Regulation of System B^0 amino-acid-transport activity in the renal epithelial cell line NBL-1 and concomitant changes in SAAT1 hybridizing transcripts

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α-(Methylamino)isobutyric acid (MeAIB) insensitive Na^+-dependent alanine transport activity in the bovine kidney cell line NBL-1 was increased upon amino acid starvation ($\geq 20\%$ over control levels). When L-phenylalanine (3 mM) was included in the starvation medium the increase was further enhanced ($\geq 85\%$ over control levels). In cells grown in control medium the $V_{max.}$ for MeAIB-insensitive Na^+/alanine co-transport was found to be 6.0 ± 0.7 nmol/3 min per mg (K_m 41 ± 12 μM) and for L-phenylalanine-treated amino-acid-starved cells the $V_{max.}$ was 21 ± 5 nmol/3 min per mg (K_m 92 ± 40 μM). The increase in $V_{max.}$ was prevented by cycloheximide. Substrate specificity analysis identified the L-phenylalanine-induced transport system as System B^0. [^{35}S]Methionine labelling of cells during the amino acid starvation/phenylalanine treatments resulted in the differential labelling of a protein of 78 kDa. Northern-blot analysis using a SAAT1-specific probe revealed the presence of a new transcript (3.2 kb) in RNA extracted from cells incubated in amino acid starvation medium with L-phenylalanine included. The present findings suggest a novel means of control for System B^0 by the use of physiological stress. It is also proposed that SAAT1 and System-B^0 transcripts have considerable sequence similarity.

INTRODUCTION

Amino acid uptake in mammalian cells is mediated by a number of transporters with overlapping substrate specificities. The diversity and regulation of amino-acid-transport systems operative in the bovine renal epithelial cell line NBL-1 have been previously characterized in this laboratory. Neutral amino acids are co-transported with Na^+ ions via the broad specificity system, System B^0 [1]. System B^0 is also the main neutral-amino-acid transporter of renal brush-border membranes [2] and is characterized by its ability to catalyse the Na^+-dependent transport of branched-chain (e.g. leucine), aromatic (e.g. phenylalanine) as well as the small, neutral (e.g. alanine) amino acids [3]. The non-metabolizable analogue α-(methylamino)isobutyric acid (MeAIB) is not transported by System B^0.

The MeAIB-sensitive transport system, System A, is absent in NBL-1 cells, but emerges on amino acid deprivation [4] and is further induced by hyperosmotic shock following amino acid deprivation [5]. System A has become the classic adaptive amino-acid-transport system [6,7]. Its induction by starvation, diabetes, glucagon and repression on removal of these stimulants has been well documented (see, e.g., [8]). However, the high affinity Na^+-dependent aspartate and glutamate transport system, System X_{AG}^-, has also recently been shown to be up-regulated in this cell line on amino acid starvation [9]. That study also followed the fluctuations in the transcript pool of the related, cloned System X_{AG}^- transporter EAAC1 [10] on amino acid deprivation.

The regulation of amino-acid-transporter transcript levels have been little studied since, until recently, few amino acid transporters had been cloned. Kong et al. [11] described the cloning of an Na^+-dependent neutral-amino-acid transporter cDNA (SAAT1) related to the Na^+-dependent glucose transporter (SGLT1, [12]). When SAAT1 was expressed in COS cells, uptake of MeAIB was increased. This clone was therefore described as characteristic of System A.

Here we characterize the regulation of the broad-specificity transport system of NBL-1 cells and show that it is subject to induction by the presence of L-phenylalanine during amino acid starvation. Northern-blot analysis using a SAAT1-specific probe also reveals the induction of a new transcript during the increase in phenylalanine up-regulated transport activity.

MATERIALS AND METHODS

Cell culture

The establishment and maintenance of the NBL-1 cell line has been described previously [4]. After trypsin treatment of a 75 cm^2 flask, cells were diluted to 80–90 ml in HAM's F-12 (Gibco BRL) supplemented medium (complete medium, CM) and 2 ml aliquots were seeded into 35 mm-diameter dishes. These were fed every other day and used when confluent, typically after 4–6 days. Amino acid starvation medium (AASM) was made up as described in the Gibco BRL catalogue for HAM's F-12 salts medium, but was supplemented with 0.1% BSA, 10 mM glucose and 0.01% Phenol Red. In some experiments single amino acids (as stated) were included.

Transport assays

Transport was measured using a modification of the method of Quamme et al. [13] and as described previously [9]. MeAIB-insensitive alanine transport was routinely measured with radioisotopically labelled alanine (0.1 mM) mixed with MeAIB (5 mM). For equilibration of cells prior to transport assays, the medium was tipped off the monolayers and fresh complete medium (2 ml) was added. The dishes were placed back in the incubator (37 °C, 15 min). Cells were then washed (3 × 1 ml) with transport medium before assaying uptake as described above. Protein was measured by the method of Bradford [14], using BSA as standard.

