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Tyrosine phosphorylation-and epidermal growth factor-dependent regulation of the sodium-coupled amino acid transporter B^0 in the human placental choriocarcinoma cell line JAR

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Abstract

We have recently cloned an amino acid transporter from the human placental choriocarcinoma cell line JAR which, when functionally expressed in HeLa cells, induces an amino acid transport activity with characteristics known to be associated with the amino acid transport system B^0 (R. Kekuda, P.D. Prasad, Y.J. Fei, V. Torres-Zamorano, S. Sinha, T.L. Yang-Feng, F.H. Leibach, and V. Ganapathy, J. Biol. Chem. 271, 18657–18661, 1996). The presence of the amino acid transport system B^0 (ATB^0) has however not been previously described in these cells by functional studies. In the present investigation, we have obtained evidence for the existence of ATB^0 in JAR cells and delineated the functional characteristics of the transporter. The identifying characteristics include Na^+-dependence and preference for neutral amino acids. In addition, we have used the JAR cells as a model system to investigate the regulatory aspects of ATB^0. Treatment of the cells with the neuroprotective agent aurintricarboxylic acid (ATA) for 16 h leads to a significant increase in ATB^0 activity. This increase is associated with enhanced maximal velocity of the transporter and with increased steady state levels of the transporter mRNA. The effect of ATA is blocked by the tyrosine kinase inhibitor genistein. ATA treatment results in increased tyrosine phosphorylation of two major proteins, 180 kDa and 140 kDa in size. The 180 kDa protein is likely to be the epidermal growth factor (EGF) receptor because exposure of the cells to EGF also leads to enhanced tyrosine phosphorylation of a protein of similar molecular size. Furthermore, the effects of ATA on ATB^0 activity and on ATB^0 mRNA levels can be reproduced by EGF. Treatment of the cells with EGF for 24 h results in a significant increase in ATB^0 activity and this effect is associated with an increase in the maximal velocity of the transporter and with an increase in the steady state levels of the transporter mRNA. These data suggest that ATA influences ATB^0 activity in JAR cells most likely by activating the EGF receptor through tyrosine phosphorylation. It is concluded that the human placental choriocarcinoma

Abbreviations: ATA, aurintricarboxylic acid; EGF, epidermal growth factor; ATB^0, amino acid transporter B^0; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MeAIB, α-methylaminoisobutyric acid; BCH, 2-aminobicyclo-[2.2.1] heptane 2-carboxylic acid; kb, kilo base pair(s).

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cells functionally express the amino acid transport system B^0 and that the expression of the system in these cells is stimulated by EGF.

Keywords: Amino acid transport; System B^0; Tyrosine phosphorylation; Epidermal growth factor; Regulation of expression; mRNA level; Choriocarcinoma cell; Placenta, human

1. Introduction

The amino acid transporter B^0 is a Na^+-dependent, broad-scope, neutral amino acid transport system expressed in the intestinal [1,2] and renal [3,4] brush border membranes. This system has also been shown to be expressed in the human intestinal cell line Caco-2 [5] and in the bovine kidney cell line NBL-1 [6]. A wide variety of zwitterionic amino acids, including branched chain (e.g. leucine), aromatic (e.g. phenylalanine), short chain (e.g. alanine) and amidated (e.g. glutamine) amino acids, are accepted as substrates for System B^0. Anionic amino acids, cationic amino acids, and N-methylated amino acids are excluded by the system. This transporter is differentiated from the closely related transport system $B^{0,+}$, which accepts zwitterionic as well as cationic amino acids as substrates [7]. Based upon the known expression of system B^0 in the intestine and the kidney and the known preference of this system for neutral amino acids, it has been proposed that the inheritable amino acid transport defect Hartnup disease may be associated with defective System B^0 [8]. This disease is characterized by impaired transport of a variety of neutral amino acids in the intestine and the kidney [9]. Recent evidence indicates that the expression of System B^0 in Caco-2 cells is regulated by protein kinase C [5]. In addition, Plakidou-Dymock et al [10] have described a novel means of control for System B^0 expression in NBL-1 cells which involves induction of the system by the presence of phenylalanine during amino acid starvation.

We have recently cloned and characterized an amino acid transporter from the human placental choriocarcinoma cell line JAR [11]. When functionally expressed in HeLa cells, this transporter mediates the Na^+-dependent uptake of several zwitterionic amino acids. These functional characteristics have led to the identification of the cloned transporter as the amino acid transporter B^0. Interestingly, System B^0 has not been described in the JAR cell line or in the normal human placenta. In this paper, we provide evidence for endogenous expression of System B^0 in JAR cells and, in addition, for regulation of its expression by epidermal growth factor and tyrosine phosphorylation.

2. Materials and methods

2.1. Materials

The human placental choriocarcinoma cell line JAR was obtained from the American Type Culture Collection (Rockville, MD, USA). Culture media (RPMI 1640), penicillin, streptomycin, and trypsin were purchased from Mediatech (Herndon, VA, USA). Fetal bovine serum, aurintricarboxylic acid (ATA[1]), prostaglandin E_1, apotransferrin, thyroxine, EGF, and amino acids were from Sigma (St. Louis, MO, USA). Human recombinant insulin was from Novo Nordisk Pharmaceuticals (Princeton, NJ, USA). Genistein was from Research Biochemicals (Natick, MA, USA). L-[2,3-^{3}H]Alanine (specific radioactivity, 50 Ci/mmol) was obtained from DuPont-NEN (Boston, MA, USA). [α-^{32}P]dCTP was from Amersham (Arlington Heights, IL, USA). Antiphosphotyrosine antibody (PY 20) was purchased from Transduction Laboratories (Lexington, KY, USA).

2.2. Culture and treatment of JAR cells

JAR cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Trypsin-released cells were seeded in 35 mm petri dishes at a density of 1.5×10^6 cells/dish and allowed to grow for 24 h in RPMI 1640 medium containing 10% fetal bovine serum. Following the 24 h subculture, the medium was replaced with a hormonally defined medium which did not contain fetal bovine serum. The defined medium consisted of RPMI 1640, supplemented with insulin (5 μg/ml), apotransferrin (5 μg/ml), prostaglandin E_1 (2.5×10^{-5} mg/ml), and thyroxine (5×10^{-12} M). Treatment with different agents including EGF was carried

out for indicated time periods in the defined medium prior to uptake measurements.

