recently been annotated (NCBI accession No. XM_291120). BLAST searches revealed that the mouse homologue of this transporter (94% similarity on the amino acid level) is a cDNA deposited under the accession number XM_127449 in the NCBI database. Because of its functional properties, which will be described below, we named the mouse cDNA mB$^\theta AT1$. There were no differences between the coding sequence of mB$^\theta AT1$ obtained by us and the coding sequence of XM_127449 in the NCBI database. The genomic localisation of mouse B$^\theta AT1$ is on chromosome 13 in cytoband C1. This region is syntenic to human chromosome 5p15. The transcript is deposited as ENSMUST00000022048 in the EMBL database (or as locus...
LOC340024 in the NCBI database) (27). The twelve coding exons span an area of 18920 bp (Fig. 1).

Alignment of all mouse members of the SLC6 family reveals that mB⁰AT1, together with XT2 and XT3, another orphan member of the SLC6 family (26), form a subfamily (Fig. 2). mB⁰AT1 has 51% and 43% identical amino acids with XT3 and XT2, respectively; all other SLC6 members having between 21% and 35% identical amino acids with mB⁰AT1. The mouse cDNA has a coding sequence of 1904 bp and encodes a protein of 634 amino acids. Hydropathy analysis indicates the presence of 12 transmembrane domains (Fig. 3A), which is in agreement with the general structure of transporters in the SLC6 family (28). However, B⁰AT1, XT2 and XT3 are predicted to have a larger loop between helix 7 and 8 compared to other members of the SLC6 family. The predicted structure was supported by the exon/intron boundaries, which in membrane proteins, are usually found in the sequence between transmembrane domain encoding regions (Fig. 3B).

**Tissue distribution and cellular distribution of mB⁰AT1**

The successful cloning of mB⁰AT1 from mouse kidney cDNA indicated its presence in this tissue. This was confirmed by RT-PCR experiments, which showed significant expression of mouse B⁰AT1 only in kidney and small intestine (Fig. 4). The RT-PCR experiments were confirmed by analysis of the EST database. Of 62 deposited EST’s corresponding to XM_127449, 19 are found in the kidney and 14 are found in the small intestine. Further EST’s (numbers in brackets) were detected in skin [9], thymus [7], spleen [5], uterus [4], hematopoietic stem cells [1], diencephalon [1], inner ear [1] and blood vessels [1].

In order to localize the transcriptional activity of mB⁰AT1 in mouse organs we performed radioactive in situ hybridization assays on paraffin-embedded tissue sections. As
depicted in Fig. 5 and 6, hybridization with the $^{35}$S-labeled mB$^0$AT1-specific antisense RNA probe revealed significant expression of B$^0$AT1 mRNA only in small intestine (Fig. 5, panel A) and kidney (Fig. 5, panel C) of all investigated mouse tissues. Specific autoradiographic signals were not observed in other organs, such as lung (Fig. 5, panel E), skeletal muscle (Fig. 5, panel F), brain (Fig. 5, cerebellum shown in panel G), liver (Fig. 5, panel H), and pancreas (Fig. 5, panel I). No autoradiographic signals were detected in tissue specimens of all organs when hybridized with the $^{35}$S-labeled sense mB$^0$AT1 control probe (Fig. 5 panel B (small intestine), panel D (kidney)). In kidney, mB$^0$AT1 transcripts were localized in the renal cortex but not in the medulla as exemplarily shown in Fig. 5 panel C. Analysis of hybridization positive cells at higher resolution (Fig. 6) revealed substantial amounts of mB$^0$AT1 mRNA in the proximal tubules (Fig. 6A). The distal nephron segments and the glomeruli were consistently hybridization negative. In the small intestine, mB$^0$AT1 transcripts were found to be exclusively localized in villus enterocytes. As shown in Fig. 6B, high resolution of the hybridization-positive villi reveals a gradient of mB$^0$AT1 transcription with highest levels of expression in apical cells. The mB$^0$AT1 mRNA was not detected in crypt cells or in any other cell types of the small intestine (Fig. 5A and 6B).

