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Characterisation and cloning of a Na^+-dependent broad-specificity neutral amino acid transporter from NBL-1 cells: a novel member of the ASC/B^0 transporter family

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Abstract

Na^+-dependent neutral amino acid transport into the bovine renal epithelial cell line NBL-1 is catalysed by a broad-specificity transporter originally termed System B^0. This transporter is shown to differ in specificity from the B^0 transporter cloned from JAR cells [J. Biol. Chem. 271 (1996) 18657] in that it interacts much more strongly with phenylalanine. Using probes designed to conserved transmembrane regions of the ASC/B^0 transporter family we have isolated a cDNA encoding the NBL-1 cell System B^0 transporter. When expressed in *Xenopus* oocytes the clone catalysed Na^+-dependent alanine uptake which was inhibited by glutamine, leucine and phenylalanine. However, the clone did not catalyse Na^+-dependent phenylalanine transport, again as in NBL-1 cells. The clone encoded a protein of 539 amino acids; the predicted transmembrane domains were almost identical in sequence to those of the other members of the B^0/ASC transporter family. Comparison of the sequences of NBL-1 and JAR cell transporters showed some differences near the N-terminus, C-terminus and in the loop between helices 3 and 4. The NBL-1 B^0 transporter is not the same as the renal brush border membrane transporter since it does not transport phenylalanine. Differences in specificity in this protein family arise from relatively small differences in amino acid sequence.

Keywords: Amino acid; Transport; Broad specificity; Sodium dependence; NBL-1; ASC; B^0

1. Introduction

Na^+-dependent neutral amino acid transport into mammalian cells is mediated by System A and/or by one or more of a family of broad-specificity transporters which have been termed System ASC or System B^0 (see, e.g., [1] for a review). These transport activities are differentiated by the fact that System A takes *N*-methylaminoisobutyrate (MeAIB) as a substrate while the others do not. The term System ASC describes a transport activity which recognises only small aliphatic amino acids, and excludes glutamine, branched chain amino acid and phenylalanine. Alanine transport via System ASC is inhibited by glutamate and aspartate at pH 5.5. The term System B^0 was originally applied to the broad-specificity transporter in the bovine renal epithelial cell line NBL-1 [2] This differs from System ASC in that alanine transport is competitively inhibited by glutamine, leucine and phenylalanine, but is not inhibited by glutamate and aspartate at pH 5.5. A similar activity

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has been described in JAR cells [3]. Phenylalanine and leucine are not themselves transported by System B^0 in NBL-1 cells, although they are good inhibitors of alanine transport.

In the past few years a number of clones have been isolated which, when expressed heterologously catalyse Na^+-dependent broad-specificity neutral amino acid transport activity. The first of these was ASCT1 [4,5], which was later shown to catalyse a Na^+-dependent obligatory antiport of amino acids and to have chloride channel activity [6]. Utsonomiya-Tate et al. [7] isolated a further ASC clone, designated ASCT2, which catalysed electroneutral Na^+-dependent obligatory antiport of amino acids and was suggested to correspond to System ASC. The first System B^0-like transporter (ATB^0) was cloned from the human choriocarcinoma cell line JAR [8]. Finally, Kekuda et al. cloned another broad-specificity transporter from rabbit intestine [9]. This was a brush border membrane protein [10] with broad specificity. Alanine uptake by this transporter was inhibited by glutamate and aspartate at pH 5.5, but this clone also has been termed ATB^0 [9]

ASCT1, ASCT2, ATB^0 from JAR cells and the rabbit intestinal transporter are all closely related members of a family of mammalian Na^+-dependent broad-specificity transporters which is part of the glutamate transporter superfamily. However, little is known about the structural basis of the differences in amino acid specificity between these various transporters. In order to attempt to address this question, we report here the cloning of the broad-specificity transporter from NBL-1 cells which was originally designated as System B^0. This is of interest because preliminary experiments in our laboratory had already shown some differences in specificity between alanine uptake into NBL-1 cells and into JAR cells via System B^0 transporters.

2. Materials and methods

2.1. Materials

All reagents were obtained from Sigma (Poole, Dorset, UK) unless otherwise stated. Radiolabelled L-[2,3-^{3}H]alanine and L-phenyl-[2,3-^{3}H]alanine were from Amersham (Amersham, UK).

2.2. Cell culture

Cell lines were cultured in Hams F12 nutrient medium (Gibco, Paisley, UK) supplemented with 10% (v/v) foetal bovine serum, antibiotics (100 U/ml penicillin G, 0.1 mg/ml streptomycin) and 2 mM glutamine prior to use. Cells were routinely trypsinised and seeded onto 35-mm Petri dishes for experiments. The cells were fed every other day until confluent, typically after 4 days.

2.3. Measurement of amino acid uptake by cell monolayers

Uptake of amino acids into cell lines was measured at room temperature in the presence of 0.5 mM amino-oxyacetate to inhibit alanine metabolism as described by Doyle and McGivan [2].