Abbreviations used: MeAIB, α-(methylamino)isobutyric acid; BCH, 2-aminonorbornane-2-carboxylic acid; CM, complete medium; AASM, amino acid starvation medium; Cho^+, choline chloride; 1 × SSC, 0.15 M NaCl/0.015 M sodium citrate.

Total RNA isolation

Total RNA isolation was carried out from individual 35 mm-diam.-dish-grown cell cultures as described previously [9]. Typically 100 μg of total RNA was recovered from one Petri dish of control fed cells (1.1 mg of protein).

Northern-probe preparation

The plasmid pCR318 [11] was kindly given by Dr. J. Lever (University of Texas Medical School, Houston, TX, U.S.A.) and contains a 318 bp (cDNA) SAAT1-specific insert. The plasmid was amplified in the *Escherichia coli* host XL1 (Stratagene) and purified using a Qiagen column (Qiaex; Hybaid). The insert was excised by *Hin*dIII/*Eco*RI double digestion [Pharmacia (U.K.)] and the inset separated from the plasmid on a 1.5 % -agarose gel by electrophoresis. The gel fragment containing the insert was purified (Qiaex procedure; Qiagen) according to the manufacturer's instructions). The purified insert (typically 1 μg) was radioisotopically labelled using the Random Primed DNA Labelling Kit (USB) and [^{32}P]dCTP (3000 Ci/mmol; Amersham International).

Northern analysis

The total RNA prepared as described above was denatured (15 min, 70 °C) in formamide [15]. After rapid chilling on an ice slurry, RNA sample buffer (7.5 μl, [15]) was added and the samples loaded on to a 1.7 % formaldehyde gel prepared and run as described by Sambrook et al. [15]. RNA size markers were from Promega Corp. The transfer to nylon membrane (Hybond N; Amersham International) was carried out as recommended by the manufacturer. Transfer was allowed to proceed for 14 h, then the membrane was dried and cross-linked (5 min) on a u.v. transilluminator (UV Products). The membrane was prehybridized (2 h, 65 °C) in hybridization solution made up as recommended by the membrane manufacturers (Hybond N handbook; Amersham International) and then hybridized (20 h, 45 °C) in the same solution with added denatured label (50 ng/ml). The posthybridization washes were 2 × SSC/0.1 % SDS, 20 °C; 1 × SSC/0.1 % SDS, 20 °C; 1 × SSC/0.1 % SDS, 55 °C (20 min, repeated twice); and where stated, 0.5 × SSC/65 °C (20 min) and finally 2 × SSC, 20 °C. The membranes were sealed in polythene bags and autoradiographed (−70 °C, 2–6 days; Hyperfilm-MP, Amersham International). (Note: 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate.)

The actin probe was made by radioisotopic labelling (as described above) of an *Eco*RI (Pharmacia)-linearized plasmid (pAT153) containing part of the human actin sequence. Northern blots were re-probed with the actin label after stripping the membranes with a boiling SDS (0.1 %) solution. Autoradiography was carried out for 2–10 days.

[^{35}S]Methionine labelling of cells

Confluent monolayer cultures of NBL-1 cells were incubated in 2 ml of fresh complete medium (CM), amino acid starvation medium (AASM) and (AASM + one amino acid), as stated (3 mM). Cells were incubated for 60 min, and then [^{35}S]methionine (Amersham) was added (10 μCi/ml). Incubation was continued for a further 24 h, after which the medium was removed, the cells harvested by scraping them off into PBS (1 ml) and microcentrifuging (12000 rev./min; $r_{av.}$ 5 cm; 30 min). Cell pellets were lysed in 50 μl of 5 % decanoyl *N*-glucamide, and 200 μl of SDS/PAGE sample buffer [16]; 5 μl aliquots were separated by SDS/10 %-PAGE as described in [16] and the gels were soaked in Amplify (Amersham), dried, and fluorographed overnight.

Gel scanning

Fluorographs were scanned using a Chromoscan 3 (Joyce Loebl, Newcastle, Tyne and Wear, U.K.).

Statistical analysis

All experiments shown were performed between three and six times. Each Table or Figure shows a single representative experiment. Data are means ± S.E.M. of three determinations for each point unless stated otherwise. Significance was assessed by Student's *t* test.

