Regulation of the glutamate transporter by amino acid deprivation and associated effects on the level of EAAC1 mRNA in the renal epithelial cell line NBL-1

Stella PLAKIDOU-DYMOCK* and John D. McGIVAN

Department of Biochemistry, University of Bristol, School of Medical Sciences, University Walk, Bristol BS8 1TD, U.K.

The glutamate transport system of the bovine renal epithelial cell line NBL-1 was studied. The K_m for Na⁺-dependent glutamate transport was found to be $13.8 \pm 2.4 \mu\text{M}$ (V_{max} , $365 \pm 19.2 \text{ pmol/3 min per mg}$) and for Na⁺-dependent aspartate transport $4.5 \pm 1.1 \mu\text{M}$ (V_{max} , $108 \pm 6.3 \text{ pmol/3 min per mg}$). The K_m values are in close agreement with those expected for high-affinity Na⁺-dependent glutamate transport by System X_{AG}⁻. Upon deprivation of amino acids, the V_{max} for Na⁺/aspartate co-transport rose to $203 \pm 6.0 \text{ pmol/3 min per mg}$

(K_m $3.8 \pm 0.5 \mu\text{M}$). A probe was constructed to the high-affinity excitatory amino acid carrier (EAAC1) [Kanai and Hediger (1992) *Nature* (London) **360**, 467–471]. The probe hybridized to a 3.5 kb transcript. On deprivation of amino acids, the level of EAAC1 mRNA decreased sharply before the measurable increase in transport levels, but was subsequently restored to control levels. A motif, which we propose is linked to amino acid deprivation, was found in the EAAC1 primary sequence.

INTRODUCTION

Glutamic acid, as well as being essential for protein synthesis, is involved in energy production, movement of reducing equivalents, urea and glutathione synthesis (Meister, 1979) and is also a neurotransmitter. The plasma-membrane transport systems that operate to ensure adequate intracellular levels of glutamate have been much studied (for a review see Guidotti and Gazzola, 1992). Three transport systems for glutamate have been described in fibroblasts (Dall'Asta et al., 1983) and hepatocytes (Gebhardt and Mecke, 1983): a Na⁺-independent system which also transports cystine (x_c⁻), a low-affinity (K_m 2–4 mM) Na⁺-dependent system which is also predominant in intestine (Wingrove and Kimmich, 1988), and the Na⁺-dependent high-affinity (K_m 2–50 μM) system, designated X_{AG}⁻ (Dall'Asta et al., 1983). System X_{AG}⁻ is characterized by its stereospecificity for L-glutamate, but non-selectivity for aspartate, since both the L- and D-isomers are transported. There is considerable heterogeneity in the high-affinity transporters (Ferkany and Coyle, 1986), and this has been exemplified recently by the cloning of three Na⁺-dependent high-affinity glutamate transporters whose cDNA sequences are all unique (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992).

High-affinity Na⁺-dependent glutamate transport has been much studied in neuronal and glial tissue, kidney and liver. Glutamate is taken up avidly by glial cells to terminate the transmitter action of glutamate released by neurons (Brew and Attwell, 1987). In the kidney, high-affinity glutamate transport is present in both the brush-border membrane and the basolateral membrane of the proximal tubule (Sacktor et al., 1981; Samarzija and Fromter, 1976). In liver, the Na⁺-dependent high-affinity glutamate transport system is localized in the canalicular membrane (Ballatori et al., 1986) and located mainly in perivenous hepatocytes (Burger et al., 1989; Taylor and Rennie, 1987). That portal glutamate is preferentially taken up by glutamine-synthesizing hepatocytes in the perivenous area of the liver was first demonstrated by Häussinger and Gerok (1983), whose findings

suggested metabolic zonation of the glutamate transport system in the plasma membrane. Hepatocyte heterogeneity in Na⁺-independent glutamate uptake was also described by this group (Häussinger et al., 1989).

Whole-animal studies have shown that glutamate transport is subject to regulation. An increase in Na⁺-dependent glutamate uptake was found by Low et al. (1992) in liver membrane vesicles from dexamethasone-treated rats. This group also found that streptozocin-diabetes led to an increase in V_{max} for Na⁺-dependent glutamate transport in sinusoidal (but not bile-canalicular) rat liver membrane vesicles.

The use of tissue culture has enabled further studies on the regulation of glutamate transport. In cultured fibroblasts, the contribution of the various entry systems for glutamate has been studied (Dall'Asta et al., 1983). This group concluded that increased cell density promoted uptake by system X_{AG}⁻; however, deprivation of amino acids led to an increase in glutamate transport via increased V_{max} of System x_c⁻. Using cultured rat hepatocytes, Gebhardt and Mecke (1983), observed stimulation of Na⁺-dependent glutamate uptake after dexamethasone treatment, and they also found that Na⁺-dependent glutamate uptake increased spontaneously during cultivation.