**Functional properties of mouse B$^0$AT1**

Expression of mB$^0$AT1 in *Xenopus laevis* oocytes resulted in a significant increase of leucine uptake activity compared to control oocytes. On day 5 of expression, for example, mB$^0$AT1 expressing oocytes took up 100 μM $^{[14]}$Cleucine at a rate of $56 \pm 10$ pmol/15 min. This was more than five times higher than the activity of non-injected oocytes, which amounted to $10 \pm 1$ pmol/15 min. Uptake of $^{[14]}$Cleucine was Na$^+$-dependent; replacement of NaCl by NMDG-Cl or LiCl completely abolished the transport activity (Fig. 7A). In contrast to other members of the SLC6 family, leucine uptake via mB$^0$AT1 was not significantly affected by replacement of chloride with gluconate (Fig. 7A). Transport of amino acids via
mB<sup>0</sup>AT1 was driven by the membrane potential. Addition of 50 mM KCl to the transport buffer, a manipulation that reduces the membrane potential of oocytes from $-40 \pm 7$ mV to $-16 \pm 3$ mV, reduced leucine uptake by 52% (Fig. 7B), addition of 50 mM NH<sub>4</sub>Cl reduced transport activity further (68%) consistent with its stronger depolarizing effect (-8 ± 3 mV) as compared to 50 mM KCl (Fig. 7B). Reduction of the transport activity was not caused by the increased osmolarity in these solutions as evidenced by unaltered transport activity observed when 50 mM NMDG-Cl or 100 mM sucrose was added to the ND96 buffer, which contains 96 mM NaCl (Fig. 7B). Transport of leucine via mB<sup>0</sup>AT1 was pH dependent, strongly increasing with alkaline pH (Fig. 7C).

The data from Figure 7A/B suggest that mB<sup>0</sup>AT1 is an electrogenic Na<sup>+</sup>-dependent transporter, but in contrast to other members of the neurotransmitter transporter family does not appear to be chloride dependent. This suggests that at least 1 Na<sup>+</sup> is co-transported together with neutral amino acids. An activation analysis of leucine transport as a function of the Na<sup>+</sup> concentration showed a sigmoidal dependence. Half-maximal transport velocity was reached at a Na<sup>+</sup> concentration of 54 ± 4 mM (Fig. 8A). Linearisation of the data according to the Hill equation yielded a Hill coefficient of 1.5 ± 0.2 (Fig. 8B), suggesting that either two Na<sup>+</sup>-ions are cotransported with neutral amino acids or that 1 Na<sup>+</sup> is cotransported and a second Na<sup>+</sup> ion binds to a modifier site on the transporter.

In order to determine the substrate specificity of the transporter, uptake of 100 µM [<sup>14</sup>C]leucine was measured in the presence of a 200-fold excess of unlabelled L-amino acids (Fig. 9A). Leucine uptake was inhibited by all neutral amino acids. The transporter was strongly (>80 %) inhibited by leucine, cysteine, glutamine, valine, isoleucine, methionine, phenylalanine, alanine, serine, and asparagine. Medium inhibition (50-80 %) was exerted by threonine, glycine, proline, histidine, tyrosine and tryptophan and the amino acid analogue 2-
aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH). No significant inhibition was observed on addition of arginine, lysine, aspartate, glutamate and N-methyl-aminoisobutyric acid (MeAIB). Thus it appears that almost all neutral L-amino acids bind to this transporter. D-amino acids, by contrast, were almost not recognised by the transporter. D-alanine, D-glutamine and D-aspartate did not inhibit L-leucine transport when added at 200-fold excess; partial but significant inhibition was observed with D-leucine and D-serine (Fig. 9B). Some amino acids, however, may be inhibitors of the transporter albeit not being actually translocated. To test for active transport we used a variety of [14C]labelled amino acids (Fig. 10A). Of the tested amino acids, leucine appeared to be the best substrate, followed by isoleucine, glutamine, phenylalanine, alanine and proline. Histidine and glycine transport activity in mB0AT1 expressing oocytes was small but significant (Fig. 10A). Glutamate and arginine were neither inhibitors of leucine transport, nor accepted as substrates of the transporter. The basic characteristics of mB0AT1 as determined by flux measurements were confirmed by electrophysiological recordings of substrate-induced transporter currents. Superfusion of oocytes with saturating concentrations of leucine (5 mM) generated inward currents of about 30-40 nA, confirming the electrogenic nature of the transport process (Fig. 10B). When tested at a concentration of 5 mM, currents were induced in the following order: Leu > Gln > Phe = Ala > His = Trp >> Glu = Arg, confirming the order of transport activity as determined with labelled amino acids. Non-injected oocytes displayed currents of 2-3 nA in response to the same amino acids (not shown).