RESULTS AND DISCUSSION

Amino-acid-starvation effect on alanine uptake

The bovine kidney cell line NBL-1 has been used by us as a model system for analysing amino-acid-transport systems and their regulation. System A is up-regulated in this cell line upon amino acid deprivation. Inclusion of at least one amino acid from the repertoire of System A-transported amino acids or analogues (e.g. alanine, glutamine or MeAIB) suppresses the starvation-induced up-regulation [4]. As a continuation of this study, the decay rate of amino-acid-starvation-induced System A activity was determined by transferring cells back into CM.

Starved cells grown in the presence of a System A repressor (L-alanine) and a non-repressor (L-phenylalanine, hereafter called L-Phe) were transferred to CM, and L-alanine (0.1 mM)-uptake rates determined. To distinguish between System A and the other main alanine-transport system, System B^0, alanine transport was allowed to proceed in the presence of excess MeAIB (5 mM). Inclusion of L-Phe in the AASM prior to transfer to CM (15 min) led to an unexpected increase in MeAIB-insensitive, Na^+/alanine co-transport rates. The results are presented in Table 1. The increase in total alanine-transport activity after incubation in AASM is due almost entirely to System A (94 % was MeAIB-sensitive relative to CM). However, a significant (26 %) increase in MeAIB-insensitive Na^+/alanine transport was observed in cells incubated in AASM relative to cells incubated in CM. This increase was much the same after equilibration in CM (29 %). The addition of L-alanine to the AASM repressed the MeAIB-insensitive increase to a level similar to that of CM cultures. When L-Phe was included in the AASM, it appeared that System A was not repressed by this amino acid as it was with L-alanine. However, once the equilibration step was carried out it was apparent that not all the increase in total alanine uptake was due to System A. Inclusion of L-Phe in AASM increased MeAIB-insensitive transport by 91 % relative to CM-incubated cells and by an extra 48 % relative to AASM-incubated cells. The L-Phe-increased 'latent' MeAIB-insensitive alanine-transport activity was suppressed on inclusion of L-alanine.

Hybridization of amino-acid-starved-cell RNA with SAAT1

A neutral-amino-acid transporter cDNA clone, SAAT1, has recently been isolated [11] and has been associated with System A. Since System A transport activity was observed to increase 2-fold on starvation of NBL-1 over 24 h (as described above; see also [4]), total RNA was extracted from fed and starved NBL-1 cells at 5 h intervals over 30 h and Northern hybridization

Table 1 Effect of equilibration in complete medium on MeAIB-sensitive and -insensitive Na^+-dependent alanine uptake by NBL-1 cells

Monolayer cultures of NBL-1 cells were switched to fresh medium (2 ml) or AASM (2 ml) with or without addition of an amino acid (3 mM). The Na^+-dependent alanine (0.1 mM)-transport rate and MeAIB(5 mM)-insensitive rates were measured after 20 h further incubation in the switch media, either before (non-equilibrated) or after (equilibrated), replacing the switch medium with fresh CM (2 ml) for 15–30 min. The Na^+-dependent rate is the rate in Na^+-transport medium minus that in choline chloride (Cho^+-transport medium. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus CM value in same column; †$P < 0.05$; ††$P < 0.01$ versus corresponding non-equilibrated value.

	Na^+-dependent alanine uptake (nmol/3 min per mg of protein)			
	Non-equilibrated		Equilibrated	
Switch medium	−MeAIB	+MeAIB	−MeAIB	+MeAIB
CM	5.58 ± 0.79	5.10 ± 0.20	5.96 ± 0.07	4.86 ± 0.28
AASM	11.27 ± 0.34**	6.45 ± 0.37*	10.35 ± 0.55**	6.30 ± 0.13**
(AASM + Ala)	7.22 ± 0.34	5.97 ± 0.05*	6.32 ± 0.63	5.44† ± 0.13
(AASM + Phe)	11.96 ± 0.38**	5.35 ± 0.38	14.56† ± 0.81***	9.31†† ± 0.37***
(AASM + Ala, + Phe)	7.76 ± 0.14	6.85 ± 0.32*	7.20 ± 0.24**	5.9 ± 0.29

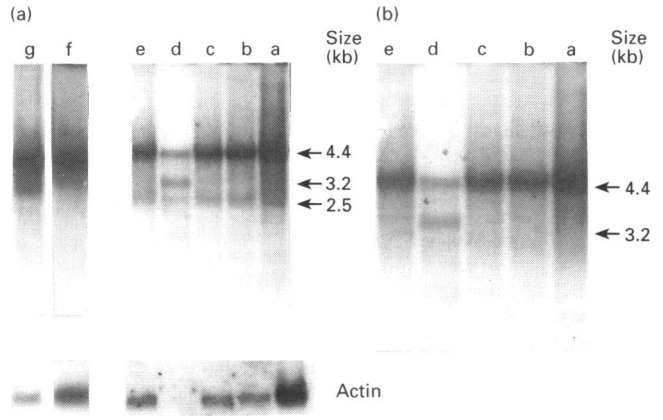

Figure 1 Northern blot of total RNA from NBL-1 cells grown in CM, AASM with and without L-alanine or L-Phe