Aspects of the regulation of amino acid transport 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 B⁰ (Doyle and McGivan, 1992). System A is absent from these cells, but emerges on amino acid starvation (Felipe et al., 1992) and is further induced by hyperosmotic shock after amino acid deprivation (Soler et al., 1993). Adaptive regulation of system A by an increase in transport rate produced by amino acid starvation is of great physiological importance and has been conserved in this cell line (Felipe et al., 1992). In this paper we characterize the glutamate transport system of NBL-1 cells and show that it is subject to regulation by amino acid starvation, in an analogous manner to System A.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Cell culture

The establishment and maintenance of the NBL-1 cell line has been described previously (Felipe et al., 1992). After trypsin treatment of the contents of a 75 cm² flask, cells were diluted to 80–90 ml in HAM's F-12 (GIBCO) supplemented medium, and 2 ml portions were seeded into 35 mm-diameter Petri dishes (30). These were fed every other day, and used in experiments typically after 4–6 days. Amino acid-free medium made up as described by Felipe et al. (1992) was supplemented in some experiments, where shown, with single amino acids at 0.5 mM.

Transport assays

Transport was measured by a modification of the method of Quamme et al. (1989). Briefly, cells in 35 mm Petri dishes were washed (3 × 1 ml) with basic transport medium (137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgSO₄, 10 mM Hepes adjusted to pH 7.4 with Tris). Amino acid transport was initiated by addition of 1 ml of basic transport medium to which had been added amino-oxyacetate (0.5 mM) (to inhibit cell aspartate metabolism) and the radioisotope-labelled amino acid. L-[1-¹⁴C]Glutamic acid (Amersham) was added to 0.1 µCi/ml (1.7 µM) and the specified concentration was made up with unlabelled glutamate (pH 7.4). L-[U-¹⁴C]Aspartate (Amersham) was similarly added to transport medium at 0.05 µCi/ml (0.23 µM) and made up to the specified concentration with unlabelled aspartate (pH 7.4). Uptake was carried out at 20 °C. The uptake was terminated by washing (6 × 1 ml) with ice-cold 137 mM NaCl/10 mM Tris/Hepes, pH 7.4. Plates were drained and lysed by addition of Triton X-100 (0.5 ml; 0.5 %) for 30 min before sucking up and down with a 1 ml pipette tip. Samples (200 µl) were added to scintillation fluid for counting of radioactivity. Protein was measured by the method of Bradford (1976). Na⁺-independent transport was measured by replacing NaCl with 137 mM choline chloride in the transport medium.

EAAC1 probe construction

Primers were made (in house) to the amino acid sequence IIEVEDWEIF spanning amino acids 270–279 and to YFNGGFAVDK spanning amino acids 503–512 of the published sequence of EAAC1 (Kanai and Hediger, 1992). The forward primer was:

5'ATT ATT GAA/GGTX GAA/GGAT TGG GAA/G ATT TT 3'

and the reverse primer was:

5'TT G/ATCXACTGC AAA TCCTCC G/ATT TAT G/ATA 3'

Isolation of total RNA

This was carried out from individual 35 mm-Petri-dish-grown cell cultures. The growth medium was drained and replaced by lysis medium (0.9 ml; 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol, 0.5 % *N*-lauroylsarcosine). Cells were disrupted by being drawn up and down a 1 ml pipette tip (15 times) and finally dispensed into an Eppendorf tube (2 ml capacity) containing 100 µl of sodium acetate (2 M, pH 4.5). Phenol/chloroform/3-methylbutan-1-ol (1 ml; 25:24:1, by vol.) was added and the tube was vortex-mixed for 30 s. The tubes were incubated at 4 °C for 15 min, and the solvent and aqueous layers were separated by spinning in a Microfuge (10 min, 12000 rev./min). The aqueous layer was

removed, mixed with an equal volume of propan-2-ol and incubated for 30 min (–20 °C). The RNA was pelleted (20 min, Microfuge) and resuspended in lysis buffer (300 µl). The RNA was precipitated again by addition of propan-2-ol (300 µl) and incubation (30 min, –20 °C). The RNA was pelleted (20 min, Microfuge) and resuspended in 18 µl of water for RNA to be used in Northern-blot analysis or 100 µl of water for use in cDNA synthesis.

First-strand cDNA synthesis

First-strand cDNA synthesis was primed with random hexamers [as oligo(dT)priming failed to give the required product after PCR amplification]. Total RNA (2 µg, 10 µl) was denatured (10 min, 65 °C) and added to the reaction mixture (total 40 µl) for Moloney Murine reverse transcriptase catalysis as recommended by the manufacturer (Gibco-BRL).