2.3. Uptake measurements

The culture medium from the dishes was removed by aspiration and the cells were washed once with the uptake buffer. One ml of uptake buffer containing radiolabeled alanine was added to the cells and incubated for 2 min. Uptake was terminated by aspirating the uptake buffer from the dish and subsequently washing the cells thrice with uptake buffer. The cells were lysed with 1 ml of 1% SDS–0.2 N NaOH and the lysate was transferred to counting vials for measurement of radioactivity. The composition of the uptake buffer was 25 mM Hepes/Tris (pH 7.5), 140 mM NaC1, 5.4 mM KC1, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. In some experiments, the composition of the uptake buffer was modified by replacing 25 mM Hepes/Tris with 25 mM Mes/Tris (pH 5.5). Alanine uptake that occurred in the absence of Na^+ was subtracted from total uptake to calculate Na^+-dependent uptake. The Na^+-free uptake buffer was prepared by replacing NaC1 with choline chloride. In some experiments, a Cl^--free uptake buffer was used and its composition was 25 mM Hepes/Tris (pH 7.5), 140 mM sodium gluconate, 5.4 mM potassium gluconate, 1.8 mM calcium gluconate, 0.8 mM Mg SO_4, and 5 mM glucose.

2.4. Isolation of Poly(A)$^+$ RNA and Northern blot analysis

Poly(A)$^+$ RNA was isolated from JAR cells using FastTrack mRNA isolation Kit (Invitrogen, San Diego, CA, USA). Northern blot hybridization was carried out under high stringency conditions using ^{32}P-labeled human ATB^0 cDNA as the probe. The same membrane blot was used for probing with either ^{32}P-labeled human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA or ^{32}P-labeled mouse β-actin cDNA as an internal control for RNA loading and transfer efficiency. Quantification of transcript signals in autoradiograms was done with a Hewlett Packard densitometer (Scan Jet II CX). The ATB^0 cDNA probe was a 1.4 kb fragment obtained by digestion with *Xho*I and *Bam*HI from the human ATB^0 clone [11]. The GAPDH cDNA probe was a 0.78 kb fragment obtained from a human GAPDH clone (American Type Culture Collection, Rockville, MD, USA) by digestion with *Pst*I and *Xba*I. The β-actin cDNA probe was a 1.2 kb fragment obtained from a mouse β-actin clone by digestion with *Pst*I. The β-actin clone was kindly provided by Dr. V.B. Mahesh, Department of Physiology and Endocrinology, Medical College of Georgia. The cDNA probes were radiolabeled with [α-^{32}P]dCTP by random priming using ready-to-go oligolabeling kit from Pharmacia Biotech (Piscataway, NJ, USA).

2.5. Immunoblot analysis of phosphotyrosine-containing proteins

JAR cells were cultured for 24 h in the presence of fetal bovine serum and for another 24 h in the hormonally defined medium. Cells were then treated with either ATA (150 μM) for different time periods (0–6 h) or EGF (100 ng/ml) for 5 min. Control cells were treated in a similar way in the absence of these reagents. Following the treatment, the medium was removed and the cells were lysed with 400 μl of SDS-PAGE sample buffer (62.5 mM Tris/HCl, pH 6.8, 50 mM dithiothreitol, 2.5% SDS, 10% glycerol) containing 1 mM orthovanadate. The samples were boiled for 5 min at 100°C and centrifuged. The supernatant was used for SDS-PAGE. The size-fractionated proteins were transferred onto a nitrocellulose membrane. The blots were blocked with 3% fetal bovine serum, 1% nonfat dry milk, and 0.5% Tween-20. The blots were probed with anti-phosphotyrosine antibody (1 μg/ml) and the immunoreactive proteins were detected using the Enhanced chemiluminescence Western blotting detection kit from Amersham (Arlington Heights, IL, USA).

2.6. Data analysis

Uptake experiments were done in duplicate or triplicate and each experiment was repeated two or three times. Kinetic analysis was done using commercially available computer programs Fig. P, version 6.0 or Sigma Plot. Northern blot analysis was done twice in the case of ATA and thrice in the case of EGF. Data are presented as means $\pm$ S.E.

3. Results and discussion

3.1. Evidence for expression of amino acid transport system B^0 in JAR cells

Alanine has been shown to be an excellent substrate for the amino acid transport system B^0 that is expressed in the human colon carcinoma cell line Caco-2 [5], the bovine renal epithelial cell line NBL-1 [6], and the rabbit intestine [12]. Therefore, we used this amino acid as a test substrate to determine whether the B^0 system is expressed in the human placental choriocarcinoma cell line JAR. Fig. 1 describes the uptake of alanine in JAR cells in the presence and in the absence of Na^+. Uptake of alanine was rapid from a NaCl-containing uptake medium. In contrast, the uptake was reduced markedly when NaCl in the uptake medium was replaced by choline chloride. When the concentration of alanine was 2.5 nM, uptake in the presence of Na^+ was at least 10-fold greater than in the absence of Na^+ at all time periods. Similar results were obtained when the concentration of alanine was 50 μM instead of 2.5 nM. Thus, the uptake of alanine in JAR cells occurs predominantly via a Na^+-dependent amino acid transport system.

Alanine is a potential substrate for at least three Na^+-dependent amino acid transport systems known to be expressed in epithelial cells. These three systems are A, ASC, and B^0. These systems can however be differentiated from their substrate specificity. We characterized the substrate specificity of the transport system responsible for alanine uptake in JAR cells by studying the ability of various amino acids to inhibit the uptake of [^{3}H]alanine. Fig. 2 describes the dose-response relationship for the inhibition of [^{3}H]alanine uptake by selected amino acids and Table 1 gives the IC_{50} values (the concentration of the amino acid which causes 50% inhibition of [^{3}H]alanine uptake) for those amino acids which showed marked inhibition within the concentration range studied (1 μM–10 mM). Even at a concentration as high as 10 mM, the System A-specific substrate MeAIB caused only about 10% inhibition of alanine uptake. The bicyclic amino acid BCH was similarly less effective, causing approx. 25% inhibition at 10 mM. The cationic amino acids arginine and lysine which are substrates for the Na^+-dependent system $B^{0,+}$ known to be expressed in certain cell types [7] were also found to be not effective as inhibitors of alanine uptake in JAR cells. In contrast, the short-chain neutral amino acids alanine, serine,