The transporter showed rather low affinity for its substrates. For leucine transport a K_m of 630 ± 150 µM was determined (average of n = 3 experiments, representative curve shown in Fig. 11); other neutral amino acids were transported with similar affinity (Table 1). The V_max-values varied between oocyte batches and the day of analysis. Nevertheless, we
observed a consistently higher $V_{\text{max}}$ for leucine transport relative to other substrates (Table 1). Thus it appears that leucine is the preferred substrate of mB$^0$AT1.

**Discussion**

*General properties*: amino acid transport in mammalian cells is mediated by a large variety of amino acid transporters with overlapping substrate specificity (1,6,29). The corresponding transport activities have been classified by their substrate specificity (30). The molecular correlates of most of these activities have been identified in recent years (see Ref. (6,31) for recent summaries). In general it has been observed that uptake of neutral amino acids across the apical membrane of epithelial cells is largely mediated by Na$^+$-dependent transport mechanisms (32), whereas Na$^+$-independent transport mechanisms prevail in the plasma membrane other cell types, particularly for large, hydrophobic amino acids (29). The molecular correlate of the major transport activity for neutral amino acids in the brush border membrane of kidney and intestinal epithelial cells (named system B$^0$ for broad neutral, sometimes also referred to as NBB for neutral brush border) has remained elusive until now. In this study we have characterized a transporter that has all hallmarks of system B$^0$. It transports most neutral amino acids but does not recognise glutamate and arginine, moreover, transport is Na$^+$-dependent, Cl$^-$-independent and electrogenic. Mouse B$^0$AT1 belongs to the SLC6 family of Na$^+$ and Cl$^-$ dependent transporters, which comprises transporters for neurotransmitters, osmolytes and amino acids (28,33). B$^0$AT1 forms a small subfamily together with two orphan transporters of the SLC6 family, namely XT2 and XT3 (26), which is characterised by a large loop between helix 7 and 8. Surprisingly, B$^0$AT1 is less closely related to amino acid transport system B$^{0,+}$ (cDNA ATB$^{0,+}$), which also is a general amino acid transporter, but transports neutral and cationic amino acids (34). It has to be noticed, however, that the SLC6 family has two major branches. The upper branch comprises transporters for amino acid related substrates, such as GABA, taurine, creatine and
catecholamines. By contrast, the lower branch comprises only transporters for amino acids, including $B^0$AT1 and $ATB^{0+}$ and a number of orphan transporters, the function of which remains elusive. It is tempting to speculate that the orphan transporters of this family, which are all located in the lower branch are amino acid transport related proteins.

**Functional properties:** The $mB^0$AT transporter displays rather low affinity for its substrates. For the three amino acids tested, $K_m$-values were found to lie between 500 µM and 700 µM. These figures correspond well with the reported $K_m$-values for system $B^0$ (9,12,35).