PCR amplification

The template (first-strand cDNA; 4 µl) was added to the *Taq* DNA polymerase mixture (total 40 µl) made up as recommended by the enzyme manufacturer (Boehringer Mannheim). Cycling (Thermal Reactor, Hybaid) was carried out at 94 °C (5 min), 55 °C (1 min), 72 °C (2 min) for one cycle, followed by 30 cycles at 94 °C (1 min), 55 °C (1 min), 72 °C (2 min). The single product (726 bp) was gel-purified (Qiaex procedure; Qiagen), blunt-ended with T₄ DNA polymerase (Pharmacia) at 11 °C for 20 min after treating with T₄ polynucleotide kinase (Pharmacia) (60 min, 37 °C). The PCR product was ligated (T₄ DNA ligase, Pharmacia) into *EcoRV*-cut pBluescript (Stratagene).

DNA sequencing

Sequencing of the 726 bp fragment cloned into pBluescript was carried out with dATP[α³⁵S] (Amersham) and the Sequenase version 2.0 Sequencing Kit (USB) according to the manufacturers' instructions.

Northern analysis

The total RNA prepared as described above was denatured (15 min, 70 °C) in formamide (Sambrook et al., 1989). After rapid chilling on ice slurry, RNA sample buffer (7.5 µl, as in

Sambrook et al., 1989) was added and the samples were loaded on a 1 %-formaldehyde gel prepared and run as described in Sambrook et al. (1989). RNA size markers were from Promega. The transfer to nylon membrane (Hybond N, Amersham) was carried out as described in the accompanying handbook. Transfer was allowed to proceed for 14 h, and the membrane dried and cross-linked (10 min) on a u.v. trans-illuminator (UV Products). The membrane was pre-hybridized (2 h, 65 °C; Hybond N handbook, Amersham) and then hybridized (20 h, 65 °C) in the above solution with added denatured label (2 µg/ml). The label was obtained by cutting out the cloned PCR product (double digest with *EcoRI* and *HindIII*) from pBluescript, separating it from the plasmid (1 %-agarose-gel electrophoresis), Qiaex (Qiagen)-purifying the DNA and using the Random Primed

Labelling Kit (USB) and [32 P]dCTP (Amersham). The post-hybridization washes were $1 \times$ SSC (SSC = $0.15 \text{ M NaCl}/0.015 \text{ M sodium citrate}$)/ 0.1% SDS, at 65°C , for 10 min, repeated twice, and finally $2 \times$ SSC, 20°C . The membranes were sealed and autoradiographed (Hyperfilm-MP, Amersham).

The actin probe was made by radioisotope-labelling (as described above) a *EcoRI*-linearized plasmid (pAT153), containing part of the human actin sequence. Northern blots were either re-probed with the actin label or labelled at the same time as hybridization with the EAAC1 probe.

Autoradiograph scans

The autoradiographs were scanned with a Chromoscan 3 (Joyce-Loebl), and the ratios of the calculated areas of the actin signal were compared with the EAAC1-probe signal.

RESULTS

The time course of the uptake of L-[^{14}C]aspartate ($0.12\text{--}50 \mu\text{M}$) into control monolayers of NBL-1 cells was linear for at least 10 min and was stimulated in the presence of an inward gradient of Na^+ (results not shown). Typical values for uptake at $5 \mu\text{M}$ aspartate were $57.9 \text{ pmol}/3 \text{ min per mg}$ in the presence of Na^+ and $5 \text{ pmol}/3 \text{ min per mg}$ in the absence of Na^+ ; at $50 \mu\text{M}$ aspartate, the corresponding rates were $117 \text{ pmol}/3 \text{ min per mg}$ and $17 \text{ pmol}/3 \text{ min per mg}$. In all the experiments, the Na^+ -dependent rate was relatively very low.

In order to compare the glutamate/aspartate transport system operative in NBL-1 cells with those previously characterized in other cell types, a concentration-dependence study was carried out for glutamate transport (Figure 1). The data described a single saturable system. Non-linear regression to the Michaelis-Menten equation gave values of $13.8 \pm 2.4 \mu\text{M}$ for the K_m for glutamate, and $365 \pm 19.2 \text{ pmol}/3 \text{ min per mg}$ for V_{max} . For Na^+ -dependent aspartate transport, K_m was $4.5 \pm 1.1 \mu\text{M}$ and V_{max} was $108 \pm 6.3 \text{ pmol}/3 \text{ min per mg}$. These K_m values are in close agreement with published data for high-affinity Na^+ -dependent glutamate transport in other cell types.