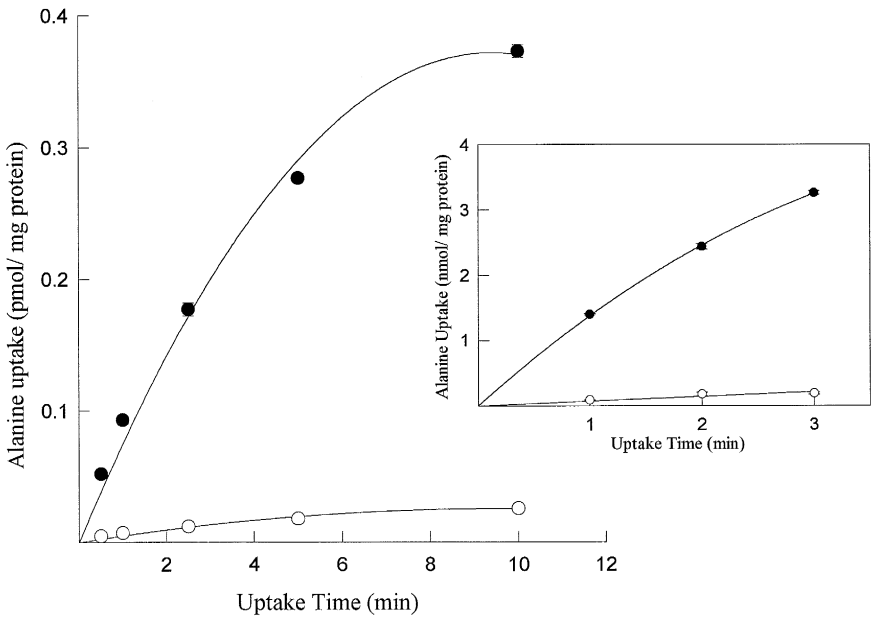

Fig. 1. Time-course of alanine uptake in the presence and absence of Na^+. Uptake of alanine at 2.5 nM or at 50 μM (inset) in confluent cultures of JAR cells was measured from uptake medium containing NaCl (●) or choline chloride (○). Composition of the uptake medium was (in mM) 25 Hepes/Tris (pH 7.5), 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 5 glucose and 140 NaCl or choline chloride.

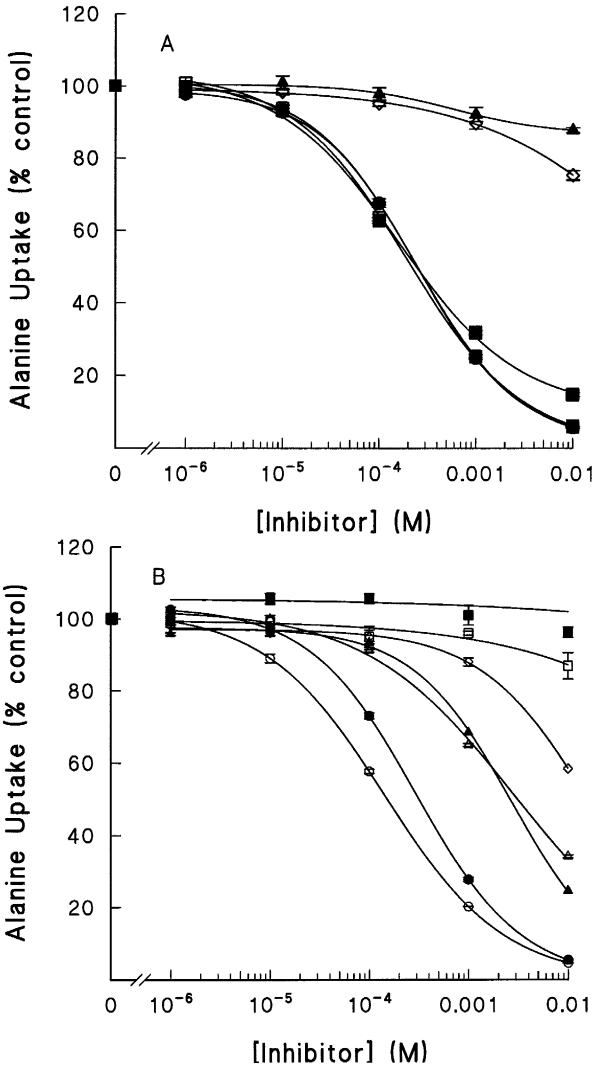

Fig. 2. Substrate specificity of alanine transport process. Uptake of [^{3}H]alanine (2.5 nM) in confluent cultures of JAR cells was measured with a 2-min incubation from uptake medium (pH 7.5) containing NaCl in the presence or absence of indicated unlabeled amino acids. Concentration range for unlabeled amino acids were 1 μM–10 mM. Results are given as percent of control uptake (0.20 ± 0.02 pmol/mg of protein/2 min) measured in the absence of unlabeled amino acids. Key: (A) Alanine (○), serine (●), cysteine (□), threonine (■), BCH (◇), and MeAIB (▲); (B) Glutamine (○), asparagine (●), lysine (□), arginine (■), glycine (▲), leucine (△), and phenylalanine (◇).

cysteine, and threonine as well as the amidated amino acids glutamine and asparagine were highly potent in interacting with the alanine-transporting system in JAR cells. The IC_{50} values for these amino acids for the inhibition of [^{3}H]alanine uptake ranged between 144 μM (for glutamine) and 295 μM (for asparagine). Other neutral amino acids such as glycine, leucine (a branched-chain amino acid), and phenylalanine (an aromatic amino acid) also inhibited [^{3}H]alanine uptake, though with much lesser potency than seen in the cases of short-chain and amidated amino acids. These substrate specificity studies effectively rule out participation of System A and System $B^{0,+}$ in the observed uptake of alanine in JAR cells.

The inhibition of alanine uptake by glycine and by leucine was competitive (Fig. 3A). In this experiment, alanine uptake was measured over a concentration range of 25–750 μM and, when present, the concentrations of glycine and leucine were 5 mM each. In the absence of glycine and leucine, the values for K_t and V_{max} for alanine uptake were 174 ± 6 μM and 11.2 ± 0.2 nmol/mg of protein/2 min. The corresponding values were 461 ± 20 μM and 10.3 ± 0.3 nmol/mg of protein/2 min in the presence of glycine and 330 ± 10 μM and 9.6 ± 0.2 nmol/mg of protein/2 min in the presence of leucine. There are conflicting data in the literature with regard to the kinetic nature of glycine-induced inhibition of alanine uptake via System B^0. In bovine renal epithelial cell line NBL-1, the inhibition is noncompetitive [6] whereas in human intestinal cell line Caco-2, the inhibition is competitive [5]. Therefore, we verified the kinetic nature of glycine-induced inhibition of alanine uptake in JAR cells by Dixon plot (Fig. 3B). Again, the inhibition was competitive. The K_i value for glycine calculated from this experiment (3.5 mM) closely resembled the K_i value calculated from the

Table 1
Relative potencies of various amino acids for the inhibition of [^{3}H]alanine uptake in JAR cells

Amino acid	IC_{50} value (μM)
Glutamine	144 ± 1
Threonine	158 ± 4
Cysteine	192 ± 2
Alanine	238 ± 2
Serine	242 ± 6
Asparagine	295 ± 9
Glycine	2790 ± 218
Leucine	2869 ± 380
Phenylalanine	16890 ± 1166

The IC_{50} values were calculated from the data given in Fig. 2.