Activation analysis of leucine transport at different $Na^+$ concentrations yielded a Hill coefficient of 1.5, suggesting that at least 1 $Na^+$ is cotransported together with substrates and that a second $Na^+$ either is cotransported or modulating the transport activity. In the electrophysiological recordings inward currents of 30-40 nA were observed at -50 mV. This corresponds to a transport activity of $1080 - 1440$ pmol charges/h ($1A = 1C/s$), which is approximately twice the $V_{max}$ of $[^{14}C]$leucine transport of 760 pmol/h. Although uptake was not studied under voltage clamp conditions, this calculation also supports a stoichiometry of $2Na^+$/amino acid. The reduction of the transport activity observed under depolarising conditions combined with the substrate-induced inward currents, in addition suggests that the observed currents are largely generated by the influx of $Na^+$ coupled to amino acid translocation. This has to be seen, for example, in comparison to the amino acid transporter SN1 (SNAT3), which is also $Na^+$-dependent, but transport associated currents are generated by a cation conductance whereas the overall transport process is electroneutral (36). The difference between both transporters becomes apparent when oocytes are depolarised by addition of KCl, which reduces transport activity of $mB^0$AT1 but does not affect the activity of SN1 (36). In contrast to most other members of the SLC6 family $mB^0$AT1 is not chloride dependent. This feature appears to be unique among the mammalian members of this family. However, a recently identified insect member cloned from the midgut of the caterpillar
Manduca sexta was also found to be unaffected by chloride replacement (37). As a further hallmark of mB^0AT1, we also observed a strong pH dependence, which has been reported previously for system B^0 in bovine brush-border membranes (38). This pH-dependence might contribute to the strong inhibition observed after addition 50 mM NH₄Cl. NH₄Cl at this concentration not only depolarises the oocyte but also causes intracellular acidification (39).

Physiological function: The functional properties of mB^0AT1 suggest that it is the molecular correlate of system B^0. This view is supported by the in situ hybridization data showing strong expression in the kidney and intestine. The cortical localisation of the in situ hybridization signal in the kidney allocates the transporter to the proximal tubule where amino acid resorption occurs in the kidney (40). In the intestine a gradient was observed of the in situ hybridization signal towards the tip of the villi, again supporting the role of this transporter in nutrient resorption. The functional characteristics and cellular distribution of mB^0AT1 fits well with the proposed tertiary transport of cationic amino acids in the kidney [Ref. (7) and Fig. 12]. Uptake of cationic amino acids in kidney and intestine is mediated by the heteromeric amino acid transporter rBAT/b^0AT in exchange for neutral amino acids (41). As a result removal of neutral amino acids is thought to provide an additional driving force for the accumulation of cationic amino acids in the kidney (Fig. 12). In agreement with this notion, leucine is the preferred efflux substrate for rBAT/b^0AT (42) and the preferred uptake substrate for mB^0AT1.

In addition to the conspicuous expression in kidney and intestine, analysis of the expressed sequence tag database further suggests significant expression of the transporter in the skin. Pigmentation of melanocytes involves synthesis of melanins. Tyrosine is the major precursor of melanin biosynthesis (43). Thus, B^0AT1 may play a role to protect skin cells against UV
light. Furthermore, enzymes of serotonergic and melatonergic systems have been detected in
the skin, which require transport of their precursor tryptophan (44).

Relation of \( mB^0AT1 \) to Hartnup disorder: Hartnup disorder is characterized by a striking
increase of neutral amino acids in the urine (15,45). Because of the pattern of amino acid
excretion in Hartnup disorder it is generally assumed that mutations in system \( B^0 \) underlie its
pathogenesis (1). Recently, SLC1A5 (named ASCT2 or \( ATB^0 \)) has been suggested to be the
molecular correlate of system \( B^0 \) (46,47). Three lines of evidence clearly refute this
assignment. First, ASCT2/\( ATB^0 \) does not transport phenylalanine or tryptophan (48,49), two
well-established substrates of system \( B^0 \), which are transport by \( mB^0AT1 \). Second,
ASCT2/\( ATB^0 \) is an obligatory amino acid exchanger (50) and as a result cannot mediate net
fluxes of neutral amino acids across the apical membrane, whereas \( mB^0AT1 \) mediates net
uptake of amino acids. Third, our analysis of Hartnup disorder families did not reveal the
presence of potentially causative mutations in the coding sequence or splice donor/acceptor
sites in the SLC1A5 gene on chromosome 19 (45). As a result, the name ASCT2 appears to
be more appropriate for SLC1A5.