To establish the specificity of the glutamate/aspartate transporter in NBL-1 cells, the effect of various amino acids on the Na^+ -dependent rate of aspartate transport was determined and

Table 1 Amino acid inhibition of Na^+ -dependent L-aspartate transport

The uptake of $20 \mu\text{M}$ L-aspartate (in cells 5 days after trypsin treatment) was determined in the absence and presence of unlabelled amino acids at 1 mM , in both Na^+ - and choline-transport medium. Data presented are Na^+ -dependent rates and are means $\pm$ S.E.M. of three determinations: $**P \leq 0.001$, $*P \leq 0.05$ versus control (by Student's *t* test).

Addition	Rate (% of control)
None (control)	100 ± 11.3
D-Aspartate	$6.9 \pm 0.9^{**}$
L-Glutamate	$12.4 \pm 1.3^{**}$
D-Glutamate	91.5 ± 0.7
L-Lysine	106.0 ± 3.2
L-Leucine	109.2 ± 7.0
L-Alanine	$129.5 \pm 1.5^*$

the results are presented in Table 1. The order of inhibition was D-Aspartate > L-Glutamate > D-Glutamate, with no inhibitory effect seen with lysine, leucine or alanine. Since these findings suggest that the operative system in NBL-1 cells was similar to the well-characterized System X_{AG}^- , the rest of the experiments were performed with aspartate as substrate. The transaminase inhibitor amino-oxyacetate was included in the transport medium as a precaution against metabolism of labelled aspartate; however, over the 3 min during which transport was typically measured, the presence or absence of this inhibitor made no difference to the radioactivity measured.

Since glutamate/aspartate transport has been reported to be dependent on cell density (Dall'Asta et al., 1983), a time course of aspartate uptake was studied with freshly trypsin-treated cells (24 h) and monitored for 5 days (Figure 2). Typically, experiments were performed 4–5 days after trypsin treatment when rates of transport were most consistent and Na^+ -dependent aspartate ($20 \mu\text{M}$) uptake was between 40 and 60 $\text{pmol}/3 \text{ min per mg}$. Continued incubation (7–8 days) of cells under these conditions led to a sharp increase in Na^+ -dependent aspartate uptake (results not shown), but this represented a post-confluent state.

When cells 4–5 days after trypsin treatment were deprived of amino acids for 12 h, it was found that Na^+ -dependent aspartate

Figure 1 Glutamate uptake kinetics in NBL-1 cells

Initial rates of glutamate uptake (in cells 4 days after trypsin treatment) were measured at 3 min over a range of concentrations in Na^+ - or choline-containing media. The Na^+ -dependent rate was derived by subtracting the rate in the presence of Na^+ from that in choline medium. The data points are means $\pm$ S.E.M. for 3–6 determinations.

Figure 2 Uptake of aspartate in NBL-1 cultures after trypsin treatment

A flask of cells was treated with trypsin as described in the Materials and methods section, and seeded Petri-dish cultures were tested for uptake of aspartate ($20 \mu\text{M}$), over a period of 5 days. Data points are means $\pm$ S.E.M. of 3 cultures.

Figure 3 Time course of the induction of Na^+ -dependent aspartate transport by monolayers of NBL-1 cells 5 days after trypsin treatment, in the presence or absence of cycloheximide

NBL-1 cell monolayers were incubated in the presence or absence of amino acids with and without cycloheximide ($8 \mu\text{g/ml}$), for up to 30 h. The initial rate of L-aspartate ($20 \mu\text{M}$) transport was measured in the presence and absence of Na^+ over 3 min. The values shown are of the Na^+ -dependent rate (the rate in the presence of Na^+ minus that in the presence of choline). $\blacktriangle$, Rate for amino-acid-starved cells; $\blacksquare$, rate for control fed cells; $\blacktriangledown$, rate for amino-acid-starved cells plus cycloheximide ($8 \mu\text{g/ml}$). Results are means $\pm$ S.E.M. of 3–6 different Petri-dish monolayers.

uptake was increased about 2-fold. The time course of aspartate (0.12 – $100 \mu\text{M}$) uptake in starved cells remained linear over at least 10 min, and the Na^+ -independent rate was unaffected (results not shown). To characterize the starvation-enhanced aspartate uptake effect further, cells 4 days after trypsin treatment were switched to fresh control (fed) medium or amino-acid-free medium with and without cycloheximide. The initial rate of uptake of aspartate ($20 \mu\text{M}$) at intervals over 30 h is presented in Figure 3. Deprivation of amino acids led to an increase in Na^+ -dependent aspartate uptake, from about $60 \text{ pmol/3 min per mg}$ to about $120 \text{ pmol/3 min per mg}$, with an optimum between 11 and 20 h. Addition of cycloheximide prevented a sustainable increase in transport activity upon starvation, showing that the induction of the transport activity was a protein-synthesis-dependent phenomenon.