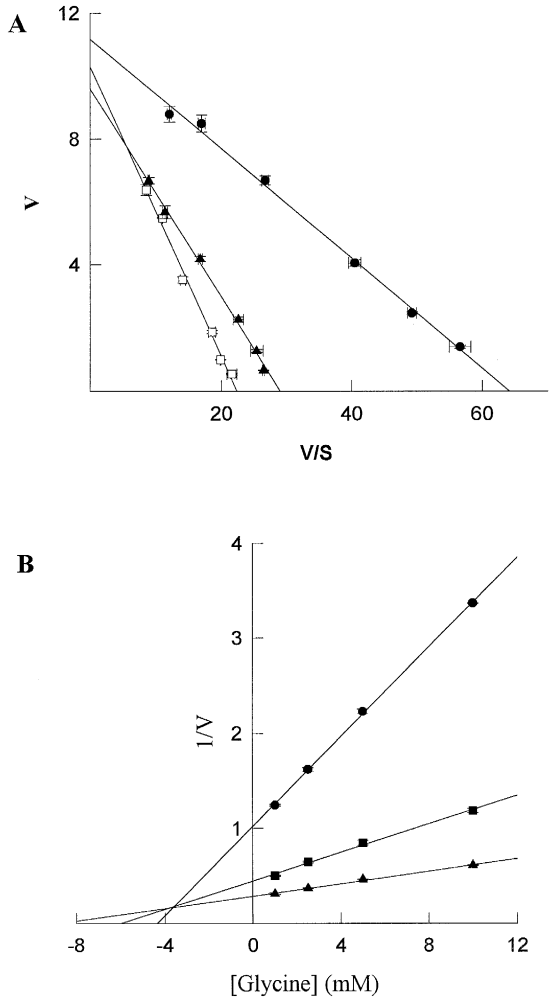

Fig. 3. Kinetics of inhibition of alanine uptake by leucine (A) and by glycine (A and B). (A) Uptake of alanine was measured in confluent cultures of JAR cells over a alanine concentration range of 25–750 μM and with a 2-min incubation from uptake medium containing NaCl. Uptake of alanine measured in the absence of Na^+ was subtracted from total uptake to calculate Na^+-dependent uptake which was used in data analysis. Alanine uptake was measured in the absence (●) or presence of 5mM glycine (□) or 5 mM leucine (▲). Results are given as Eadie-Hofstee plots. Key: V, alanine uptake rate in nmol/mg of protein/2 min; s, alanine concentration in mM. (B) Uptake of alanine was measured with a 2-min incubation at three concentrations: 25 μM (●), 75 μM (■), and 150 μM (▲). Concentration of glycine was varied between 1 and 10 mM. Only Na^+-dependent alanine uptake was used in data analysis. Results are given as Dixon plots. Key: V, alanine uptake rate in nmol/mg of protein/2 min.

IC_{50} data (2.8 ± 0.2 mM). Apparently, the kinetics of interaction of glycine with System B^0 are different between the bovine cells and the human cells.

System ASC and System B^0 exhibit significant overlap in substrate specificity. System B^0 that is expressed in Caco-2 cells shows high affinity towards short-chain neutral amino acids and amidated amino acids [5]. The substrate specificity of the alanine transporting system in JAR cells is markedly similar to that described for System B^0 in Caco-2 cells. However, System ASC can also transport the amidated amino acids glutamine and asparagine, in addition to the short-chain neutral amino acids alanine, serine, cysteine, and threonine [13]. Even though the ASC system cloned from human brain (designated as ASCT 1) has been shown not to interact with glutamine and asparagine [14,15], a more recently cloned ASC system from mouse testes (designated as ASCT 2) has been shown to transport glutamine and asparagine [16]. Therefore, additional studies were needed to determine whether the uptake of alanine in JAR cells is mediated by System B^0 or by System ASC. A unique characteristic of the ASC system is that even though it does not exhibit measurable affinity for the acidic amino acids aspartate, glutamate, and cysteate at pH 7.5, its affinity for these amino acids increases markedly at acidic pH [17]. This characteristic has recently been demonstrated un-

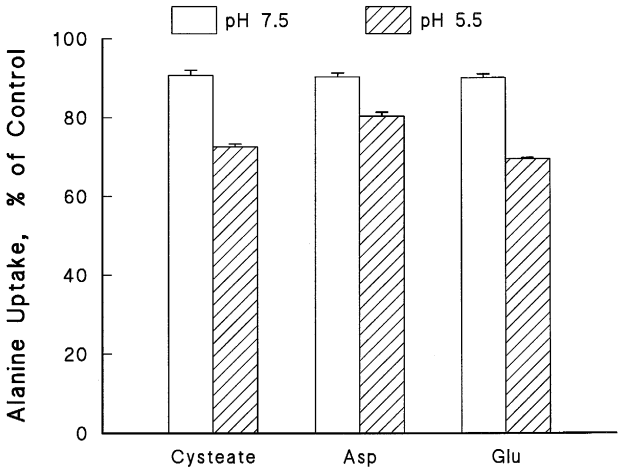

Fig. 4. Influence of acidic amino acids on [^{3}H]alanine uptake at pH 7.5 and 5.5. Uptake of [^{3}H]alanine (2.5 nM) in confluent cultures of JAR cells was measured with a 2-min incubation from uptake medium (pH 7.5 or pH 5.5) containing NaCl in the presence or absence of 5 mM acidic amino acids. Results are given as percent of control uptake measured at respective pH values in the absence of acidic acids (0.20 ± 0.01 pmol/mg of protein/2 min at pH 7.5 and 0.12 ± 0.01 pmol/mg of protein/2 min at pH 5.5).

equivocally for the ASC systems cloned from human brain (ASCT 1) [18] and from mouse testes (ASCT 2) [16]. Therefore, we investigated the interaction of acidic amino acids with alanine-transporting system in JAR cells at two different pH, 7.5 and 5.5 (Fig. 4). At both pH, the acidic amino acids cysteate, aspartate, and glutamate failed to cause appreciable inhibition of alanine uptake in JAR cells. The maximal inhibition observed at pH 5.5 with these amino acids at a concentration of 5 mM was 25–30%. In contrast, the uptake of alanine mediated by the cloned human ASC system was completely inhibited by these amino acids under comparable experimental conditions [18]. These data demonstrate convincingly that alanine uptake in JAR cells in mediated predominantly by System B^0. It is not clear whether the small inhibition caused by acidic amino acids at pH 5.5 is due to participation of system ASC to a small extent in alanine uptake in JAR cells because system B^0 may itself have the ability to interact with acidic amino acids at pH 5.5 to some extent, though not as prominently as System ASC. However, the possible contribution of System ASC to alanine uptake in JAR cells cannot be entirely ruled out at this time. But, if this system exists in JAR cells, its contribution to alanine uptake is not expected to be greater than 25–30% at the maximum.

Since alanine uptake in JAR cells occurs primarily via System B^0, we were able to study the dependence of System B^0 activity on Na^+. Alanine uptake in these cells was found to be stimulated by increasing concentrations of Na^+ in a hyperbolic manner (data not shown), indicating a Na^+/alanine coupling ratio of 1. The $K_{0.5}$ for Na^+, i.e., the concentration of Na^+ necessary to stimulate alanine uptake to half-maximal level, was 10.2 ± 0.3 mM. Anions such as Cl^- had no role in System B^0 activity (data not shown).