2.4. Screening of an NBL-1 cell cDNA library

A commercial NBL-1 cell lambda-Zap II bacteriophage cDNA library (Stratagene, Cambridge, UK) was screened according to manufacturer's instructions. Briefly, appropriate cDNA fragments (see results) were radiolabelled using the 'Redi-prime' cDNA labelling kit (Amersham) and [α-^{32}P]dCTP (ICN, Basingstoke, UK) according to the manufacturer's directions. Filters were prehybridised for 4 h and hybridised to radiolabelled cDNA probes overnight at 60°C in a buffer containing 6× SSC (1× SSC contains 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 5× Denhardt's reagent (1% Ficoll (type 400), 1% polyvinylpyrrolidone and 1% BSA), 0.2% SDS and 50 μg/ml sonicated salmon sperm DNA. Filters were subsequently washed three times in 1× SSC, 0.1% SDS at room temperature and a further three times in 0.5× SSC, 0.1% SDS at 50°C prior to autoradiography. Positive clones were subjected to further rounds of screening prior to excision and subsequent culture according to manufacturers' instructions.

2.5. Transporter expression in oocytes

Isolation of *Xenopus laevis* oocytes and expression of NBL-1 cell B^0 mRNA in this system was performed essentially as described previously [11]. Oo-

cytes were maintained at 18°C in modified Barth's medium (89 mM NaCl, 1 mM KCl, 0.42 mM $MgSO_4$, 0.84 mM $NaHCO_3$, 0.82 mM $CaCl_2$, 5 mM HEPES (pH 7.6), 5 mM sodium pyruvate, 50 μg/ml gentamycin (Fluka)) after type II collagenase separation and manual defollication. Healthy stage V and VI oocytes were selected for microinjection with either 50 ng of cRNA (T7 mMessage mMachine in vitro transcription kit, Ambion) encoding the transporter or water as a control. Oocytes were then used in transport assays 4 days post injection.

2.6. Oocyte amino acid uptake experiments

Oocytes (minimum of five per data point) were washed for 1 min at room temperature in the appropriate incubation medium (either 95 mM NaCl or 95 mM LiCl with 2 mM KCl, 0.82 mM $MgCl_2$, 1 mM $CaCl_2$ and 20 mM Tris–HEPES at pH 7.4). This medium was then changed for one supplemented with the required concentration of unlabelled substrate spiked with 0.5 mCi/ml of ^{3}H-labelled substrate, as indicated in the legends to the figures. Uptake was terminated by five sequential washes in ice-cold uptake medium, prior to lysis of single oocytes in scintillation vials with 100 μl SDS (2% w/v), addition of scintillation fluid (Emulsifier Safe, Packard) and liquid scintillation counting for 10 min/vial (Canberra Packard 4430 Series, Berkshire, UK).

3. Results and discussion

3.1. Comparison of alanine uptake into NBL-1 cells and JAR cells

The initial rate of alanine uptake into both cell lines was linear with time over 5 min and was observed to be 90% sodium dependent (results not shown). Uptake of alanine was measured in the presence of a 100-fold excess of single unlabelled amino acids. Alanine uptake was not significantly inhibited by the system A substrate MeAIB nor by the charged amino acids glutamate, aspartate and lysine in either cell line. Glutamine and to a lesser extent leucine inhibited sodium dependent alanine uptake into both cell lines. However, phenylalanine was a more potent inhibitor of alanine uptake into NBL-1 cells than into JAR cells (Table 1). Fig. 1 shows the effect of phenylalanine concentration on the initial rate of alanine uptake into both cell lines. Phenylalanine was a weak inhibitor of alanine transport into JAR cells but was more effective in NBL-1 cells. These direct comparisons of phenyalanine inhibition of alanine transport are consistent with previous reports. Thus phenylalanine competitively inhibited Na^+-dependent alanine transport (100 μM) into NBL-1 cells with a K_i of approximately 4 mM [2] whereas the IC_{50} for the inhibition of 2.5 nM alanine uptake into JAR cells by phenylalanine was 16.9 mM [3]. In neither cell line was alanine transport inhibited by glutamate and aspartate at pH 5.5 (results not shown).