The concentration-dependence of the amino-acid-starvation-enhanced aspartate transport activity was compared with the basal level in control cells (Figure 4). The K_m of the starvation-enhanced Na^+ -dependent aspartate transport was similar to the basal value, at $3.8 \pm 0.5 \mu\text{M}$, but the V_{max} had almost doubled, to $203 \pm 6.0 \text{ pmol/3 min per mg}$. To determine the specificity of the starvation-enhanced Na^+ -dependent transporter, the rates of transport in 22 h-starved cells were measured in the presence of different amino acids at 1 mM . The results are presented in Table 2. The specificity of the enhanced transporter is similar to the basal-level transport, with greatest inhibition by D-aspartate, followed by L-glutamate and much less inhibition by D-glutamate. Some inhibition (12–28%) is seen on addition of the other amino acids. The data suggest that it is the activity of the control (basal)-level Na^+ -dependent aspartate transporter which is increased upon starvation, and not synthesis of a novel transporter.

The increase in the activity of the Na^+ -dependent aspartate transporter upon starvation was preventable, wholly or in part, by the addition of single amino acids (at 0.5 mM) to the starvation medium. The results are presented in Table 3. Greatest inhibition of the onset of the starvation effect was seen on inclusion of D-aspartate (the most specific amino acid for System X_{AG}^-), followed by L-glutamate and L-aspartate. D-Glutamate, glutamine and alanine also significantly inhibited induction. The small apparent

Figure 4 Concentration-dependence of uptake of aspartate in amino acid-starved NBL-1 cells

Cells 4 days after trypsin treatment were switched to fresh control medium or amino-acid-free medium. After 20 h, the uptake of various concentrations of aspartate was measured in Na^+ medium and choline medium. The data presented are the Na^+ -dependent rates and are means $\pm$ S.E.M. of 3–6 determinations. $\blacktriangle$, Control cells; $\blacksquare$, 20 h-amino-acid-starved cells.

Table 2 Inhibition of Na^+ -dependent L-aspartate transport ($20 \mu\text{M}$) in amino-acid-starved NBL-1 cells (22 h) by addition of various amino acids (at 1 mM) to the transport medium

Data are means $\pm$ S.E.M. of 3 samples: $**P \leq 0.001$ versus control (by Student's *t* test).

Addition (1 mM)	Uptake in amino-acid-starved cells	
	($\text{pmol of aspartate/}$ 3 min per mg)	(% of control)
Starved control	137 ± 6.7	100
D-Aspartate	$9.3 \pm 3.0^{**}$	6.8
L-Glutamate	$10.0 \pm 1.1^{**}$	7.3
D-Glutamate	128.9 ± 4.1	94.1
N-Methylaminoisobutyrate	142.0 ± 7.7	103.6
L-Alanine	117.3 ± 6.9	85.6
L-Leucine	125.1 ± 8.2	91.3
L-Lysine	131.4 ± 2.5	95.9

Table 3 Effect on the induction of Na^+ -dependent aspartate transport in amino-acid-starved NBL-1 cells by the inclusion of various amino acids to the incubation medium

Cells 4 days after trypsin treatment were switched to fresh medium (Fed control), amino-acid-free medium (Starved control) and amino-acid-free medium in which had been included one amino acid (as indicated) to 0.5 mM final concn. Data are the Na^+ -dependent rates of 3–4 samples and are means $\pm$ S.E.M.: $**P \leq 0.001$ versus starved control (by Student's *t* test).

Addition	Initial rate of transport		
	($\text{pmol of aspartate/}$ 3 min per mg)	(% of fed control)	(% of starved control)
Fed control	$40.5 \pm 1.3^{**}$	100	33.1
Starved control	122.2 ± 7.1	301.7	100
L-Aspartate	$51.4 \pm 3.1^{**}$	126.9	42.1
D-Aspartate	$27.2 \pm 1.2^{**}$	67.2	22.3
L-Glutamate	$47.7 \pm 5.1^{**}$	117.8	39.0
D-Glutamate	$56.6 \pm 1.8^{**}$	139.8	46.3
L-Glutamine	$57.7 \pm 1.3^{**}$	142.5	47.2
L-Leucine	196.0 ± 31	490.0	161.0
L-Alanine	$64.2 \pm 3.6^{**}$	158.5	52.5

NBL-1.
ATT.ATT.GAA.GTA.GAA.GAT.TGG.GAA.ATT.TTC.CGC.AAA.CTG.
ile.ile.glu.val.glu.asp.trp.glu.ile.phe.arg.lys.leu.

 GGC.CTT.TAC.ATG.GTC.ACC.GTC.CTG.AGT.GGG.CTT.GCA.ATC.
 gly.leu.tyr.met.val.thr.val.leu.ser.gly.leu.ala.ile.