Hartnup disorder has been mapped to human chromosome 5p15 (17), allowing a targeted
approach to the molecular identification of the corresponding transporter. A hydropathy
screen for putative membrane proteins in 5p15 yielded two uncharacterized members of the
neurotransmitter transporter family as candidates. One has been termed XT2 or ROSIT, an
orphan member of the SLC6 family (25,26). Several studies and our own unpublished
observations indicate that XT2 is expressed in the plasma membrane but does not transport
amino acids or other compounds. The second has been annotated as protein XP_291120 in the
NCBI database, the mouse homologue of which we have characterized here as \( mB^0AT1 \). In
further support of this notion the genomic sequence of \( mB^0AT1 \) is located in an area of mouse
chromosome 13 that is syntenic to human chromosome 5p15. Our data suggest that the annotated protein XP_291120 is a likely candidate for mutations associated with Hartnup disorder.

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Figure legends

Figure 1: Genomic structure of mouse B\textsuperscript{0}AT1.
The mouse B\textsuperscript{0}AT1 sequence was compared to the mouse genome sequence using the ENSEMBL genome server. The gene is localized on chromosome 13 in cytoband C1 and its sequence is deposited as transcript ENSMUST0000022048 in the EMBL database. The coding region comprises twelve exons that span 18920 bp. Exons are given as black boxes or upper case letters, introns are shown as lines or in lower case letters.

Figure 2: Relationship of mB\textsuperscript{0}AT1 to other members of the SLC6 family.
Peptide sequences of all mouse members of the SLC6 family were used for a sequence alignment and tree building. Accession numbers are serotonine transporter (SERT), Q60857; noradrenaline transporter (NET), O55192; dopamine transporter (DAT), NP_034150; GABA transporter (GAT1), NP_848818; creatine transporter (CT1), NP_598748; taurine transporter (TauT), NP_033346; GABA/betaine transporter (GAT2), P31651; GABA transporter (GAT4), P31650; GABA transporter (GAT3), P31649; proline transporter (ProT), XP_140385; glycine transporter (GlyT1) NP_032161; glycine transporter (GlyT2) NP_683733; amino acid transporter B\textsuperscript{0,+} (ATB\textsuperscript{0,+}), AAK43541; orphan transporter (NTT5), XP_145587; orphan transporter (v7-3), NP_780537; orphan transporter (NTT4), NP_758475; orphan transporter (XT3), NP_035861; amino acid transporter (B\textsuperscript{0}AT), XP_127449; orphan transporter (A12, XT2) AAC27757.
Figure 3: Hydropathy plot and topological model of mB\textsuperscript{0}AT1.

A) Hydropathy plot of mB\textsuperscript{0}AT1 as calculated by the TMpred program. Predicted transmembrane spanning domains are numbered. B) Topological model of mB\textsuperscript{0}AT1 derived from the combined analysis of several topology predictions. The location of the amino- and carboxyterminus were both postulated to be intracellular in accordance with other members of this family. The transporter is predicted to have 12 transmembrane spanning domains. Charged residues in the membrane are shaded in grey. Exon/intron boundaries, translated into the peptide sequence, are indicated by arrows.

Figure 4: RT-PCR analysis of mB\textsuperscript{0}AT1 mRNA in mouse tissues

Total RNA was isolated from different mouse tissues, reverse transcribed into cDNA. Upper panel: a mB\textsuperscript{0}AT1 specific fragment of 1597 bp was amplified. Tissues are labeled as follows: Sp, spleen; Ki, kidney; P, parotis; T, testis; B, brain; Si, small intestine; Lu, lung; H, heart; Li, liver. Markers were loaded on each side of the gel showing bands of 1000 bp, 1500 bp and 2000 bp. Lower panel: Equal loading was tested by amplifying an actin specific fragment in the same tissue samples. Markers shown in the lower panel are 750 bp and 1000 bp.

Figure 5: In situ hybridization of mB\textsuperscript{0}AT1 in mouse tissues.