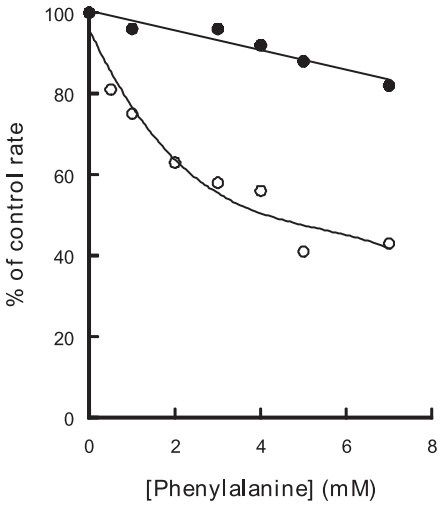

Fig. 1. Inhibition of Na^+-dependent alanine uptake by phenylalanine in NBL-1 and JAR cells. The initial rate of Na^+-dependent uptake of 0.1 mM alanine by confluent monolayer cultures of NBL-1 cells (○) and JAR cells (●) was measured at room temperature after 3 min in the presence of 0.5 mM amino-oxyacetate. Phenylalanine was present at the concentrations shown. The results are presented as a percentage of the uninhibited rate which was 0.94 ± 0.09 nmol/mg protein per min for NBL-1 cells and 1.51 ± 0.12 nmol/min per mg protein for JAR cells. Results shown are the overall means obtained from two or more experiments for each phenylalanine concentration and individual values for each experimental point were the means of three Petri dishes. S.E.M.s (not shown) were less than 10% of the mean value in each case.

3.2. Cloning of NBL-1 cell B^0

Two pairs of oligonucleotide primers were designed to conserved transmembrane spanning regions

Table 1
Inhibition profile of alanine uptake into NBL-1 cells and JAR cells

Inhibiting amino acid	Initial rate of alanine uptake (% of control)	
	NBL-1 cells	JAR cells
None	100	100
Lysine	82 ± 5.5	96 ± 4
MeAIB	97 ± 3	98 ± 4
Serine	7 ± 31	10 ± 3
Glutamine	10 ± 4	5 ± 3
Glutamate	87 ± 7	91 ± 4
Leucine	40 ± 3.5	43 ± 2
Phenylalanine	54 ± 5	89 ± 2*
Aspartate	90 ± 6	97 ± 4.5

The initial rate of Na^+-dependent alanine uptake into confluent monolayer cultures of NBL-1 and JAR cells was measured after 3 min. Alanine was present at 50 μM and inhibitory amino acids were added at 5 mM. Rates are shown as the percentage of the rates in the absence of inhibitor which were 0.83 ± 0.04 nmol/min per mg protein for NBL-1 cells and 1.6 ± 0.11 nmol/min per mg protein for JAR cells. Results are the means ± S.E.M. for triplicate Petri dishes. *$P < 0.05$ vs. NBL-1 cells.

of a multiple sequence alignment of ASCT1, ASCT2 and the JAR cell B^0 (not shown). These primer pairs, designated F1/R1 and F2/R2 were used in reverse transcription–polymerase chain reactions (RT–PCR) on NBL-1 cell total RNA in order to amplify two fragments of NBL-1 cell B^0. The resulting PCR products (of 400 and 370 bp, respectively) were ligated into pGEM T-Easy (Promega) and used to transform $CaCl_2$-competent *E. coli*. Plasmid DNA was extracted (Qiaprep spin kit, Qiagen) and subjected to automated sequencing (Applied Biosystems, Warrington, UK) using vector-specific M13 primers to confirm the identity of the PCR fragments.The primers used were as follows. F1: 5′-CCA TCA TGG TGG CCG ATC CTC-3′; F2: 5′-CTG CTC TTT TTC CTG GTC ACC AC-3′; R1: 5′-GTG GTG ACC AGG AAA AAG AGC AG-3′; R2: 5′-AGG AGT TGA AGA AGC GAA TGA GCA GC-3′.

These radiolabelled PCR fragments were subsequently used to screen 800 000 plaques of a lambda-Zap II bacteriophage NBL-1 cDNA library as described above. One full-length 2.9 kb clone was detected by both probes and subsequently sequenced in its entirety. Fig. 2 shows the nucleotide sequence

