Amino Acids (1996) 11: 107–116

Amino
Acids

Regulation of amino acid transport in the renal epithelial cell line NBL-1

Review Article

J. D. McGivan, J. Burston, and **B. Nicholson**

Department of Biochemistry, University of Bristol, Bristol, United Kingdom

Accepted December 15, 1995

Summary. The activities of the transport systems A, B° and X_{AG}^- are induced by various forms of stress in renal epithelial cells. Amino acid deprivation induces System A and X_{AG}^- in a protein-synthesis dependent process. In the case of System X_{AG}^- evidence is presented that induction of transport does not involve an increase in the amount of mRNA for the transporter or of the amount of transport protein. Preliminary evidence for the existence of a novel glycoprotein which is induced in parallel to the induction of these transport systems is presented. It is suggested that the induction of amino acid transport proteins and of some of the so-called stress proteins may be triggered by a common molecular mechanism.

Keywords: Amino acids – Regulation – Stress – Amino acid deprivation

Introduction

It is becoming clear that the expression of mammalian amino acid transporters is subject to rather complex regulation. At present, few of the mechanisms involved in the regulation of the expression and activity of these transporters have been elucidated at the molecular level. In this article, we describe some of the factors which regulate amino acid transport in renal epithelial cells and provide preliminary evidence for some of the mechanisms which may be involved.

Renal epithelial cells provide a convenient model system for the study of the regulation of transporter expression. These cells catalyse the accumulation of a number of different substrates, the extracellular medium can be defined and responses occurring over periods of hours can be easily studied. In our laboratory, we have carried out an extensive characterisation of amino acid transport and its regulation in the bovine renal epithelial cell line NBL-1. These cells grow rapidly and are easy to maintain. The cells express minimal activities of the typical proximal tubule marker enzymes alkaline phosphate

and aminopeptidase and do not catalyse the Na^+-dependent transport of glucose or of nucleosides. Therefore it is probable that these cells are not of proximal tubule origin. In general the cells share many of the properties of the well characterised canine kidney epithelial cell line MDCK. The kinetic properties of the amino acid transporters in NBL-1 cells are similar to those observed in renal epithelial cells from other species.

Methods

NBL-1 cells were routinely cultured in Hams-F12 medium containing antibiotics and either 10% fetal calf serum or 10% new born calf serum. In experiments where amino acid deprivation was studied the cells were cultured in a medium containing the inorganic salts of Hams F-12 plus glucose, antibiotics and either dialysed new born calf serum or bovine serum albumin as described in the appropriate references. The measurement of amino acid transport was performed as described in (Doyle and McGivan, 1992). Other methods are given in the legends to the figures where appropriate.

Results

Characteristics of basal amino acid transport in NBL-1 cells

In confluent monolayers of NBL-1 cells cultured in normal Ham's F12 medium alanine transport is highly Na^+-dependent, and is not inhibited by excess concentrations of N-methylaminoisobutyrate (MeAIB), indicating the absence of System A. Kinetic analysis of the inhibition of alanine transport by other amino acids indicates that alanine transport is competitively inhibited by related amino acids such as serine and threonine, by branched chain amino acids such as leucine and the leucine analogue BCH and also by aromatic amino acids such as phenylalanine, but not by lysine or glutamate (Doyle and McGivan, 1992). These results are consistent with the presence in NBL-1 cells of a single broad-specificity Na^+-dependent transport system for neutral amino acids similar to that previously characterised in bovine renal brush border membrane vesicles. We have termed this system System B° to indicate a system of broad specificity which transports only amino acids with no net charge. A transport system of similar specificity has been characterised in intestine and has been termed System B (Stevens et al., 1984); these renal and intestinal systems may be closely related or identical proteins. It appears that such broad-specificity systems are expressed only in epithelial cells and not for example in hepatocytes. NBL-1 cells also express a substantial Na^+-independent amino acid transport activity which has properties corresponding to those of System L.

Glutamate transport in NBL-1 cells is nearly completely Na^+-dependent. The specificity of transport corresponds closely to that of the transport system X_{AG}^- which has been characterised in other cell types. The Km of the transporter for glutamate, and the substrate and inhibitor specificity of glutamate transport in NBL-1 cells corresponds closely to that obtained in Xenopus Oocytes injected with EAAC1 cRNA Kanai and Hediger, (1992) indicating

that the cDNA clone EAAC1 probably encodes the Na^+-dependent glutamate transporter in these cells.