3.2. Role of tyrosine phosphorylation in the regulation of system B^0 activity in JAR cells

The successful isolation of the System B^0 cDNA clone from a cDNA library derived from JAR cell mRNA [11] indicates that this system is expressed in these cells. But, there has been no previous report describing the existence of System B^0 in these cells by functional studies. The present study represents the first evidence for functional expression of this amino acid transport system in JAR cells. Very little is known at present on the regulatory aspects of System B^0. Since the uptake of alanine in JAR cells is mediated primarily by this system, this cell line provides an excellent experimental model to investigate the regulation of System B^0.

ATA has been shown to be a very useful agent to study the role of multiple signalling pathways involving tyrosine phosphorylation in cell function [19]. ATA is a neuroprotective agent which can prevent cell death induced by deprivation of serum and NGF in PC12 cells [20], and also protect hippocampal neurons from NMDA − and ischemia-induced death [21]. This compound induces tyrosine phosphorylation of several proteins which lie in prominent signalling pathways [19]. Therefore, we chose this compound in our initial studies to determine whether tyrosine phosphorylation plays any role in the regulation of System B^0 in JAR cells.

When confluent cultures of JAR cells were treated without or with ATA (150 μM) at 37°C for various time periods (1–16 h) and then the cells were used for measurement of alanine uptake, it was found that ATA caused a significant stimulation of alanine uptake (Fig. 5A). The stimulation was not significant when the treatment time was 1 h, but the stimulation became highly significant when the treatment time was 2 h or greater. The increase in alanine uptake was about 30% when the treatment time was 16 h. Fig. 5B describes the influence of ATA treatment on the kinetic parameters of alanine uptake. Uptake was measured with a 2-min incubation in control cells and in cells treated with ATA (150 μM, 16 h treatment). The range of alanine concentration used in this experiment was 25–750 μM. The Eadie-Hofstee plot of the uptake data in control cells gave a straight line ($r^2 = 0.99$), indicating that there was only a single transport system kinetically discernable which was responsible for alanine uptake in JAR cells. The Michaelis-Menten constant, K_t, for the system was 239 ± 5 μM and the maximal velocity, V_{max}, was 16.1 ± 0.2 nmol/mg of protein/2 min. Kinetic analysis of alanine uptake in ATA-treated cells revealed that the K_t value remained almost the same (271 ± 2 μM) as in control cells but the V_{max} increased significantly (22.0 ± 0.1 nmol/mg of protein/2 min).

To determine whether the ATA-induced stimula-

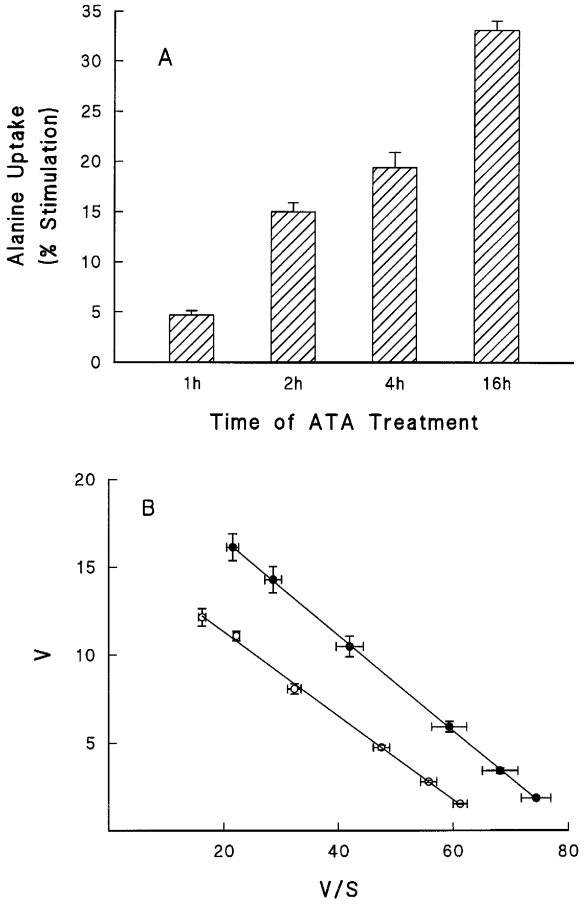

Fig. 5. Stimulation of alanine uptake by ATA. (A) Confluent cultures of JAR cells were treated with or without ATA (150 μM) for indicated time periods at 37°C, following which uptake of [^{3}H]alanine (2.5 nM) was measured with a 2-min incubation from uptake medium containing NaCl. Results are given as percent stimulation compared to corresponding control uptake measured in cells treated similarly in the absence of ATA. Control uptake did not vary significantly among cultures treated in the absence of ATA for indicated time periods (0.18 ± 0.01 pmol/mg of protein/2 min). (B) Confluent cultures of JAR cells were treated with (●) or without (○) ATA (150 μM) for 16 h at 37°C. Uptake of alanine was measured in these cells over a alanine concentration range of 25–750 μM and with a 2-min incubation from uptake medium containing NaCl. Uptake of alanine measured in the absence of Na^+ was subtracted from total uptake to calculate Na^+-dependent uptake which was used in data analysis. Results are given as Eadie-Hofstee plots. Key: v, alanine uptake rate in nmol/mg of protein/2 min; s, alanine concentration in mM.

tion of alanine uptake was due to ATA-induced tyrosine phosphorylation of specific cellular proteins, we studied the influence of genistein, an inhibitor of several classes of tyrosine kinases, on the ATA effect on alanine uptake (Fig. 6). Genistein was found to block completely the stimulatory effect of ATA.

The increase in the V_{max} of the transport system and the requirement of long periods of treatment for maximal effect suggested that the ATA-induced stimulation of alanine uptake might involve increased synthesis of the transport protein in response to ATA treatment. Since alanine uptake in these cells occurs primarily via the amino acid transport system B^0, we determined the steady state levels of B^0 mRNA in control and in ATA-treated cells (Fig. 7; lanes 1 and 3 respectively). The GAPDH mRNA levels were used as internal control to normalize for RNA loading and transfer efficiency during Northern blot hybridization. In control cells as well as in ATA-treated cells, the ATB^0 cDNA identified a 2.9 kb mRNA transcript and the GAPDH cDNA identified a 1.4 kb mRNA transcript. The arbitrary B^0 mRNA/GAPDH mRNA ratio for control cells was 0.47 and this value increased to 0.76 for ATA-treated cells. This represents a 60% increase in the steady state levels of B^0 mRNA as a result of ATA treatment. This increase