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   1 AATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCC 60
  61 GCGCTCGGGTGGTTTGGTGTAGGCTAGCAGCTGTTTACTCCTGAGGTCTCGGGCCGCCGC 120
 121 CTTCGGCACCCCGGGGCTTGAATCTCCCCTCTTGGACCCAAGAACCTGGCCCCCTCTCTT 180
 181 CCCGGAGCTCCGTCTTCTAGCCCCCAGACTCAGACTCCCCAGCCCTCTGTTTCCAGGACC 240
 241 CCGCCAACCGCCAGGTTCCCGCCTCCCGGATCCAGGCGTCCCGGATCTGAGCCACCAGGA 300
 301 CCTAGTCTCTTGAAGACCTCCGCCGCCCCGGACGTCTTCAACACTTCCGGAGCCGAGGACC 360
 361 CCAGGGTCCCATTTCGAGAACCTCCCGTATTCCTAACCCCTACGGTCAAGAACGCCAGCG 420
 421 CTCCCACCTCCCAGCTTTCAGCGCCGGACACAGACAGAGAACAGAGGAACCCCAGCGCCC 480
 481 AGCCAAGGGACTCTCAAACATTTCAGCTTCTAAGAGCCAAGGAACTTGAGCGCTGTGAAC 540
 541 TCACAACTGCAAAGAATTCTTGAAAGTTCTAGCCTCCGGACTCTGTTAAAGTTTTAAGGA 600
 601 ACGTCAGCGTCCTGAGAAGGAACCCCAAGCGCTCCCAACTTGCAGGCGCTAAAGGAACCC 660
 661 CAGCGGCGTTCATCATGGTGGCCGATCCGCCTAAGGGAGACCCCAAAGGGTACGCGGCGG 720
                 M  V  A  D  P  P  K  G  D  P  K  G  Y  A  A  A
 721 CGGAACCCACCGCCAACGGTGTCTCGATGTTGGTCCCCATAGAGGACGTAGGCTCGTTAA 780
      E  P  T  A  N  G  V  S  M  L  V  P  I  E  D  V  G  S  L  K
 781 AAGGCGGCCGTTGCGGTTCCGGGGATCAGGTGCGTCGCTGCCTTCGCGCCAACTTGCTGG 840
      G  G  R  C  G  S  G  D  Q  V  R  R  C  L  R  A  N  L  L  V
 841 TGCTGCTGACGGTAGTGGCCGTGGTGGCCGGCGTGGCGCTGGGGCTGGGGGTCTCGGGAG 900
      L  L  T  V  V  A  V  V  A  G  V  A  L  G  L  G  V  S  G  A
 901 CCGGCGGCGCGTTCGCCCTGGGCCCCGCGCGCCTGGAAGCCTTCTCCTTTCCGGGAGAGC 960
      G  G  A  F  A  L  G  P  A  R  L  E  A  F  S  F  P  G  E  L
 961 TGCTGCTGCGCCTGTTAAAGATGATCATCTTGCCGCTGGTGGTGTGCAGCTTGATCGGCG 1020
      L  L  R  L  L  K  M  I  I  L  P  L  V  V  C  S  L  I  G  G
1021 GCGCCGCCAGCCTGGATCCGAGCGCGCTCGGGCGCCTTGGCGCCTGGGCGCTGCTCTTTT 1080
      A  A  S  L  D  P  S  A  L  G  R  L  G  A  W  A  L  L  F  F
1081 TCCTTGTCACCACACTGCTAGCGTCGGCGCTCGGCGTGGGCTTGGCGCTCGCGCTGCAGC 1140
      L  V  T  T  L  L  A  S  A  L  G  V  G  L  A  L  A  L  Q  P
1141 CGGGCGCCGCCTTCGCCGCCATCAACACCTCGGTCGGGGCCCCGGTGGAAGAGGCCCCCA 1200
      G  A  A  F  A  A  I  N  T  S  V  G  A  P  V  E  E  A  P  S
1201 GCAAGGAGGTGCTCGATTCGTTCCTGGATCTTGTGAGAAATATTTTCCCCTCCAACCTGG 1260
      K  E  V  L  D  S  F  L  D  L  V  R  N  I  F  P  S  N  L  V
1261 TATCTGCAGCCTTCCGCTCATACACTACCTCCTATAAGGAGAGATTGTTCAACGGCACTC 1320
      S  A  A  F  R  S  Y  T  T  S  Y  K  E  R  L  F  N  G  T  L
1321 TGGTGAAGGTGCCCACTGGGGGCGAGGTTGAGGGTATGAACATTCTGGGCCTGGTGGTGT 1380
      V  K  V  P  T  G  G  E  V  E  G  M  N  I  L  G  L  V  V  F
1381 TTGCCATCATCTTTGGTGTGGCCCTGCGGAAGTTGGGGCCCGAGGGAGAGCTGCTCATTC 1440
      A  I  I  F  G  V  A  L  R  K  L  G  P  E  G  E  L  L  I  R
1441 GCTTCTTCAACTCCTTCAATGATGCCACCATGGTGCTGGTCTCCTGGATCATGTGGTACG 1500
      F  F  N  S  F  N  D  A  T  M  V  L  V  S  W  I  M  W  Y  A
1501 CCCCTGTGGGAATCTTGTTCCTGGTGGCCGGCAAGATTGTGGAGATGGAGAACGTGGGGC 1560
      P  V  G  I  L  F  L  V  A  G  K  I  V  E  M  E  N  V  G  L
1561 TGCTCTTTGCTAGTCTCGGCAAATACATCCTGTGCTGCCTGCTCGGCCATGCCATCCATG 1620
      L  F  A  S  L  G  K  Y  I  L  C  C  L  L  G  H  A  I  H  G
1621 GGCTCCTGACACTGCCCCTCATCTACTTTCTCTTCGCCCGCAAGAACCCCTACCGCTTCC 1680
      L  L  T  L  P  L  I  Y  F  L  F  A  R  K  N  P  Y  R  F  L
1681 TGTGGGGCATCATGACGCCGCTGGCCACCGCCTTCGGGACCTCCTCCAGCTCCGCCACGC 1740
      W  G  I  M  T  P  L  A  T  A  F  G  T  S  S  S  A  T  L
1741 TGCCGCTGATGATGAAGTGTGTGGAGGAGAAGAATGGAGTGGCCAGACACATCAGCCGCT 1800
      P  L  M  M  K  C  V  E  E  K  N  G  V  A  R  H  I  S  R  F
1801 TCATTCTGCCCATCGGTGCCACGGTCAACATGGACGGTGCCGCCCTCTTCCAGTGTGTGG 1860
      I  L  P  I  G  A  T  V  N  M  D  G  A  A  L  F  Q  C  V  A
1861 CTGCAGTGTTCATTGCACAGCTCAACCACCGGTCCTTGGACTTCGTGAAGATTATCACCA 1920
      A  V  F  I  A  Q  L  N  H  R  S  L  D  F  V  K  I  I  T  I
1921 TCCTGGTCACGGCCACAGCATCCAGTGTGGGTGCGGCGGGCATCCCATCTGGAGGGGTGC 1980
      L  V  T  A  T  A  S  S  V  G  A  A  G  I  P  S  G  G  V  L
1981 TCACTCTGGCCATCATCCTCGAGGCGGTCAACCTGCCGGTTCACGACATCTCCTTGATCT 2040
      T  L  A  I  I  L  E  A  V  N  L  P  V  H  D  I  S  L  I  L
2041 TGGCCGTGGACTGGCTAGTGGACCGGTCCTGTACCGTCCTCAACGTGGAAGGTGATGCCT 2100
      A  V  D  W  L  V  D  R  S  C  T  V  L  N  V  E  G  D  A  F
2101 TTGGGGCGGGACTCCTCCAGAGTTACCTGGATCGCACAGAGAACTGCAACTCCGTGCCGG 2160
      G  A  G  L  L  Q  S  Y  L  D  R  T  E  N  C  N  S  V  P  E
2161 AGCTGATCCAGGTGAAGAGTGAGATGCCCCTGGCCGCGCTGCCGGTCCCCGGCGAGGAGG 2220
      L  I  Q  V  K  S  E  M  P  L  A  A  L  P  V  P  G  E  E  G
2221 GGAACCCTCTCCTCAAAGGCTGCCCGGGACCTGCTGGGGATGCTGACACCTGTGAGAAGG 2280
      N  P  L  L  K  G  C  P  G  P  A  G  D  A  D  T  C  E  K  E
2281 AATCAGTCATGTGAATCCCTGCAGAGACGTTCCCTGCCCCATGGGGGTGCTCTGGACATT 2340
      S  V  M  *