Regulation of amino acid transport in NBL-1 cells

Induction of System A activity by amino acid deprivation

It was shown by Felipe et al. (1992) that alanine transport is induced on culturing NBL-1 cells in an amino acid-free medium for several hours. The increased transport activity was dependent on protein synthesis. As previously described, the basal rate of transport was insensitive to inhibition by excess Me-AIB. However, it was found that the adaptive increase in alanine transport activity was completely abolished by addition of Me-AIB to the assay medium. This indicated that the endogeneous System B° transport activity was not induced under conditions of amino acid starvation, but that System A activity was induced from a very low initial level under these conditions. As previously noted by others in various cell types, readdition of various single amino acids to the amino acid-free medium caused repression of the induced System A activity. Induction of System A activity was abolished by the inhibitor of glycoprotein synthesis tunicamycin, at concentrations at which the effect of this compound should be specific. These results are broadly consistent with observations on the induction of System A in liver performed some years ago (Barber et al., 1983). However, a major difference in the two cell types is that in NBL-1 cells System A activity is negligible until amino acids are removed whereas in liver system A activity is endogenously present at a high level and is further increased by amino acid starvation. System A activity is also increased by hyperosmotic shock in NBL-1 cells, but only if the cells are first exposed to amino acid-free medium (Soler et al., 1993).

A novel protein induced by amino acid deprivation in NBL-1 cells

The emergence of System A activity on amino acid starvation of NBL-1 cells may provide a route to the molecular characterisation of this transport protein. It is assumed from the inhibitory effect of tunicamycin that the induction of transport activity involves the de novo synthesis of a so far identified glycoprotein which may be either the transporter itself or a protein which activates the transporter. Identification and characterisation of this glycoprotein is a necessary prerequisite for the understanding of the mechanism underlying the induction of System A activity. It has already been shown that in NBL-1 cells, induction of transport is accompanied by the tunicamycin-sensitive incorporation of ^{3}H-mannose into a membrane protein fraction of Mr 110–130kD (Felipe et al., 1992).

NBL-1 cells were cultured for 18 hours in the presence or absence of amino acids, and a membrane protein fraction was prepared. When membrane proteins were separated on by SDS-PAGE no significant differences in

the protein composition of membranes from fed and amino acid starved cells were observed. However solubilisation of the cell membranes in the neutral detergent Mega-10 followed by chromatography on Concanavalin A indicated a considerable increase in a glycoprotein band of about 110kD in the starved membranes compared with those from fed cells. This protein was not observed if the membranes were dissolved in Triton, or if the chromatography was performed at 0.5M salt concentrations.

NBL-1 cells were then cultured and fractionated on a large scale and the N-terminal sequence of this protein was determined to be VDRINFKT. This does not correspond to any protein sequence in the database at present and may therefore represent a novel protein species. The peptide VDRINFKTAG was linked to keyhole limpet haemocyanin and injected into rabbits. Antipeptide antibodies were purified on a column containing the immobilised peptide. These antibodies reacted on Western blots with a single protein band at 110kD from NBL-1 cell membranes. There was an approximately three-fold increase in the amount of this 110kD protein in membranes from normal NBL-1 cells compared with amino acid starved cells (Fig. 1). The time course of the increase in the amount of the 110kD protein is similar to the induction of System A by amino acid deprivation (Fig. 2). Further, the antibody did not react with bovine kidney brush border membranes, which have no System A activity (results not shown). These observations are consistent with the hypothesis that there may be some association between this novel glycoprotein and the regulation of System A, but further progress will require the cloning of this protein and evaluation of its function by heterologous expression in a suitable cell type; such investigations are in progress.