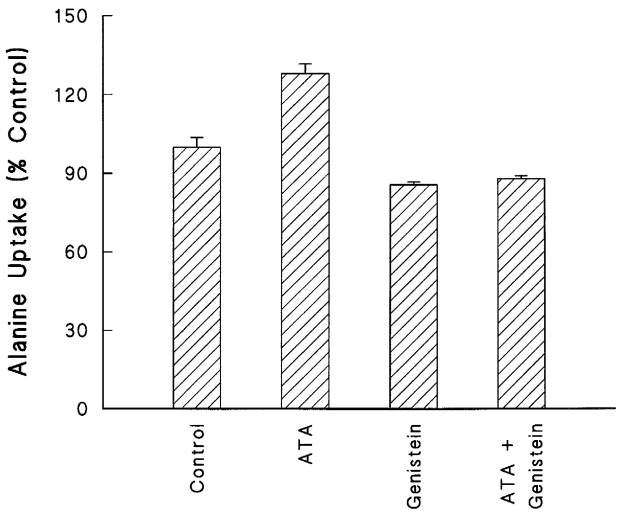

Fig. 6. Influence of genistein on ATA-induced stimulation of [^{3}H]alanine uptake. Confluent cultures of JAR cells were treated with or without (control) ATA (150 μM), genistein (60 μM) or ATA (150 μM) plus genistein (60 μM) for 16 h at 37°C. Uptake of [^{3}H]alanine (2.5 nM) was measured at pH 7.5 with a 2-min incubation from uptake medium containing NaCl. Results are given as percent of uptake measured in control cells (0.21 ± 0.01 pmol/mg of protein/2 min).

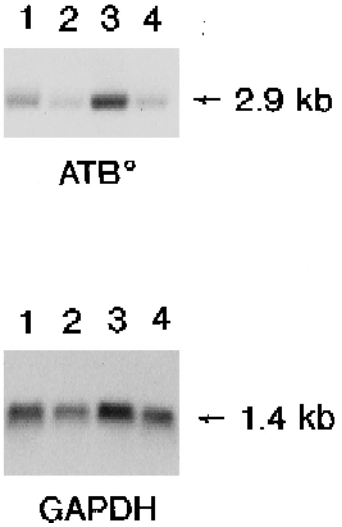

Fig. 7. Influence of ATA and genistein on steady state levels of ATB^0 mRNA and GAPDH mRNA. Confluent cultures were treated without (lane 1) or with genistein (60 μM) (lane 2), ATA (150 μM) (lane 3) or genistein (60 μM) plus ATA (150 μM) (lane 4). Poly$(A)^+$ RNA was prepared from the cells, size-fractionated, and probed with ATB^0 cDNA or GAPDH cDNA by sequential hybridization.

was reproducible in a second experiment in which β-actin mRNA levels were used as internal control (data not shown). The increase in this experiment was 57%. Since cotreatment of the cells with ATA and genistein was found to abolish the stimulatory effect of ATA on alanine uptake, we also investigated whether genistein could block the ATA-induced increase in B^0 mRNA levels. The steady state levels of B^0 mRNA and GAPDH mRNA were quantified in cells treated either with genistein alone or with genistein plus ATA (Fig. 7; lanes 2 and 4 respectively). The arbitrary B^0 mRNA/GAPDH mRNA ratio for genistein-treated cells was 0.38. The corresponding value for cells treated with ATA plus genistein was 0.37. These data show that genistein was able to block the stimulatory effect of ATA on B^0 mRNA levels. Genistein treatment itself was found to decrease the B^0 mRNA/GAPDH mRNA ratio by about 20% compared to control cells. This agreed well with a small decrease observed in alanine uptake in genistein-treated cells compared to control cells (Fig. 6).

The biological actions of ATA are cell type-specific. ATA protects PC12 cells from cell death, but it does not prevent NIH3T3 cells from similar cell death [19]. Interestingly, ATA is able to induce tyrosine phosphorylation of several proteins in cell signalling pathways in PC12 cells, but it has no effect on tyrosine phosphorylation in NIH3T3 cells [19]. It appears that the ability of ATA to induce tyrosine phosphorylation is closely linked to its biological effects and that ATA is not able to influence tyrosine

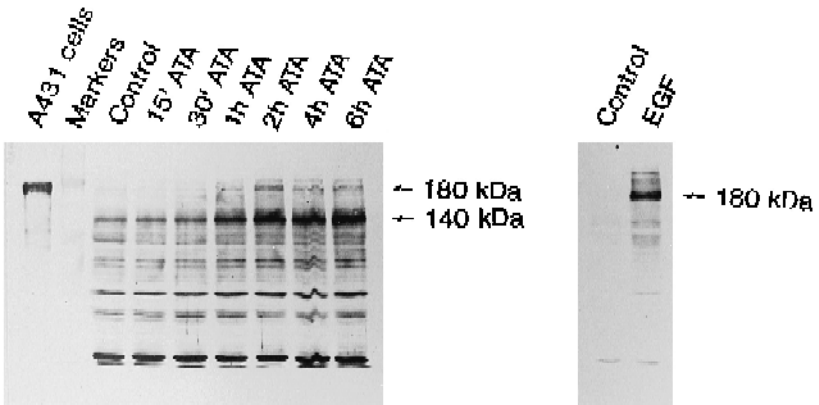

Fig. 8. Influence of ATA and EGF on tyrosine phosphorylation of cellular proteins in JAR cells. Confluent cultures of JAR cells were treated without or with ATA (150 μM) for indicated time periods (left panel) or with EGF (50 ng/ml) for 5 min (right panel). Proteins in cell lysates were separated by SDS-PAGE and then immunoblotted using anti-phosphotyrosine antibody (PY 20). Immunoreactive proteins were detected by using commercially available Enhanced Chemiluminescence Western blotting detection kit. Proteins in the cell lysate of A431 cells stimulated with 100 ng/ml EGF for 5 min were run in parallel (lane 1 in the left panel). Lane 2 in the left panel contains mol wt. marker proteins. Exposure time for left panel was much longer than for right panel.

phosphorylation in all cell types. These observations suggest that ATA may initiate its effects by interacting with a specific cellular protein which might be expressed in a cell type-specific manner. Since ATA induces alanine uptake activity and increases steady state levels of B^0 mRNA in JAR cells, we investigated the influence of ATA on tyrosine phosphorylation in these cells. JAR cells were treated with ATA for different time periods (0–6 h) and phosphotyrosine-containing proteins in cell lysates were detected by immunoblotting using a monoclonal antibody specific for phosphotyrosine residues. As shown in Fig. 8, left panel, treatment of the cells with ATA increased tyrosine phosphorylation of specific proteins in a treatment time-dependent manner. At least two proteins were clearly identified. A protein of 180 kDa exhibited markedly increased phosphotyrosine content as a result of ATA treatment. This increase was noticeable at 1 h of treatment, reached the maximum at 2 h, but declined at longer periods of incubation. The second protein had a molecular mass of 140 kDa and its phosphotyrosine content was found to increase starting at 1 h of treatment with ATA. However, unlike in the case of the 180 kDa protein, the increase in the phosphotyrosine content of the 140 kDa protein persisted even after 2 h of treatment with ATA.