2341 GGAATCATGGGGGATGGATGAACGGACAAACAAGGCCTCTTGGAGGGCCCTGGCCACACA 2400
2401 CTCGGGAGCCATGGGCCTCAGCTTCCCTCCCTGTCGTCAGATTCGGGAAGCCTCGCTGCT 2460
2461 GGGGTGTATGTTGCTTGTGTGTGAATGAGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT 2520
2521 GTGTGTGACTGTTTCCAAATCTGCCCAGTCCTCAACTCCTGGCCCCCACCCAAGGCTAGG 2580
2581 AAACCATATAAGACGGAGAAGTAGCATGGCCTCCACGCCCCATCCTGGCAGCCTGCCTGG 2640
2641 CCTTCCTGTCTCAGGGCATAGGGAATCATGGGAAATTCTGCTCTCTGAGAGCAAGGATGT 2700
2701 TCTGACAGGTTGCTGGCTCCTTTTCTGGTTATTTTAGTGGCTGTGTGTGTGTGTGT 2760
2761 GCATGTGTGTGTGTGTGTGTTCTGTGACTCTCCACGGTACATCCCACCCTGTCCCC 2820
2821 GGGCCCTCTGTCCCCTCCACAATACGAAACACTCCTTGGGAACACTGAAGGGAGACTCAT 2880
2881 AACACGTTGTTGTTACTCTGAGGATGTTTATAACAATAAAACTGTCAGTTTGTAGTCTAA 2940
2941 AAAAAAAAAA 2951
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Fig. 2. Full-length cDNA sequence and derived amino acid sequence for System B^0 from NBL-1 cells. This sequence has been submitted to GenBank (accession no. AY039236).

and derived amino acid sequence of this clone. The putative transporter clone was 2892 bp in length possessing an open reading frame of 539 amino acids.

3.3. Expression of the NBL-1 transporter in Xenopus oocytes

In order to characterise the properties of alanine transport into oocytes expressing the NBL-1 B^0 clone alanine was used at 50 μM – the same concentration as used in the cell experiments. The initial rate of sodium dependent alanine (50 μM) uptake into B^0 cRNA-injected oocytes was linear with time over 30 min. Fig. 3 shows that the initial rate of 50 μM alanine transport in sodium-containing medium was approximately 20 times greater for B^0 cRNA injected oocytes than their water-injected or non-injected counterparts. In order to determine the extent to which transport was Na^+-dependent, the experiments were repeated in a medium where Na^+ was replaced by an equal concentration of Li^+. This resulted in a smaller induction of alanine transport.