Confluent monolayer cultures of NBL-1 cells were switched to fresh CM (a), AASM (b), AASM + L-alanine (3 mM) (c), AASM + L-Phe (3 mM) (d) or AASM + L-alanine-L-Phe (3 mM each) (e) for 20 h. Total RNA extractions were carried out as described in the Materials and methods section from one 35 mm-diam.-dish culture for each treatment. Half the total RNA extracted from each treatment was denatured and separated on an agarose gel (1%), transferred to a membrane and probed with the 318 bp SAAT1-specific cDNA. (**a**) (Tracks a–e) is the result after a final wash of 1 × SSC/0.1% SDS at 55 °C; (**b**) is the result after a further final wash of 0.5 × SSC/1% SDS at 65 °C. (**a**) Tracks f (AASM) and g (AASM + L-Phe, 3 mM) are included to show a second Northern (65 °C, final wash) with all the total RNA extracted from one 35 mm-diam.-Petri-dish culture loaded. The actin-probe hybridization is indicated.

analysis carried out with the 318 bp SAAT1-specific cDNA probe. The probe hybridized to a 4.4 kb and a 2.5 kb transcript. However, no increase in transcript levels was observed over 30 h of amino acid deprivation, although System A transport increased 2-fold (results not shown). Northern-blot analysis was also carried out with total RNA extracted from CM-fed cells or AASM-treated cells with and without L-alanine, L-Phe or both. One culture dish was used for each treatment, and half the total RNA extracted was denatured and loaded on to an agarose gel. The relative RNA transcript levels observed were therefore directly comparable with the respective transport levels. The Northern blot is presented in Figure 1. Since the 318 bp probe was of porcine origin and NBL-1 cells were of bovine origin, the final hybridization wash was initially carried out at 1 × SSC/0.1% SDS (55 °C) (a medium–high stringency wash for a probe of 300 bp and of different species origin). This result is presented in Figure 1(a). The SAAT1 probe hybridized to a 4.4 kb transcript and a 2.5 kb transcript in all the samples, but also hybridized to a new transcript (3.2 kb) present in RNA from AASM + L-Phe-treated cells only. The blot was rewashed at a higher stringency (0.5 × SSC/1% SDS, 65 °C), and this is presented in Figure 1(b). The 2.5 kb transcript was lost from all the samples, but the L-Phe-induced transcript (3.2 kb) was retained. Inclusion of L-alanine in the AASM + L-Phe treatments prevented synthesis of the 3.2 kb transcript. These findings mirror the effects seen with latent MeAIB-insensitive alanine transport presented in Table 1.

Actin probing of the filters (Figure 1) revealed that the L-Phe-treated sample was not overloaded with extracted RNA relative to the other samples (autoradiographed for 2 days). The actin RNA levels were found to be generally lower in AASM-treated cells over a 24 h period, but were severely depleted in L-Phe-treated cells. A 50% reduction in transcription of the β-actin gene was reported for cells stressed in hypertonic medium [17]. The lack of actin RNA labelling in the AASM + L-Phe-treated cells was due to the fact that the RNA was isolated from less cells (as determined by protein levels) relative to control (fed) cells, and was not due to degradation, since no smaller actin-hybridizing transcripts were visible even after prolonged autoradiography (10 days). Tracks from a second Northern blot with greater loadings of RNA are presented to show that the actin from AASM + L-Phe-treated cells is of full size ($\approx$ 2 kb) and not degraded. Further Northern analysis of cells grown in AASM plus L-serine, L-lysine, L-leucine or L-glutamine showed that inclusion of these amino acids did not lead to induction of the 3.2 kb transcript (results not shown).

Analysis of the equilibration step

In order to determine how necessary the equilibration medium constituents were, and for how long the equilibration step had to be carried out, initial tests were performed. AASM + L-Phe-treated cells were resuspended for 15 min in various media. The results of the effect on MeAIB-sensitive Na^+/alanine co-transport is presented in Table 2. Resuspension in fresh AASM led to a decrease in transport which was prevented by inclusion of amino acids. However, addition of amino acids to AASM did not significantly increase transport, and therefore amino acid exchange was not affecting the increase in transport. The most significant increase was observed after equilibration in CM, and

Table 2 Effect of equilibration-medium constituents on MeAIB-insensitive Na^+-dependent alanine-transport activity

Monolayer cultures of NBL-1 cells were switched to AASM + Phe (3 mM) for 20 h. Transport assays were then carried out (non-equilibrated) or the medium was replaced by fresh AASM; fresh (AASM + aa) (alanine, glutamine and leucine at 2 mM each); fresh (AASM + newborn-calf serum) (NCS, 10%); HAM's F-12 + NCS (CM) or HAM's F-12 salts medium for 15 min prior to transport assays. $^*P < 0.05$; $^{**}P < 0.01$ versus non-equilibrated control.