 CAC.TCC.ATT.GTC.ATT.CTC.CCA.CTG.ATA.TAT.TTC...
 his.ser.ile.val.ile.leu.pro.leu.ile.tyr.phe...

Figure 5 Partial sequence of probe constructed to EAAC1 clone

Arrow indicates end of primer. Points in the sequence are merely to separate each codon or its corresponding amino acid.

stimulation observed with leucine is not significant at $P < 0.05$. All in all, these results show that there is not a complete correspondence between substrate specificity and ability to prevent induction of this transport activity by amino acid starvation. This phenomenon has been observed previously for induction of System A (Handlogten et al., 1982; Felipe et al., 1992). This experiment was also performed with additions of amino acids at 5 mM (results not shown), and similar results were obtained. In order to eliminate interference from *trans*-inhibition by added amino acids, cells were also grown in complete medium with single extra additions of amino acids (at 0.5 mM and 5 mM). No lowering of the basal level of System X_{AG-} was observed (results not shown).

The glutamate transporter (EAAC1), cloned recently by Kanai and Hediger (1992) from rabbit small intestine, showed a wide tissue distribution. Its amino acid specificity when expressed in *Xenopus* oocytes is similar to that of the glutamate transporter characterized above in NBL-1 cells. It was decided to construct a probe to the EAAC1 sequence and determine whether there was any homology to the X_{AG-} transporter present in fed and starved NBL-1 cells. An internal probe was constructed by PCR, spanning amino acids 270–512 of the EAAC1 sequence (as described in the Materials and methods section). The cloned PCR product (726 bp) was partially sequenced to confirm its similarity. The sequence obtained is presented in Figure 5. The amino acid sequence was found to be identical with that published for EAAC1 (Kanai and Hediger, 1992).

The EAAC1 probe, constructed as above, hybridized to a transcript of 3.5 kb from NBL-1 total RNA. The tissue distribution of EAAC1 determined by Kanai and Hediger (1992) revealed that superficial kidney cortex contains primarily a 3.5 kb transcript. To determine whether there were any changes to the level of the EAAC1-hybridizing transcript during starvation, NBL-1 cultures 4 days after trypsin treatment were starved, and some were used to monitor the onset of the starvation effect on transport (Figure 6a), and others were used for total RNA extraction. Northern-blot analysis, hybridizing with the probe to EAAC1, was carried out, and the result of a typical blot is presented in Figure 6(b). The autoradiograph was scanned, the areas under the actin and EAAC1 peaks were measured, and the ratios of the two areas at different time points are presented in Figure 6(c). The sharp decline in the EAAC1 signal relative to that of actin between 2 and 4 h after switching to starvation medium was reproducible in a duplicate experiment, as was the recovery of the signal at 11 h, although the duplicate Northern blot showed a slight overshoot in the EAAC1-hybridizing transcript relative to the control cells at 11 h. Since RNA was extracted from the equivalent amount of cells in which transport was carried out (35 mm Petri dish), the transcript signals observed

Figure 6 Time course of the effect on EAAC1 mRNA level during amino acid starvation of NBL-1 cells

Cells 5 days post-trypsinized were switched to fresh control medium or amino-acid-free medium. The Na^+ -dependent rate of aspartate ($20 \mu\text{M}$) transport was measured (mean of two samples) (a). Duplicate samples were used for total RNA extraction (as described in the Materials and methods section) at the same time points, and Northern-blot analysis was performed (b). Actin (2 kb) and EAAC1-hybridizing transcript (3.5 kb) are shown by arrows. The tracks represent probed RNA extracts at various time points after shifting to new medium: 1, control cells at 2 h; 2, starved cells at 2 h; 3, control cells at 4 h; 4, starved cells at 4 h; 5, control cells at 11 h; 6, starved cells at 11 h; 7, control cells at 24 h; 8, starved cells at 24 h; 9, control cells at 30 h; 10, starved cells at 30 h. Scans of the areas of actin and EAAC1 signals were made, and the ratios of the areas of EAAC1 signal to actin signal ($\times 100$) is presented in (c). ■, control fed cells; ▲, amino-acid-deprived cells.

can be directly compared with the transport rates measured. The actin levels declined by about 20% and over 30 h of starvation, whereas the cell protein level declined by about 40%. When the EAAC1/actin ratio was expressed for equivalent amounts of protein, a similar effect of a sharp fall in EAAC1 signal between 2 and 4 h of starvation, followed by an overshoot relative to the control at 11 h, and subsequent recovery to control levels, was confirmed.

DISCUSSION

The uptake of glutamate by the NBL-1 cell line is mediated mainly by a Na⁺-dependent high-affinity transport system. The kinetics and stereospecificity of this transporter are similar to those of the high-affinity Na⁺-dependent glutamate transport System X_{AG}⁻ already described in a number of other tissues.