The effects of high concentrations of single unlabelled amino acids on the initial uptake of 50 μM alanine into NBL-1 B^0 cRNA-injected oocytes were tested (Table 2). Glutamine, serine, leucine and phenylalanine inhibited alanine uptake, whilst lysine and Me-AIB had no effect. Fig. 4 shows concentration dependence of the inhibition of alanine transport by phenylalanine in NBL-1 B^0 cRNA-injected oocytes. These results are consistent with inhibition data in NBL-1 cells. In further experiments, it was shown that NBL-1 B^0 cRNA injection into oocytes did not induce any significant uptake of radiolabelled phenylalanine in the presence of sodium (results not shown).

Table 2
Inhibition profile of alanine uptake into NBL-1 B^0 cRNA-injected *Xenopus* oocytes

Inhibiting amino acid	Initial rate of alanine uptake (% of control)
None (control)	100.0
Glutamine	−0.5 ± 0.7**
Serine	0.5 ± 0.8**
Leucine	13.9 ± 2.6**
Phenylalanine	56.1 ± 8.0*
Lysine	81.8 ± 9.9
MeAIB	93.7 ± 11.5

The effects of a 100-fold excess of single unlabelled amino acids (5 mM) on the sodium-dependent uptake of alanine (50 μM) into NBL-1 B^0 cRNA-injected oocytes were tested. Rates as determined from a minimum of five oocytes per experiment are shown as a percentage of the control rate in the absence of inhibiting amino acids (mean ± S.E.M.) which was found to be 78 ± 7 pmol/oocyte per 25 min. Rates were corrected for the uptake measured in water-injected oocytes. *$P < 0.05$ vs. control, **$P < 0.001$ vs. control.

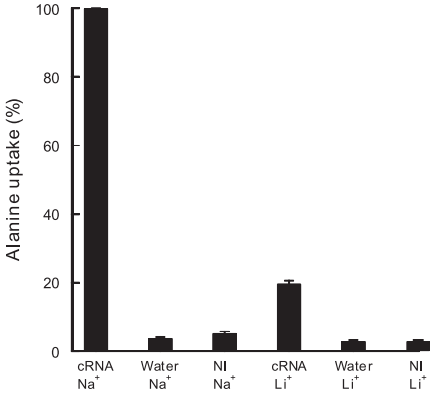

Fig. 3. Uptake of alanine (50 μM) into *Xenopus* oocytes. Oocytes were injected with NBL-1 B^0 cRNA or H_2O and uptake of alanine was measured in the presence of Na^+- or Li^+-containing medium after 25 min as described in Section 2. Results are the mean ± S.E.M. of at least five oocytes for each point. Results are expressed as a percentage of the rate with cRNA-injected oocytes in the Na^+ medium which was 86 ± 6 pmol/oocyte per 25 min. NI, non-injected.

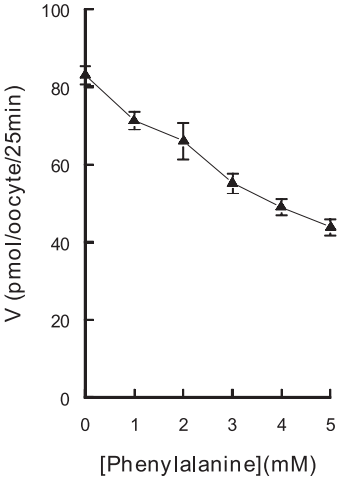

Fig. 4. Inhibition of alanine transport by phenylalanine in oocytes injected with NBL-1 System B^0 cRNA. The initial rate of uptake of alanine (25 μM) was measured after 25 min in Na^+-containing medium in the presence of the concentrations of phenylalanine shown. Each value is the mean of five oocytes.

These kinetic characteristics of alanine transport catalysed by oocytes expressing NBL-1 B^0 are very similar to those of System B^0 originally reported in NBL-1 cells. In both cases alanine uptake was observed to be largely sodium dependent and was inhibited by small aliphatic amino acids, leucine and phenylalanine, but not by Me-AIB or lysine. This relatively strong inhibition by phenylalanine is a distinctive feature of this NBL-1 cell broad-specificity transporter which differentiates it from the weak inhibition observed in JAR cells. Although phenylalanine was an inhibitor of the NBL-1 System B^0 transporter in injected oocytes these oocytes did not catalyse phenylalanine uptake. Similarly phenylalanine was not transported into NBL-1 cells by a Na^+-dependent process [2]. It appears that phenylalanine may compete with alanine for the substrate binding site but once bound, cannot be transported. Similar behaviour has been observed for the intestinal peptide transporter where 4-aminomethylbenzoic acid acts as a competitive inhibitor of peptide transport but is not itself a substrate [11]. However, it is clear that the B^0 clones from JAR cells and NBL-1 cells do not correspond to the Na^+-dependent broad-specificity amino acid transporter activity located in renal brush border membranes, for which phenylalanine and leucine are good substrates. Indeed, System B^0 in NBL-1 cells is predominantly expressed on the basolateral membrane when these cells are grown as confluent monolayers on filters [2].