Equilibration medium	MeAIB-insensitive Na^+/alanine uptake (nmol/3 min per mg of protein)
Non-equilibrated	5.35 ± 0.38
Fresh AASM	2.51 ± 0.54*
Fresh AASM + aa	6.16 ± 0.16
Fresh AASM + NCS	7.25 ± 0.62
HAM's salts medium	6.95 ± 0.48
CM	9.31 ± 0.37**

so this was used throughout the experiments. This finding was unexpected, and it remains difficult to explain until extensive analysis is conducted to determine which of the constituents (or combination of these) in CM are needed for optimum transport.

A time course for the equilibration in CM step showed that optimum equilibration was attained within 5 min of resuspension in CM and there was no further significant increase over the 60 min of continued sampling (results not shown). The need for only such a short period of equilibration eliminates the possibility that further protein synthesis was required for the transport rate to increase.

Effect on latent transport rate by other amino acids and analogues

In order to determine whether the increase in MeAIB-insensitive Na^+/alanine co-transport resulted specifically from the presence of L-Phe in AASM, a range of other amino acids and analogues were tested. The results are presented in Table 3. The only additions that were found to significantly increase uptake were D-Phe and the arginine analogue canavanine. However, the proline analogue L-azetidine-2-carboxylic acid had no effect on alanine uptake. Addition of L-cysteine and L-tryptophan decreased the transport rate, most likely because they caused extensive cell-lifting from the plates, and remaining cells may have been impaired in transport.

Effect of the inclusion of further amino acids to AASM + L-Phe-treated cells on alanine-uptake rates

to confirm the necessity for amino acid deprivation for the inclusion of L-Phe to increase transport, a number of other amino acids were co-included in the AASM medium with L-Phe. The effect on transport rate is presented in Table 4. Inclusion of a single amino acid in the AASM acted to prevent induction of transport. In every case the transport rate was reduced to that in AASM-treated cells. Addition of L-leucine did not reduce the level as much as the other amino acids. This lack of total inhibition by L-leucine has been observed by us previously in similar studies [9].

The observations in Table 4 eliminate the possibility that L-Phe was *trans*-stimulating exchange, since its presence plus further amino acids leads to loss of increased transport.

Table 3 Addition of various amino acids and analogues to AASM and subsequent effect on MeAIB-insensitive Na^+/alanine uptake

Monolayer cultures of NBL-1 were switched to AASM and AASM + one amino acid (3 mM) or analogue (1 mM) and incubated for 18 h. The medium was replaced by CM (2 ml) 15 min prior to carrying out MeAIB-insensitive alanine-uptake assays. Results are means ± S.E.M. of four determinations. $^{**}P < 0.01$; $^{***}P < 0.001$ versus AASM.

Switch medium	MeAIB-insensitive Na^+/alanine uptake (nmol/3 min per mg of protein)
AASM	4.66 ± 0.15
+ L-Phe	7.05 ± 0.17***
+ L-Cysteine	2.34 ± 0.10
+ L-Serine	3.51 ± 0.20
+ L-Tryptophan	1.21 ± 0.16
+ L-Lysine	4.42 ± 0.22
+ L-Leucine	4.49 ± 0.04
+ L-Glutamine	3.03 ± 0.29
+ D-Alanine	4.78 ± 0.16
+ D-Phe	5.98 ± 0.34**
+ Canavanine	6.31 ± 0.23***
+ Azetidine	3.70 ± 0.22

Table 4 Addition of a second amino acid to the amino-acid-starved phenylalanine-treated NBL-1 cells and the effect on MeAIB-insensitive Na^+/alanine uptake

Monolayer cultures of NBL-1 cells were switched to AASM with or without L-Phe (3 mM) and with or without a second amino acid (3 mM). After 20 h the switch media were replaced by CM (2 ml) 15 min prior to assaying for MeAIB-insensitive Na^+/alanine uptake. $^{***}P < 0.001$ versus AASM.