Probes corresponding to the sense and antisense strand of mB\textsuperscript{0}AT1 cDNA were generated by in vitro transcription. The antisense probe was hybridized to mRNA in sections from small intestine (panel A), kidney (panel C), lung (panel E), muscle (panel F), cerebellum (panel G), liver (panel H) and pancreas (panel I). Control sections from small intestine (panel B) and kidney (panel D) were incubated with the sense probe. No signal was observed in the controls.
**Figure 6: In situ hybridization of mB\textsuperscript{0}AT1 in kidney and intestine at higher resolution.**

Probes corresponding to the sense and antisense strand of mB\textsuperscript{0}AT1 cDNA were generated by in vitro transcription. The antisense probe was hybridized to mRNA in sections from kidney (panel A) and small intestine (panel B). Panel A shows strong expression in segments of the proximal tubule. In panel B a notable increase of expression is observed towards the tip of the villus.

**Figure 7: Ion dependence and voltage dependence of amino acid uptake via mB\textsuperscript{0}AT1.**

Oocytes were each injected with mB\textsuperscript{0}AT1 cRNA or remained uninjected in the controls. (A) \(^{14}\)C-leucine (100 µM) uptake was determined 5 days after injection in buffer containing NaCl, or in buffer where NaCl was replaced by LiCl, NMDG-Cl or Na-gluconate. Each bar represents the mean ± SD transport activity of m = 10 oocytes. The transport activity of non-injected oocytes is already subtracted (n = 3 experiments). (B) To investigate whether mB\textsuperscript{0}AT1 is electrogenic \(^{14}\)C-leucine uptake (100 µM) was determined in ND96 buffer (pH 7.4) with or without addition of 50 mM KCl or 50 mM NH\textsubscript{4}Cl. To test for an effect of increased osmolarity in these experiments, mB\textsuperscript{0}AT1 activity was determined after addition of 50 mM NMDG-Cl or 100 mM sucrose to ND96 (pH 7.4). Black bars display the activity of mB\textsuperscript{0}AT1 expressing oocytes, open bars represent the transport activity of non-injected oocytes. Each bar represents the mean ± SD transport activity of m = 10 oocytes (n = 2 experiments). (C) Leucine uptake (100 µM) was determined at pH values ranging from 5.0 to 8.0 in ND96 buffer containing 96 mM NaCl. Each bar represents the mean ± SD transport activity of m = 10 oocytes. The transport activity of non-injected oocytes was subtracted (n = 3 experiments).
Figure 8: Leucine transport as a function of the Na⁺ concentration in mB⁰AT1 expressing oocytes.

Oocytes were each injected with mB⁰AT1 cRNA or remained uninjected in the controls. (A) [¹⁴C]leucine uptake (100 µM) was determined at Na⁺ concentrations ranging from 0 to 100 mM (all pH 7.4). (B) Transformation of the data according to the Hill equation. Each datapoint represents the mean ± SD transport activity of m = 10 oocytes. The transport activity of non-injected oocytes was subtracted (n = 3 experiments).

Figure 9: Substrate specificity of mB⁰AT1.

A) Oocytes were each injected with mB⁰AT1 cRNA or remained uninjected in the controls. [¹⁴C]leucine uptake (100 µM) was determined 4 days after injection in the presence or absence of 20 mM of unlabelled amino acids or analogues. Each bar represents the mean ± SD transport activity of m = 10 oocytes. The transport activity of non-injected oocytes was subtracted in each case. The one letter code is used for amino acids; MeAIB, N-methylaminoisobutyric acid; BCH, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid. The experiment was performed n = 3 times with similar results.

B) Stereoselectivity of mB⁰AT1 was determined by measuring [¹⁴C]leucine uptake (100 µM) in the presence of a 200-fold excess of unlabelled D-amino acids. Black bars display the activity of mB⁰AT1 expressing oocytes, open bars represent the transport activity of non-injected oocytes. Each bar represents the mean ± SD transport activity of m = 10 oocytes, n = 3 experiments.
Figure 10: Active transport of amino acids by mB^0AT1 expressing oocytes.