3.4. Sequence comparison with other B^0/ASC transporters

Fig. 5 shows a multiple alignment of the derived amino acid sequences of rat ASCT2, the JAR cell clone, the NBL-1 B^0 clone described in this paper and the rabbit intestinal clone which has also been termed B^0. All these clones are similar in length. The ten predicted transmembrane domains are nearly

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          1            15 16           30 31           45 46           60 61           75 76           90
rASCT2    MAVDPPKADPKGVVA VDPTANCGSGLKSRE DQGAKAGGCCSSRDQ VCRCLRANLLVLLTV AAAVAGVVLGLGVSA AGGAEALGHARFTAF
JAR B0    MVADPPR-DSKGLAA AEPPPTGAWQLASIE DQGAAAGGYCGSRDL VRRCLRANLLVLLTV VAVVAGVALGLGVSG AGGALALGPGALEAF
NBL-1 B0  MVADPPKGDPKGYAA AEPTANGVSMLVPIE DVGSLKGGRCGSGDQ VRRCLRANLLVLLTV VAVVAGVALGLGVSG AGGAFALGPARLEAF
rabint    MVADPPKGDPKGLAA VEPTANGAPAQDPLE DSGAAVGRCCSSRDQ VRRCLRANLLVLLTV VAVVAGVALGLAVSG AGGALALGPARLIAF

          91          105 106         120 121         135 136         150 151         165 166         180
rASCT2    AFPGELLLRLLEMII LPLVVCSLIGGAASL DPSALGRLGAWALLF FLVTTLLSSALGVAL ALALKPGAAFAAINS SVVDS-SVHRAPTKE
JAR B0    VFPGELLLRLLRMII LPLVVCSLIGGAASL DPGALGRLGAWALLF FLVTTLLASALGVGL ALALQPGAASAAINA SVGAAGSAENAPSKE
NBL-1 B0  SFPGELLLRLLKMII LPLVVCSLIGGAASL DPSALGRLGAWALLF FLVTTLLASALGVGL ALALQPGAAFAAINT SVGAP--VEEAPSKE
rabint    AFPGELLLRLLKMII LPLVVCSLVGGAASL DPSALGRLGAWALLF FLVTTLLASALGVGL ALALQPGAAFAAMNA SLSSTGAVEQTPSKQ

          181         195 196         210 211         225 226         240 241         255 256         270
rASCT2    FLELL----RNMFPS NLVSASAAFRIPCG- -ACPQRSNATMDQPH CEMKMNILGLVVFAI VFGVALRKLGPEGEL LIRFFNSFNDATMVL
JAR B0    VLDSFLDLARNIFPS NLVSAAFRSYSTTYE ERNITGTRVKVPVGQ EVEGMNILGLVVFAI VFGVALRKLGPEGEL LIRFFNSFNEATMVL
NBL-1 B0  VLDSFLDLVRNIFPS NLVSAAFRSYTTSYK ERLFNGTLVKVPTGG EVEGMNILGLVVFAI IFGVALRKLGPEGEL LIRFFNSFNDATMVL
rabint    VLDSFLDLLRNIFPS NLVSAAFRSYSTSYE EKNFNGTLVKVPVAH EEEGMNILGLVVFAI VFGVALRKLGPEGEP LIRFFNSFNDATMVL

          271         285 286         300 301         315 316         330 331         345 346         360
rASCT2    VSWIMWYAPIGILFL VAGKIVEMKDIRQLF IGLGKYIVCCLLGHA IHGLLVLPLIYFLFT RKNPYRFLWGIVTPL ATAFGTSSSSATLPL
JAR B0    VSWIMWYAPVGIMFL VAGKIVEMEDVGLLF ARLGKYILCCLLGHA IHGLLVLPLIYFLFT RKNPYRFLWGIVTPL ATAFGTSSSSATLPL
NBL-1 B0  VSWIMWYAPVGILFL VAGKIVEMENVGLLF ASLGKYILCCLLGHA IHGLLTLPLIYFLFA RKNPYRFLWGIMTPL ATAFGTSSSSATLPL
rabint    VSWIMWYAPVGILFL VASKIVEMDDVGVLF ASLGKYILCCLLGHA IHGLLVLPLIYFLFT RKNPR-FLWGILTPL AMAFGTSSSSATLPL