Medium	MeAIB-insensitive Na^+/alanine uptake (nmol/3 min per mg of protein)
Untreated	3.80 ± 0.09
AASM	4.40 ± 0.20
AASM + L-Phe	11.83 ± 0.76***
AASM + L-Phe + L-glutamine	4.31 ± 0.14
AASM + L-Phe + L-leucine	5.40 ± 0.37
AASM + L-Phe + L-lysine	4.43 ± 0.54
AASM + L-Phe + L-glycine	4.63 ± 0.09
AASM + L-Phe + L-serine	4.51 ± 0.26

Concentration-dependence of L-Phe additions

L-Phe was routinely used at 3 mM, higher levels leading to cell-lifting from the plates. The use of much lower levels of L-Phe did not lead to a significant increase in transport rate. In a typical experiment the rates in AASM were 6.7 ± 0.26 nmol/3 min per mg; including 0.03 mM L-Phe they were 7.1 ± 0.45 nmol/3 min per mg (not significant), with 0.3 mM L-Phe, 7.6 ± 0.36 nmol/3 min per mg (not significant) and with 3 mM L-Phe, 11.7 ± 0.09 ($P < 0.001$). The need for non-physiological levels of L-Phe suggests that the increase in transport activity may be a result of cell stress, since the increase is mimicked by canavanine, which has been shown to cause a cellular-stress response [18]. The analogue *p*-fluorophenylalanine has also been implicated in causing a cell-stress response, and it is possible that phenylalanine at 3 mM has a specific effect in eliciting a similar response.

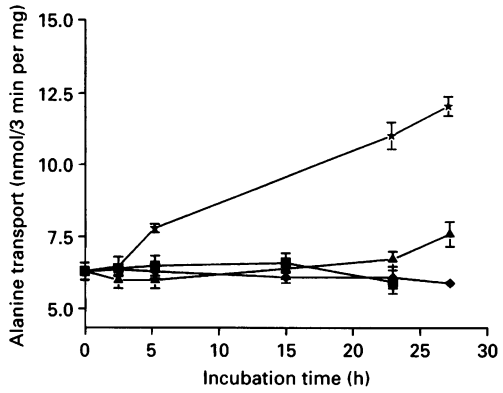

Figure 2 Time course of the induction of MeAIB-insensitive Na^+/alanine transport by NBL-1 cells in the presence or absence of cycloheximide

Confluent NBL-1 cell monolayers were switched to fresh CM or AASM + L-Phe (3 mM), with and without cycloheximide (9 μg/ml), for up to 28 h. The initial rate of alanine transport (assayed with 0.1 mM alanine and 5 mM MeAIB) was measured in the presence or absence of Na^+ over 3 min (nmol/3 min per mg of protein). The values shown are of the Na^+-dependent rate (the rate in the presence of Na^+ minus that in the presence of Cho^+). The switch medium was replaced with CM for 15 min prior to transport assay for each time-course estimation. Results are means ± S.E.M. of three to six determinations. Where error bars are not shown, they are incorporated within the symbol. ◆, Rate of cells switched to CM; ▲, rate of cells switched to AASM; ★, rate of cells switched to AASM + L-Phe (3 mM); ■, rate of cells switched to AASM + L-Phe (3 mM) + cycloheximide (9 μg/ml).

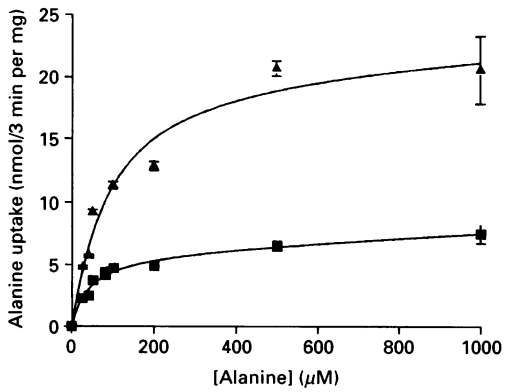

Figure 3 Concentration-dependence of MeAIB-insensitive Na^+/alanine uptake kinetics in CM-grown NBL-1 cells and AASM-L-Phe treated NBL-1 cells

Confluent monolayers of NBL-1 cells were switched to fresh CM or AASM + L-Phe (3 mM) and incubated for a further 19 h. They were then switched to fresh CM 15 min prior to carrying out assays for MeAIB (5 mM)-insensitive Na^+/alanine transport. Initial rates of alanine uptake were measured at 3 min (nmol/3 min per mg of protein) over a range of concentrations in Na^+ or Cho^+ transport media. The Na^+-dependent rate was derived by subtracting the rate in the presence of Cho^+ from that in Na^+ medium. The data points are means ± S.E.M. for three to six determinations. ■, Control (CM) cells; ▲, AASM + L-Phe (3 mM)-treated cells.

Time course of induction of transport

Kinetic analysis of the L-Phe-induced system is presented in Figure 2. The time course of induction of increased MeAIB-insensitive alanine uptake follows the expected pattern for transcriptional control and was not therefore due to exchange, which would be characterized by a much more rapid increase in transport levels. The transport rate increase was entirely prevented in the presence of cycloheximide, suggesting the need for protein synthesis.