Regulation of System X_{AG}^- activity in NBL-1 cells

The Na^+-dependent transport of glutamate into NBL-1 cells via System X_{AG}^- was also found to be stimulated by amino acid deprivation (Plakidou-Dymock and McGivan, 1993). Like that of System A, the induction of System X_{AG}^- on removal of amino acids was sensitive to inhibition by cycloheximide, implying a requirement for de novo protein synthesis. The induction of Sys-

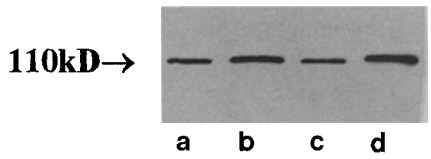

Fig. 1. Effect of amino acid deprivation on the amount of a 100kD protein in NBL-1 cell membranes NBL-1 cells were cultured in normal medium or in amino-acid starvation medium as described in Felipe et al. (1992). A crude membrane fraction was prepared by scraping the cells off the plates and passing them through an 18-gauge needle 5 times. The membrane fraction was harvested by centrifugation at 100,000g and 4°C for 30min. 25μg of membranes protein was separated on 10% SDS-PAGE and transferred to nitrocellulose before blotting with the antipeptide antibody. Lanes *a* and *c*: – normal medium (cells from two separate flasks); lanes *b* and *d*: – amino acid starvation medium

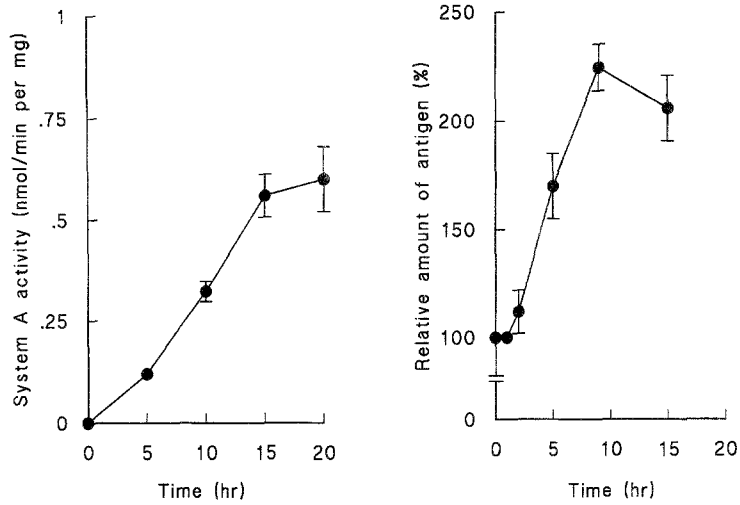

Fig. 2. Time course of the induction of System A transport activity and of the induction of the 110kD protein during amino acid starvation in NBL-1 cells. Cells were switched from normal medium to amino acid deprived medium at zero time. System A activity was measured as the initial rate of Na^+-dependent alanine transport at 0.1 mM alanine in the presence of 5mM methyl AIB. Cell samples were taken at various times and the amount of 110kD protein reacting with the antipeptide antibody was determined as in the legend to Fig. 1

Table 1. Induction of System X_{AG}^- activity in NBL-1 cells under various conditions

Medium	Initial rate of aspartate uptake (pmol/min per mg protein)
Hams F12	78 ± 2
Amino acid free	318 ± 16*
Hams F-12 + 0.2M sucrose	333 ± 8*
Hams F-12 + 0.1μg/ml tunicamycin	155 ± 6*

NBL-1 cells were incubated in the media shown for 24 hours. System X_{AG}^- activity was determined as the initial rate of Na^+-dependent uptake of aspartate (50μM) measured over 5 minutes. The detailed methods used are those described in (Plakidou-Dymock and McGivan, 1993). The results are the mean ± S.E.M. of three dishes of cells in each case. *$P < 0.001$

tem X_{AG}^- activity was prevented by the inclusion in the starvation medium of single amino acids, particularly glutamate and amino acids which can be metabolised to glutamate. We have shown also that glutamate transport is induced in a cycloheximide-dependent manner by subjecting normally fed NBL-1 cells to two further forms of stress – (i) hyperosmotic medium (Ferrer-Martinez et al., 1995) and (ii) treatment with tunicamycin. Table 1 shows an experiment illustrating the induction of glutamate transport under these various conditions.