           361         375 376         390 391         405 406         420 421         435 436         450
rASCT2    MMKCVEEKNGVAKHI SRFILPIGATVNMDG AALFQCVAAVFIAQL NGMSLDFVKIITILV TATASSVGAAGIPAG GVLTLAIILEAISLP
JAR B0    MMKCVEENNGVAKHI SRFILPIGATVNMDG AALFQCVAAVFIAQL SQQSLDFVKIITILV TATASSVGAAGIPAG GVLTLAIILEAVNLP
NBL-1 B0  MMKCVEEKNGVARHI SRFILPIGATVNMDG AALFQCVAAVFIAQL NHRSLDFVKIITILV TATASSVGAAGIPSG GVLTLAIILEAVNLP
rabint    MMKCVEERNGVAKHI SRFVLPIGATVNMDG AALFQCVAAVFIAQL NRQSLDFVKIITILV TATASSVGAAGIPAG GVLTLAIILEAVSLP

          451         465 466         480 481         495 496         510 511         525 526         540
rASCT2    VKDISLILAVDWLVD RSCTVLNVEGDAFGA GLLQSYVDRTKMPSS EPELIQVKNDVSLKP LPLATEEGNPLLKQC REPSGDSSATCEKESVM
JAR B0    VDHISLILAVDWLVD RSCTVLNVEGDALGA GLLQNYVDRTESRST EPELIQVKSELPLDP LPVPTEEGNPLLKHY RGPAGDATVASEKESVM
NBL-1 B0  VHDISLILAVDWLVD RSCTVLNVEGDAFGA GLLQSYLDRTENCNS VPELIQVKSEMPLAA -PVPGEEGNPLLKGC PGPAGDAD-TCEKESVM
Rabint    VSEISLILAVDWLVD RSCTIINVEGDAFGA GLLQHYVDRTEQRGS EPELTQVKSEVPLGS LPAPNEEGNPLLRHS PGAAGDAG-ACEKESVM
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Fig. 5. Amino acid sequence alignment of some broad-specificity amino acid transporters. The sequences shown are: rASCT2, rat ASCT2 [12], GenBank AJ132846; JAR B0, JAR cell B^0 [8], GenBank U53347; NBL-1 B0, this paper, GenBank AY039236; Rabint, rabbit intestine B^0 [9], GenBank U75284. Sequences were aligned using the ClustalW multiple sequence alignment program. Assignments of transmembrane regions are taken from [9] and are shown in bold.

identical in sequence between the four clones and the overall sequences are closely similar. Thus the clone from NBL-1 cells shows similarities of 82.6%, 87.7% and 88.3% at the amino acid level and 78%, 84.8% and 84% at the DNA level with rat ASCT2, JAR cell B^0 and rabbit intestine B^0. respectively. A comparison of the NBL-1 clone reported here with the B^0-clone from JAR cells shows some differences in the N-terminal region between amino acids 19 and 35, differences in the loop region between helices 3 and 4 and some differences also in the C-terminal domain. Some of these differences in amino acid sequence are presumably responsible for the difference in affinity of phenylalanine as an inhibitor between these clones. Differences in sequence between the B^0 and ASCT2 clones also occur mainly in these regions, and there are also quite large differences in these regions between individual ASCT2 clones isolated from mouse, human and rat sources (not shown).

This family of Na^+-dependent broad-specificity transporters encompasses a spectrum of proteins showing progression from limited to much wider substrate specificity. The ASCT2 clone catalyses the obligatory exchange of small amino acids but excludes glutamine, branched chain amino acids and phenylalanine. Transport of alanine is inhibited by glutamate and aspartate at pH 5.5. Alanine transport catalysed by the NBL-1 and JAR B^0 clones is inhibited by glutamine, leucine and phenylalanine. Inhibition by phenylalanine is much stronger for the NBL-1 clone than for the JAR cell clone. Transport by these B^0 clones is not affected by glutamate and aspartate at pH 5.5.

The rabbit intestinal clone shows rather different properties. This transports amino acids in a strongly electrogenic manner, and has a broad specificity. It interacts with branched chain and aromatic amino acids although direct evidence for transport of these amino acids is lacking. However, it also differs from the NBL-1 and JAR B^0 clones in that it does interact with glutamate and aspartate at pH 5.5. This clone may well encode the major brush border amino acid transporter involved in intestinal amino acid absorption [9], and may represent a different sub-family from the B^0 transporters in NBL-1 cells and JAR cells. The overall nomenclature of this broad-specificity transporter family may require some revision.

All these differences in specificity arise as a result of relatively subtle changes in amino acid sequence and little information about structure–function relationships can be derived simply from an inspection of sequence differences. In particular, it is not possible to deduce any structural features which differentiate ASCT2 from B^0 transporters. It is possible that site-directed mutagenesis or domain-swapping experiments based on these sequences may eventually shed more light on the molecular basis of substrate specificity in this transporter family.

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