Table 5 Effect on MeAIB-insensitive Na^+/alanine transport in amino-acid-starved phenylalanine-treated NBL-1 cells by the addition of various amino acids in the transport medium

Monolayer cultures of NBL-1 cells were switched (for 20 h) to fresh CM or (AASM + L-Phe) (3 mM), equilibrated in fresh CM (15 min) and then MeAIB (5 mM)-insensitive Na^+/alanine uptake was estimated in the presence of various inhibitors (5 mM; except cysteine, which was 2.5 mM). Measurements were carried out at 27 °C. Results are means ± S.E.M. of three determinations. Values in parentheses are percentage inhibitions relative to respective controls.

	MeAIB-insensitive Na^+/alanine uptake (nmol/3 min per mg of protein)	
Addition	CM	AASM + Phe
None (control	10.9 ± 0.27	18.3 ± 0.71
L-Serine	1.0 ± 0.19 (91)	1.6 ± 0.04 (91)
L-Lysine	11.1 ± 0.29 (0)	18.9 ± 0.23 (0)
L-Cysteine	1.2 ± 0.19 (89)	1.8 ± 0.07 (90)
BCH	8.2 ± 0.17 (24)	17.3 ± 0.81 (6)
L-Leucine	3.9 ± 0.19 (64)	7.6 ± 0.53 (59)
L-Phe	6.3 ± 0.13 (42)	12.9 ± 0.37 (29)
L-Glutamine	1.9 ± 0.06 (83)	2.4 ± 0.02 (87)

$V_{max.}$ and K_m values of increased transport

Substrate-concentration-dependence kinetics of the induced alanine-transport activity are presented in Figure 3. The control cells had MeAIB-insensitive Na^+/alanine co-transport that was saturable, with a $V_{max.}$ value of 6.0 ± 0.7 nmol/3 min per mg and that of L-Phe-treated cells was increased ≈ 2.5-fold to 21 ± 5 nmol/3 min per mg (Michaelis–Menten fit kinetics). The K_m values were similar in the control and treated cells: 41 ± 12 μM for control cells and 92 ± 40 μM for L-Phe-treated cells.

Does L-Phe induce a novel transport system?

NBL-1 cells possess a number of now-well-characterized transport systems (System B° [1], System A [4] and System X_{AG^-} [9]). The MeAIB-insensitive nature of the induced transport system reported here distinguishes it from System A. However, in order to determine whether the L-Phe-inducible system was novel or the same as the other MeAIB-insensitive Na^+/alanine-transport system inherent in this cell line (System B°), inhibition kinetics by various amino acids was carried out. The results are presented in Table 5. The main amino acids characteristic of the broad-specificity transport system, System B°, inhibit the control (fed) and AASM + L-Phe-treated cells in a similar manner. The inhibition profile for both control cells and L-Phe-treated cells was: serine > cysteine > glutamine > leucine > phenylalanine > 2-aminonorbornane-2-carboxylic acid (BCH), with no inhibition by lysine of either.

The cumulative results from the kinetic analysis suggest that L-Phe treatment of starved cells leads to an increase in existing System B° transport activity and is not due to synthesis of a new class of transporter.

[^{35}S]Methionine labelling of NBL-1 cells

Since protein synthesis was required in order for the transport activity to be expressed, *de-novo*-synthesized proteins under conditions of starvation and in the presence of single amino acids

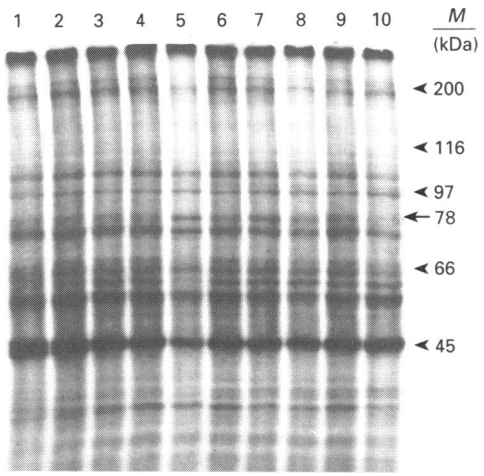

Figure 4 Fluorograph of SDS/PAGE separation of [^{35}S]methionine-labelled NBL-1 cells grown in CM or AASM with the addition of different amino acids