In PC12 cells, ATA could stimulate most of major signal transduction pathways which are influenced by the growth factors NGF and EGF [19]. This suggested that the 180 kDa protein whose phosphotyrosine content was increased following ATA treatment in JAR cells may be the EGF receptor. The lysate of A431 cells (a human epidermoid carcinoma cell line) which had been exposed to EGF was run in parallel with the JAR cell lysates to compare the mobility the EGF receptor of the A431 cells with that of the 180 kDa protein in JAR cells (Fig. 8, left panel). A431 cells express abundant EGF receptor and tyrosine phosphorylation of the receptor increases dramatically when the cells are stimulated with EGF. As shown in Fig. 8, the EGF-stimulated A431 cell lysate contained a 180 kDa phosphotyrosine-containing protein. These data suggested that ATA possibly leads to activation of the JAR cell EGF receptor by increasing tyrosine phosphorylation of the receptor. The identity of the 140 kDa protein however remains unknown.

3.3. Role of EGF on system B^0 activity in JAR cells

If the 180 kDa protein whose phosphotyrosine content was increased following ATA treatment is indeed the EGF receptor, we thought that treatment of the JAR cells with EGF could reproduce the effects of ATA on the alanine uptake and on the steady state levels of B^0 mRNA. To probe this possibility, we first investigated the effect of EGF on tyrosine phosphorylation in JAR cells for comparison with the ATA effect (Fig. 8, right panel). Treatment of the cells with EGF (100 ng/ml) for 5 min resulted in a robust increase in the phosphotyrosine content of a 180 kDA protein which is most likely the EGF receptor. These data provide evidence for the presence of EGF receptor in JAR cells and suggest that the effects of ATA on system B^0 mRNA and activity in these cells may indeed be mediated by the activation of the EGF receptor.

These results led us to investigate the influence of EGF on System B^0 activity in JAR cells. Fig. 9A describes the effect of treatment time with EGF on alanine uptake. Treatment of the cells with EGF (50 ng/ml) increased the uptake of alanine gradually as the duration of treatment increased over a period of 1–24 h. The maximal increase observed was 20–25%. Kinetic analyses of alanine uptake, performed over the alanine concentration range of 25–750 μM, in control and in EGF-treated (EGF concentration, 50 ng/ml; treatment time, 24 h) cells are described as Eadie-Hofstee plots in Fig. 9B. For control cells, K_t for the uptake process was 216 ± 6 μM and V_{max} was 14.7 ± 0.2 nmol/mg of protein/2 min. The corresponding values for EGF-treated cells were 241 ± 7 μM and 18.7 ± 0.4 nmol/mg of protein/2 min. Thus, the EGF-induced stimulation of alanine uptake was primarily due to an increase in V_{max}. The affinity of the uptake process for alanine was not significantly altered. These results are similar to those obtained with ATA.

To demonstrate unequivocally that the EGF-induced increase in alanine uptake occurred via System B^0 rather than via system ASC, we studied alanine uptake in control and EGF-treated cells at pH 7.5 and 5.5 in the presence and in the absence of 5 mM acidic amino acids (glutamate, aspartate, and cysteate). Alanine uptake occurring via System ASC should be completely inhibitable at pH 5.5 by the acidic amino

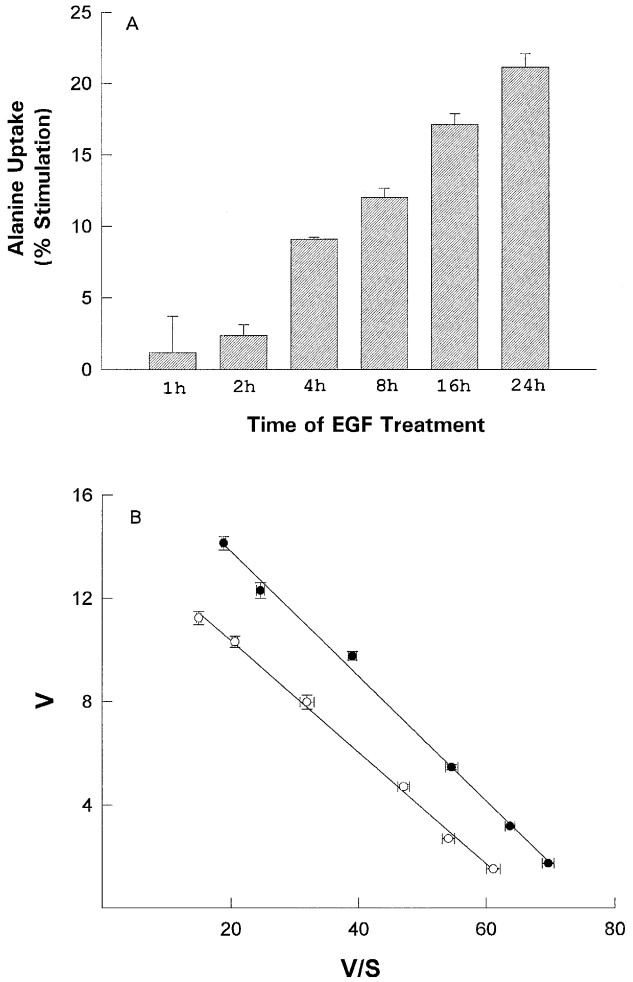

Fig. 9. Stimulation of alanine uptake by EGF. (A) Confluent cultures of JAR cells were treated with or without EGF (50 ng/ml) for indicated time periods at 37°C, following which uptake of [^{3}H]alanine (2.5 nM) was measured with a 2-min incubation from uptake medium containing NaCl. Results are given as percent stimulation compared to corresponding control uptake measured in cells treated similarly in the absence of EGF. Control uptake did not vary significantly among cultures treated in the absence of EGF for indicated time periods (0.18 ± 0.01 pmol/mg of protein/2 min). (B) Confluent cultures of JAR cells were treated with (●) or without (○) EGF (50 ng/ml) for 24 h at 37°C. Uptake of alanine was measured in these cells over a alanine concentration range of 25–750 μM and with a 2-min incubation from uptake medium containing NaCl. Uptake of alanine measured in the absence of Na^+ was subtracted from total uptake to calculate Na^+-dependent uptake which was used in data analysis. Results are given as Eadie-Hofstee plots. Key: v, alanine uptake rate in nmol/mg of protein/2 min; s, alanine concentration in mM.