Induction of this transport system by amino acid starvation has not been described previously, although this type of adaptive regulation is well established for the Na⁺-dependent neutral amino acid transport System A in many tissues (for review, see Shotwell et al., 1983). Induction of System X_{AG}⁻ occurs in cell cultures which are not fully confluent, is protein-synthesis-dependent and is prevented by the presence in the starvation medium of single amino acids. As for System A, substrates of the transport system are the most effective in this respect, but there is no complete correspondence between the ability of an amino acid to act as a substrate and its ability to prevent induction. Previous work on fibroblasts showed that increased cell density promoted uptake of glutamate by System X_{AG}⁻ (Dall'Asta et al., 1983). It would be expected that at high cell density nutrients may become limiting, and thus cells would show a tendency towards the starved state.

The present work establishes the presence in NBL-1 cells of a 3.5 kb mRNA species homologous to the high-affinity glutamate transporter cloned by Kanai and Hediger (1992). Only one high-affinity glutamate transporter was detected in NBL-1 cells, and the *K_m* values for L-glutamate and L-aspartate ($13.8 \pm 2.4 \mu\text{M}$ and $4.5 \pm 1.1 \mu\text{M}$ respectively) were very similar to the corresponding values for the EAAC1 transporter expressed in oocytes ($12.2 \pm 1.2 \mu\text{M}$ and $6.5 \pm 0.7 \mu\text{M}$ respectively). By inference therefore, the rabbit intestinal EAAC1 transporter is the same as the glutamate transporter expressed in NBL-1 cells. In support of this, the *K_m* values for L-aspartate and L-glutamate of the specific brain transporter GLAST (Storck et al., 1992) expressed in oocytes were 10-fold higher.

Amino acid deprivation led to an initial fall in the level of the EAAC1-hybridizing transcript during the initial increase in transporter activity, and this was followed by a later restoration of the transcript level. These results suggest that the initial increase in transporter synthesis is mediated at the post-transcriptional level. After the initial decrease in the level of EAAC1 mRNA, increased transcription accompanies the further increase in the activity of the transporter, leading to recovery of transcript levels.

The relationship between levels of mRNA and transport activity for Na⁺-dependent transport systems has not yet been widely explored for mammalian systems. In the sheep intestine, changes in Na⁺-dependent glucose transport activity during development or infusion of sugars correlate well with SGLT1 protein abundance, but are not accompanied by similar changes in SGLT1 mRNA levels. It has been concluded that the expression of SGLT1 is controlled primarily at the post-transcriptional level (Freeman et al., 1993; Lescale-Matys et al., 1993). On the other hand, induction of the Na⁺-dependent myo-inositol transport system by exposure of MDCK cells to hyperosmotic media is associated with greatly enhanced levels of the specific mRNA for this transporter (Kwon et al., 1992; Yamauchi et al., 1993). The regulation of the EAAC1 system appears to resemble that observed for the SGLT1 system rather than that for the myo-inositol system.

To date, two Na⁺-dependent amino acid transport systems have been shown to be induced by amino acid deprivation in NBL-1 cells. These are System A (Felipe et al., 1992) and System X_{AG}⁻ (the present paper). A common sequence motif between the

Protein	Amino acid no.		
grp78	617	K A K K K E L E E I V	627
Calreticulin	357	K K R K E E E A E D	367
SAAT1	617	K L S K E E E A Q K	627
EAAC1	464	K L S K K E L E Q M D	474

Figure 7 Partial sequences of amino-acid-starvation-regulated proteins and glucose-regulated protein grp78

The sequences aligned are the guinea-pig glucose regulated protein, grp78 (Ting et al., 1987), mouse calreticulin (Smith and Koch, 1989), the LLC-PK-1 cell-line putative System A transporter, SAAT1 (Kong et al., 1993), and the rabbit small-intestine excitatory high-affinity glutamate transporter, EAAC1 (Kanai and Hediger, 1992).

recently cloned System A transporter (SAAT) and the EAAC1 cloned sequence has been noticed by us. This is presented in Figure 7. Two other stress-related proteins show conservation of this sequence: calreticulin, which has recently been shown by us (S. Plakidou-Dymock and J. D. McGivan, unpublished work) to be enhanced 2–3-fold upon starvation, in NBL-1 cells, and the glucose-regulated protein grp78. Preliminary observations (S. Plakidou-Dymock and J. D. McGivan, unpublished work) suggest that a protein of 77 kDa, which may correspond to grp78, is also up-regulated by amino acid starvation in NBL-1 cells. The motif K–K–E–E may be an important feature of proteins up-regulated by amino acid deprivation.

This work was supported by a grant from the Wellcome Trust.