Confluent monolayer cultures of NBL-1 cells were switched to fresh CM, AASM, and AASM + one amino acid (3 mM) in the presence of [^{35}S]methionine as described in Materials and methods section. Cell lysates were separated by SDS/PAGE (10%). Tracks are: 1, AASM; 2, AASM + L-alanine; 3, AASM + MeAIB; 4, AASM + L-threonine, 5, AASM + L-Phe; 6, AASM + L-proline; 7, AASM + L-leucine; 8, AASM + L-cysteine; 9, AASM + L-lysine; 10, CM. Molecular-mass (*M*) markers (◀) are as indicated. The ratio of the peak absorbance of the polypeptide band at 78 kDa (←) relative to the 45 kDa band (actin loading guide) as determined by spectrophotometric scanning of each track was: track 1, 3.7; 2, 3.5; 3, 3.6; 4, 3.6; 5, 5.5; 6, 3.8; 7, 4.6; 8, 3.9; 9, 4.0; 10, 2.7.

were labelled with [^{35}S]methionine. A fluorograph of SDS/PAGE-separated proteins is presented in Figure 4. The fluorograph was scanned and the ratios of the loading guide (polypeptide at 45 kDa) relative to a 78 kDa polypeptide which was relatively differentially labelled, are presented in the legend. The ratios clearly demonstrate that addition of L-Phe had the greatest effect in increased differential labelling of the 78 kDa polypeptide. The synthesis of a number of other proteins was depressed in the L-Phe-treated cells.

DISCUSSION

Amino acid starvation of NBL-1 cells leads to a small, but significant, increase in MeAIB-insensitive alanine-transport activity. Such a small increase (25%) on starvation may explain why, previously, MeAIB-insensitive alanine-transport was not considered to undergo an adaptive response. On inclusion of phenylalanine, MeAIB-insensitive alanine uptake was further increased (> 80%). The need for pre-equilibration in a complete medium after cell starvation/stress before the increased transport becomes apparent may explain why this effect has not been reported before.

The fact that 3 mM L-Phe was required in order to increase uptake further and significantly may be because L-Phe accentuates the starvation effect by causing an imbalance in amino acid pools, as occurs in amino-acid-starved yeast cells on addition of a single amino acid [19]. Alternatively, 3 mM L-Phe may be causing cell stress. The activation of stress-gene expression ensures cell survival under suboptimal physiological conditions [20]. Analogues as distinct as canavanine and *p*-fluorophenylalanine have been reported to evoke rapid synthesis of a small number of polypeptides associated with stress [18]. Petronini et al. [21], using antibodies against heat-shock protein 70 (hsp70), found that this was up-regulated during hypertonic stress and down-regulated on uptake of betaine. Also, a previous study by Kelley and Schlesinger [22] noted the synthesis of stress proteins under various conditions (e.g. canavanine, heat shock) and they speculated that these proteins may be associated with a hexose-transport system.

The induction of a new transcript upon phenylalanine treatment of starved cells represents a novel means of control for mammalian transporters. Although up-regulation of amino acid transporters in NBL-1 cells by amino acid starvation has now been well documented [4,9] the specific induction of a transport system by the availability of a single amino acid is entirely novel in eukaryotic systems. The concomitant production of a transcript which hybridizes to the recently cloned neutral-amino-acid transporter SAAT1, under the same conditions, suggests SAAT1 and the L-Phe-induced System B^0 are the same or very similar.

SAAT1 is related to a number of other Na^+-dependent transporters, namely Na^+/glucose (SGLT1 [23]), Na^+/nucleoside (SNST1 [24]) and Na^+/myoinositol (SMIT [25]). Of these, SMIT has been shown to be up-regulated during hypertonic stress, with a concomitant increase in transcript levels [17]. Lescale-Matys et al. [26], using SGLT1 as a probe, found multiple transcripts in sheep intestine, and an attempt to correlate differential expression of these with Na^+/glucose transport led them to conclude that regulation of SGLT1 expression was at the translational or post-translational level. Multiple transcripts of SGLT1 were also found in human intestine (2.2, 2.6 and 4.8 kb), and cDNA clones of these were isolated [23]. The differences in the transcripts were found to be due to the length of the 3′ non-coding region. Kong et al. [11] found that SAAT1 hybridized to different transcripts in a tissue-specific manner. SAAT1 hybridized to a 2.4 kb species in kidney, to 2.4, 3.7 and 4.9 kb species in the small intestine, and to 2.4 and 3.7 kb species in the porcine renal cell line LLC-PK_1.

It is noteworthy that isolation of the Na^+/glucose transporter (SGLT1) has led to the isolation of a number of other very similar transporters for amino acid transport (SAAT1), nucleoside transport (SNST1) and Na^+/myoinositol transport (SMIT). Of those that have been looked at in terms of regulation, SMIT has been found to be up-regulated by hypertonic stress, and SAAT1 has now been shown to be up-regulated by particular amino-acid- or analogue-induced cell stress. It is of course possible that all these highly related transporters are co-regulated, and this has yet to be looked at.

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