In the case of System X_{AG}^-, molecular mechanisms can be explored in more detail, since, as described above, System X_{AG}^- activity appears to be encoded by the EAAC1 cDNA clone. It is thus possible to generate antibodies to study the correlation between changes in transporter activity and changes in transporter protein level in more detail. In this laboratory, we have generated two separate anti-peptide antibodies to the EAAC1 derived protein sequence. The antibodies were generated to the C-terminal peptide (amino acids 509–524) and to the putative extracellular loop region (amino acids 117–215). Both antibodies recognised the same protein in NBL-1 cell membranes (Mr 64kD) which is therefore likely to represent the glutamate transporter. Figure 3 shows a typical Western blot using the C-terminal antipeptide antibody on membranes from normally fed and amino acid-starved cells together with cells subjected to hyperosmotic shock and tunicamycin treatment. A quantitative measurement of the major 64kD protein reacting with the antibody is shown in Fig. 4. It is clear that while hyperosmotic shock and tunicamycin treatment increase both the transport activity and the amount of transporter protein, in the case of amino acid starvation, transport activity is increased while the amount of transport protein does not increase. Earlier work from our laboratory has shown that amino acid starvation does not increase the level of mRNA for the EAAC1 glutamate transporter in these cells (Plakidou-Dymock and McGivan, 1993). Conversely mRNA levels increase significantly on prolonged exposure of cells to hyperosmotic medium (Ferrer-Martinez et al., 1995) and to tunicamycin (B. Nicholson, unpublished results).

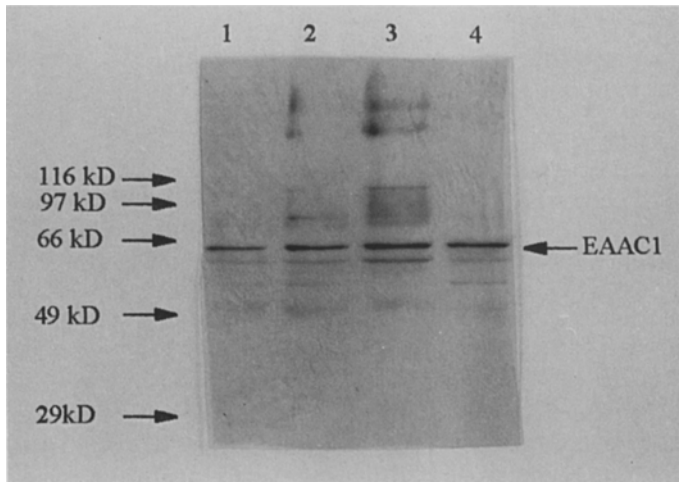

Fig. 3. Western blot showing the interaction of the C-terminal EAAC1 antipeptide antibody with proteins of NBL-1 cell membranes. Cells were incubated for 24 hours in various media, membrane proteins separated by SDS-page, transferred to nitrocellulose and blotted. The diagram shows standard molecular weight markers plus lane (*1*) normally fed cells (*2*) amino acid dpericed cells (*3*) Cells incubated in normal medium + 0.2M sucrose and (*4*) cells incubated in normal medium + 0.1μg/ml tunicamycin

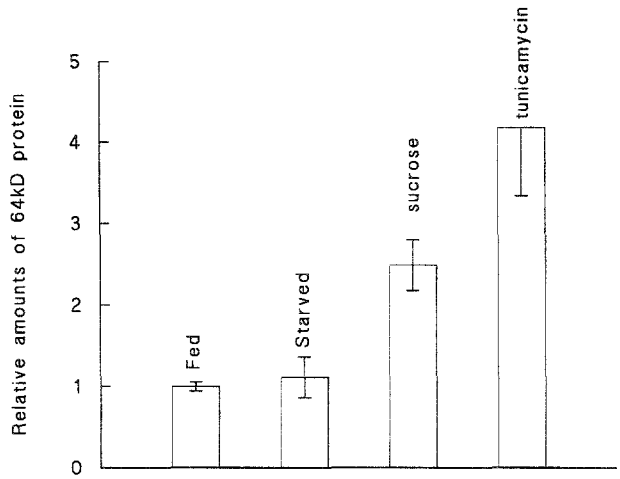

Fig. 4. Quantitation of the blot shown in Fig. 3. The blot was scanned and the area under the 64kD protein peak was measured. The area under the peak from normally fed cells was assigned a value of 1. Results are the mean ± S.E.M. of values from blots of three separate experiments