REFERENCES

- Ballatori, N., Moseley, R. H. and Boyer, J. L. (1986) *J. Biol. Chem.* **261**, 6216–6221
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Brew, H. and Attwell, D. (1987) *Nature (London)* **327**, 707–709
- Burger, H.-J., Gebhardt, R., Mayer, C. and Mecke, D. (1989) *Hepatology* **9**, 22–28
- Dall'Asta, V., Gazzola, G. C., Franchi-Gazzola, R., Bussolati, O., Longo, N. and Guidotti, G. G. (1983) *J. Biol. Chem.* **258**, 6371–6379
- Doyle, F. A. and McGivan, J. D. (1992) *Biochim. Biophys. Acta* **1104**, 55–62
- Felipe, A., Soler, C. and McGivan, J. D. (1992) *Biochem. J.* **284**, 577–582
- Ferkany, J. and Coyle, J. T. (1986) *J. Neurosci. Res.* **16**, 491–503
- Freeman, T. C., Sirinathsinghji, D. J. S., Beechey, R. B., Wood, I. S., Dyer, J. and Shirazi-Beechey, S. P. (1993) *Biochim. Biophys. Acta* **1146**, 203–212
- Gebhardt, R. and Mecke, D. (1983) *FEBS Lett.* **161**, 275–278
- Guidotti, G. G. and Gazzola, C. (1992) in *Mammalian Amino Acid Transport: Mechanisms and Control* (Kilberg, M. S. and Häussinger, D., eds.), pp. 3–29, Plenum Press, New York
- Handlogten, M. E., Kilberg, M. S. and Christensen, H. N. (1982) *J. Biol. Chem.* **257**, 345–348
- Häussinger, D. and Gerok, W. (1983) *Eur. J. Biochem.* **136**, 421–425
- Häussinger, D., Stoll, B., Stehle, T. and Gerok, W. (1989) *Eur. J. Biochem.* **185**, 189–195
- Kanai, Y. and Hediger, M. A. (1992) *Nature (London)* **360**, 467–471
- Kong, C.-T., Yet, S.-F. and Lever, J. E. (1993) *J. Biol. Chem.* **268**, 1509–1512
- Kwon, H. M., Yamauchi, A., Uchida, S., Preston, A. S., Garcia-Perez, A., Burg, M. B. and Handler, J. S. (1992) *J. Biol. Chem.* **267**, 6297–6301
- Lescale-Matys, L., Dyer, J., Scott, D., Freeman, T. C., Wright, E. M. and Shirazi-Beechey, S. P. (1993) *Biochem. J.* **291**, 435–440
- Low, S. Y., Taylor, P. M., Handal, H. S., Pogson, C. I. and Rennie, M. J. (1992) *Biochem. J.* **284**, 333–340
- Meister, A. (1979) in *Advances in Biochemistry and Physiology: Glutamic Acid* (Keler, L. J., Jr., Garattini, S., Kane, M. R., Reynolds, W. A. and Wurtman, R. J., eds.), pp. 64–84, Raven Press, New York
- Pines, G., Danbolt, N. C., Bjoras, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Storm-Mathisen, J., Seeberg, E. and Kanner, B. (1992) *Nature (London)* **360**, 464–467
- Quamme, G., Biber, J. and Murer, H. (1989) *Am. J. Physiol.* **257**, F967–F975
- Sacktor, B., Rosenbloom, I. L., Liang, C. T. and Cheng, L. (1981) *J. Membr. Biol.* **60**, 63–71

- Samarzija, I. L. and Fromter, E. (1976) *Pflügers Arch. Eur. J. Physiol.* **365**, R15
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., CSH Press, Cold Spring Harbor
- Shotwell, M. A., Kilberg, M. S. and Oxender, D. L. (1983) *Biochim. Biophys. Acta* **737**, 267–284
- Smith, M. J. and Koch, G. L. E. (1989) *EMBO J.* **8**, 3581–3586
- Soler, C., Felipe, A., Casado, J., McGivan, J. D. and Pastor-Anglada, M. (1993) *Biochem. J.* **289**, 653–658
- Storck, T., Schulte, S., Hofman, K. and Stoffel, W. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10955–10959
- Taylor, P. M. and Rennie, M. J. (1987) *FEBS Lett.* **221**, 370–374
- Ting, J., Wooden, S. K., Kriz, R. W., Kaufman, R. J. and Lees, A. S. (1987) *Gene* **55**, 147–157
- Wingrove, T. G. and Kimmich, G. A. (1988) *Am. J. Physiol.* **255**, C737–C744
- Yamauchi, A., Uchida, S., Preston, A. S., Kwon, H. M. and Handler, J. S. (1993) *Am. J. Physiol.* **264**, F20–F23

Received 14 April 1993/10 June 1993; accepted 2 July 1993