Table 2
[^{3}H]Alanine uptake in control and EGF-treated cells in the presence and absence of acidic amino acids

Acidic amino acid	[^{3}H]Alanine uptake		
	Control (pmol/mg per 2 min)		EGF (% stimulation)
pH 7.5			
None	0.392 ± 0.007	0.487 ± 0.002	24
Glutamate	0.348 ± 0.001	0.423 ± 0.007	22
Aspartate	0.360 ± 0.005	0.431 ± 0.006	20
Cysteate	0.366 ± 0.004	0.435 ± 0.016	19
pH 5.5			
None	0.198 ± 0.002	0.241 ± 0.002	22
Glutamate	0.141 ± 0.004	0.171 ± 0.002	21
Asparate	0.174 ± 0.003	0.218 ± 0.001	25
Cysteate	0.128 ± 0.001	0.162 ± 0.001	27

The JAR cells were treated with or without EGF (50 ng/ml) for 24 h at 37°C. Following the treatment, uptake of [^{3}H]alanine (5 nM) was measured with a 2-min incubation either at pH 7.5 or at pH 5.5 in the presence and in the absence of 5 mM acidic amino acids. Values are mean $\pm$ S.E. ($n = 3$)

acids. The results are given in Table 2. EGF was found to stimulate alanine uptake by about 20–25% at pH 7.5 in the absence as well as in the presence of the acidic amino acids. The EGF-induced stimulation remained the same even when the uptake was measured at pH 5.5 in the presence or in the absence of the acidic amino acids. These data clearly show that the stimulation of System B^0 activity was responsible for the observed EGF-induced increase in alanine uptake in JAR cells.

We then determined the influence of EGF on steady state levels of B^0 mRNA in these cells. Northern blot hybridization with the ATB^0 cDNA as the probe detected a single 2.9 kb mRNA species in control cells and in EGF-treated cells (Fig. 10). After normalizing the data for variations in RNA loading and transfer efficiency based upon the GAPDH mRNA signals used as an internal control, EGF treatment was found to increase the B^0 mRNA levels by 75% and 58% in two separate experiments. The experiment was repeated for the third time, using β-actin mRNA levels instead of GAPDH mRNA levels as the internal control. The increase in the steady state levels of B^0 mRNA in response to EGF treatment was again reproducible (data not shown). The increase in the third experiment was 59%.

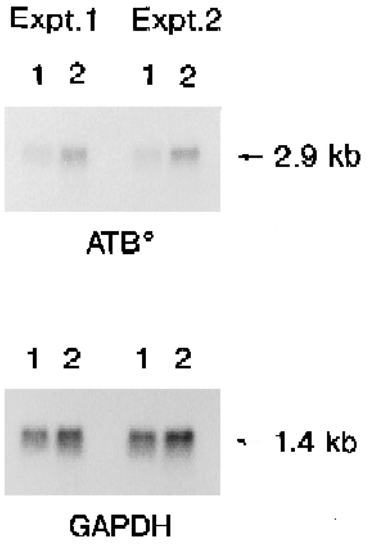

Fig. 10. Influence of EGF on steady state levels of ATB^0 mRNA and GAPDH mRNA. Confluent cultures of JAR cells were treated without (lane 1) or with (lane 2) EGF (50 ng/ml) for 24 h a t 37°C. Poly(A)$^+$ RNA was prepared from the cells, size-fractionated, and probed with ATB^0 cDNA or GAPDH cDNA by sequential hybridization.

The results of the present study provide evidence for the following: i) the human placental choriocarcinoma cell line JAR constitutively expresses the Na^+-dependent, broad-scope, amino acid transport System B^0 and this system mediates most of the Na^+-dependent alanine uptake in this cell line, ii) ATA, a neuroprotective agent which is known to induce tyrosine phosphorylation in cell type-specific manner, stimulates System B^0 activity in JAR cells and this stimulation is associated with increased tyrosine phosphorylation of specific proteins and with increased steady state levels of B^0 mRNA. iii) EGF stimulates System B^0 activity in JAR cells and this effect is associated with increased steady state levels of B^0 mRNA, and iv) ATA influences System B^0 activity by activating the EGF receptor through tyrosine phosphorylation and a common cellular signalling pathway is most likely involved in mediating the observed effects of ATA and EGF. This is the first demonstration of functional expression of System B^0 in a human placental cell line. Interestingly, there has been no report on the presence of B^0 activity in normal human placenta [22,23]. Na^+-Dependent uptake of alanine in isolated placental brush border and basal membrane vesicles is apparently mediated by amino acid transport systems A and ASC. System B^0 is prominently expressed in epithelial cells such as the intestinal and renal absorptive cells [1–6]. The syncytiotrophoblast, which is the absorptive cell in normal placenta, is also an epithelial cell and therefore it is very likely that System B^0 is functionally expressed in this cell. Northern blot analysis of human placental mRNA using the JAR cell B^0 cDNA as the probe indicates the presence of B^o mRNA in this tissue [11] The functional characteristics of System B^0 in JAR cells described in this study are very similar to those of System B^0 present in Caco-2 cells [5]. These characteristics include substrate specificity, affinity for alanine, affinity for Na^+, and Na^+/alanine coupling ratio. Furthermore, the JAR cell B^0 cDNA identifies a single, prominent 2.9 kb mRNA species in JAR cells and in Caco-2 cells under high stringency conditions [11]. The present study provides evidence for the first time for functional expression of System B^0 in a cell line of non-intestinal and non-renal origin, showing that the existence of this transport system in animal tissues is more widespread than previously thought.

Regulation of System B^0 has been demonstrated in Caco-2 cells with phorbol esters [5] and in NBL-1 cells by phenylalanine under amino acid deprivation conditions [10]. The present study describes for the first time the regulation of System B^0 by a hormone. EGF activates System B^0 in JAR cells and this effect is most likely associated with increased synthesis of the transporter protein. This is supported by the requirement of relatively long periods of EGF treatment to produce the stimulatory effect and by the facts that the EGF-induced increase in System B^0 activity is associated with an increase in the maximal velocity of the transport process and in the steady state levels of B^0 mRNA. It is not known however whether the increase in mRNA levels is due to enhanced transcription rate or increased mRNA stability. We were unable to use the transcription inhibitor actinomycin D to investigate the possible role of transcription in the observed effects of EGF because exposure of JAR cells to actinomycin D for long periods of incubation as needed to observe the EGF effect proved to be toxic to the cells. Cycloheximide, an inhibitor of protein synthesis was also found to be toxic to the cells under these conditions and therefore could not be used to determine the

participation of de novo synthesis of B^0 protein in the EGF-induced increase in B^0 activity.

The results of the present study which demonstrate stimulation of System B^0 activity by EGF may have relevance to modulation of amino acid transport across the placenta. EGF receptors are abundantly expressed in human placenta, primarily in the maternal-facing brush border membrane of the syncytiotrophoblast [24,25]. EGF in the maternal circulation may function in optimal maintenance of B^0 activity in the placenta by acting through its receptor in the placental brush border membrane.

Acknowledgements

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