Regulation of the broad-specificity System B°

In early experiments on the induction of amino acid transport by amino acid deprivation in NBL-1 cells (Felipe et al., 1992) it was noted that addition of the single amino acid phenylalanine to the amino-acid free medium caused an induction rather than a repression of amino acid transport. Further investigation (Plakidou-Dymock et al., 1994) showed that the induction of amino acid transport was insensitive to MeAIB and the induced transport had the characteristics of System B° activity. Exposure of cells to amino acid free medium with the addition of phenylalanine caused a 2.5 fold increase in the Vmax of System B° activity with no change in Km. The major increase in activity occurred between 5 and 15 hours and was sensitive to cycloheximide. System B° has not yet been cloned, so it is not possible at present to elucidate the mechanisms involved. However, Northern analysis of mRNA from phenylalanine-treated cells revealed the appearance of a novel 3.2kB transcript which was detected by hybridisation with the cDNA probe known as SAAT1. SAAT1 was originally postulated to encode an amino acid transport protein (Kong et al., 1993), but was more recently definitively shown to encode a low affinity Na^+-dependent glucose transporter (MacKenzie et al., 1994). NBL-1 cells do not express Na^+-dependent glucose transport activity and this activity is not induced by phenylalanine (McGivan, J. D., unpublished work). It is possible that the 3.2kB transcript detected specifically under conditions of phenylalanine stress in these cells encodes a protein related to the low affinity glucose transporter which may in fact catalyse System B° activity.

Table 2. Parallel induction of amino acid transport and proteins of the grp and hsp families by various forms of cell stress

Conditions	Transport activity induced	Stress proteins induced
Amino acid deprivation	System A System X_{AG}^-	grp75, grp78
Hypertonic medium	System X_{AG}^-	hsp72
Hypertonic medium + amino acid deprivation	System A	grp75, grp78, hsp72
Phenylalanine in absence of other amino acids	System B°	hsp72

See text for references

Discussion

Amino acid transporters as stress-induced proteins

The considerations outlined above suggest that at least in renal epithelial cells various amino acid transport activities are induced by different types of cell stress. Table 1 summarises the responses observed, and correlates these with the induction of various well-characterised stress proteins under the same conditions. Exposure to hypertonic media is a well-recognised form of cell stress and in NBL-1 cells is accompanied by induction of the classical stress protein hsp72 (Plakidou-Dymock and McGivan, 1994a). Amino acid deprivation has not been generally recognised as a stress situation, but we have shown that the glucose regulated proteins grp75 and grp78 are induced under these conditions (Plakidou-Dymock and McGivan, 1994b). Addition of tunicamycin to normally-fed cells induces a stress response characterised by induction of grp78 (Welch, 1992). Phenylalanine-induced stress has been characterised previously; and we have shown that exposure of cells to high levels of phenylalanine in the absence of other amino acids leads to hsp72 induction. Induction of Na^+-dependent transport systems presumably helps to maintain the intracellular amino acid pool under stress conditions.

An interesting, but still unanswered question is that of the molecular mechanism of induction of amino acid transporters by stress. This cannot be resolved until the remaining important transporters (in particular System A and System B°) are cloned and their promoter regions are characterised. Specific promoter response elements have been found to be implicated in the induction of some of the hsp protein family by stress. In the case of the EAAC1 glutamate transporter, it appears that exposure to of cells to hypertonic shock increases transporter mRNA levels prior to the detection of increased transport activity (Ferrer-Martinez et al., 1995). Specific promoter response elements have recently been implicated in the transcriptional regulation of the Na^+-dependent betaine transporter in response to hyper-tonic stress in MDCK cells (Takenaka et al., 1994). The induction of stress proteins and of amino acid transporters may be triggered by common mechanisms.

A number of considerations have suggested that amino acid deprivation is a slightly different form of stress which may not directly induce the synthesis of new transporter proteins but rather affect the synthesis of proteins which regulate transporter activity. The evidence for this in the case of System A, based on experiments with mutants of CHO cells which do not induce System A in response to amino acid deprivation (see e.g. Moffett and Englesberg, 1984) is strong and has recently been reviewed in detail (McGivan and Pastor-Anglada, 1994). The results described above clearly indicate that induction of glutamate transport by amino acid deprivation is not accompanied either by an increase in mRNA level or by an increase in the amount of transporter protein. Since this induction is sensitive to inhibitors of protein synthesis, it is likely that a novel regulatory protein is involved in the stimulation of glutamate transport activity. The hypothetical proteins involved in the induction of System A and System X_{AG}^- activity by amino acid deprivation may be the same or different.

Acknowledgement

We acknowledge financial support from the Wellcome Trust and The Medical Research Council.

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Authors' address: Dr. J. D. McGivan, Department of Biochemistry, School of Medical Sciences, University Walk, Bristol BS8 1TD, U.K.

Received November 1, 1995