A) Uptake of [^14C]leucine, [^14C]phenylalanine, [^14C]glutamine, [^14C]arginine, [^14C]alanine, 
[^14C]isoleucine, [^14C]glycine, [^14C]proline, [^14C]glutamate and [^14C]histidine, each (100 µM) 
was determined 4 days after injection. Each bar represents the mean ± SD transport activity of 
m = 10 oocytes (n = 3 experiments). Black bars display the activity of mB^0AT1 expressing 
oocytes, open bars represent the transport activity of non-injected oocytes. B) Inward currents 
generated by the influx of neutral amino acids. Oocytes were each injected with mB^0AT1 
cRNA or remained uninjected in the controls. After incubation for four days oocytes were 
held at a membrane potential of -50 mV and superfused with ND96 (pH 7.4) alone or ND96 
(pH 7.4) containing different amino acids at a final concentration of 5 mM. Superfusion with 
amino acid containing solutions is indicated by bars. The experiment was performed with m = 
7 oocytes from n = 3 different batches. Non-injected oocytes showed inward currents of 2-3 
nA in response to the same panel of amino acids.

Figure 11: Leucine transport as a function of substrate concentration in mB^0AT1 
expressing oocytes.

Oocytes were each injected with mB^0AT1 cRNA or remained uninjected in the controls. 
Uptake of [^14C]leucine was determined 6 days after injection at concentrations ranging 
between 3 µM and 3000 µM. The transport activity of non-injected oocytes was subtracted in 
each case. The experiment was performed n = 3 times, the averaged K_m-values for this and 
other substrates are presented in Table 1.
Figure 12: Resorption of neutral and cationic amino acids in kidney and intestine

Cationic amino acids are transported across the apical membrane by exchange against neutral amino acids via the heteromeric amino acid antiporter rBAT/b0AT. Leucine, the preferred neutral amino acid released by rBAT/b0AT plus other neutral amino acids already present in the urine are subsequently removed by B0AT1. B0AT1 accumulates neutral amino acids by cotransport with 1-2 Na+. Cationic amino acids are released on the basolateral side by the heteromeric amino acid transporter 4F2/y+LAT1 in exchange for neutral amino acids, neutral amino acids are released by LAT2 and a yet unidentified uniporter.
Table 1: Kinetic constants of neutral amino acid transport by mouse B\(^0\)AT.

Oocytes were each injected with 20 ng mB\(^0\)AT1 cRNA or remained uninjected in the controls. Uptake of \([^{14}\text{C}]\text{amino acids}\) was determined 3-6 days after injection at concentrations ranging between 3 \(\mu\text{M}\) and 3000 \(\mu\text{M}\). For each concentration, the activity of \(m = 10\) injected and non-injected oocytes was evaluated. The transport activity of non-injected oocytes was subtracted in each case. The \(K_m\) value in the table is the average of \(n = 3\) separate experiments. \(V_{\text{max}}\) varies between batches of oocytes and expression period and thus is given as a range observed in the different experiments.

| Substrate         | \(K_m\) (\(\mu\text{M}\)) | \(V_{\text{max}}\) (range) pmol/15 min per oocyte |
|-------------------|-------------------|---------------------------------------------|
| Leucine           | 630 ± 150         | 177-443                                     |
| Glutamine         | 522 ± 91          | 83-150                                      |
| Phenylalanine     | 589 ± 150         | 63-152                                      |
Figure 8

(A) [3H]leucine uptake (pmol/15 min per oocyte) vs Na\(^+\)-concentration (mM).

(B) log(v/V_{max} - v) vs log [Na\(^+\)].
Bröer et al., Fig. 10

A

Amino acid uptake (pmol/15 min per oocyte)

[14C]Amino acid

B

Current (nA)

Leu Glu Arg Leu Gln Phe Ala His Trp Leu

Time (min)
Bröer et al., Fig. 11

![Leucine uptake graph](image-url)
Bröer et al., Fig. 12

Apical

rBAT/ b0,+AT

AA+

Cys-

AA+

AA0

+ Leu

1-2Na+

B0AT1

Leu

Basolateral

4F2/
y+LAT1

AA+

AA0

4F2/
LAT2

AA0

AA0

AA0
Molecular cloning of mouse amino acid transport system $b^0$, a neutral amino acid transporter related to Hartnup disorder
Angelika Bröer, Karin Klingel, Sonja Kowalczuk, John E.J. Rasko, Juleen Cavanaugh and Stefan Bröer

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