Identification and partial characterization of a novel membrane glycoprotein induced by amino acid deprivation in renal epithelial cells

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We have identified a protein of 110 kDa in the renal epithelial cell line NBL-1, which is induced on incubation of the cells in an amino-acid-free medium. The protein was purified on conA–Sepharose and subjected to N-terminal sequencing. The sequence obtained, VDRINFKT, does not correspond to any protein in the databases. Antipeptide antibodies made to this sequence recognised a single protein of 110 kDa in whole cell membranes and in a conconavalin A protein extract. Using the antibody on Western blots, the protein was induced 2.5–3 fold in 10–15 h and the induction was inhibited by cycloheximide and tunicamycin. The protein was found also in rat liver plasma membranes. A procedure for the partial purification of this protein from rat liver is described, and some internal sequence is reported. The possible relationship of the induction of this novel protein to the induction of amino acid transport in these cells by amino acid deprivation is discussed.

INTRODUCTION

When mammalian cells are deprived of amino acids the overall rate of protein synthesis falls. However, the synthesis of a small number of specific proteins is increased under these conditions. In various cell types these proteins include asparagine synthase [1], the ribosomal protein L17 [2], the stress proteins grp75 [3] and grp78 [4] and the Ca^{2+}-binding protein calreticulin [4]. Two amino acid transporting systems are also induced, System A for neutral amino acids (see [5–8] for comprehensive reviews) and the high affinity glutamate transporter System X_{AG} [9]. While the physiological function of some of these proteins is not known in detail, it appears that this overall stress response is a defence mechanism which helps to maintain the intracellular amino acid content and to protect the cells from the accumulation of malfolded proteins until normal amino acid levels are restored.

The induction of System A activity by amino acid deprivation is of particular interest. In renal epithelial cells, System A activity is absent but emerges when the cells are starved of amino acids. The induction of System A activity is dependent on protein synthesis and is inhibited by tunicamycin, an inhibitor of protein glycosylation [10], in accordance with earlier results on rat liver [11]. These studies suggest that the induction of System A activity involves the synthesis of a glycoprotein. However, the regulation of amino acid transport involves additional complexities. A considerable amount of evidence, in particular that based on work using cell mutants, suggests that the induction of System A activity by amino acid deprivation in various cell types is due not to the synthesis of new transporter molecules but to the synthesis of a so far uncharacterized protein which activates the transporter (see, for example, [12]). Evidence has also been presented that the osmotic induction of System A activity is due to the synthesis of a different, osmotically induced regulatory protein [13]. Recently, it has been shown that the induction of System X_{AG}-activity by amino acid deprivation in renal epithelial cells does not involve an increase in the transporter protein, again suggesting the induction of an activator protein [14].

In a previous report [10], it was shown that the induction of System A activity by amino acid deprivation in the bovine renal epithelial cell line NBL-1 is accompanied by the incorporation of radioactive mannose into a protein fraction of 100–130 kDa. Tunicamycin inhibited both the induction of transport and the incorporation of mannose into this protein fraction. In the present paper, a novel glycoprotein of 110 kDa, which is induced by amino acid starvation in NBL-1 cells, is identified and partially characterized. The possible relationship of this protein to the induction of amino acid transport in these cells is discussed.

MATERIALS AND METHODS

Cell culture

The bovine renal epithelial cell line NBL-1 was obtained from Flow Laboratories and was used between passage numbers 130–145. All cell culture reagents were from Gibco. Cells were routinely grown in nutrient Hams's F-12 medium supplemented with 10 % (v/v) fetal calf serum, 2 mM glutamine, 100 units/ml of penicillin G, 0.1 mg/ml of streptomycin, 0.25 μg/ml of amphotericin B. In some experiments, this medium was replaced by an amino-acid-free medium which contained the salts of Ham's F-12 medium plus 0.1 % (w/v) BSA, 10 mM glucose, antibiotics and 0.01 % (w/v) Phenol Red, pH 7.4 (see [10]). Cells were grown in T-75 flasks until confluent and fed every two days. Crude cell membrane extracts were prepared as follows: cell monolayers were washed *in situ* with PBS and the cells were lysed by scraping them into 20 mM Tris/HCl, pH.7.4. The suspension was centrifuged at 100000 ***g*** for 20 min at 4 °C and the crude membrane pellet was resuspended in 20 mM Tris/HCl in the presence of the protease inhibitors pepstatin, antipain and leupeptin (1 g/ml for each). Membrane proteins were solubilized by the addition of the detergent decanoyl-*N*-methylglucamide (MEGA-10) to a final concentration of 2 %, and the solution was clarified by recentrifugation. MEGA-10 was synthesized in bulk

Abbreviations used: conA, concanavalin A; ECL, enhanced chemiluminescence; MEGA-10, decanoyl-*N*-methylglucamide.

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from *N*-methylglucamine and decanoic acid by the method of Hildreth [15]. Protein was measured by the method of Bradford [16].

Preparation of crude plasma membrane-containing fraction from rat liver

Livers were homogenized in ice-cold 0.25 M sucrose/1 mM EGTA/10 mM Tris/HCl, pH 7.4. After sedimentation of the mitochondria at 10000 ***g*** for 10 min, the post-mitochondrial supernatant was centrifuged at 40000 ***g*** for 20 min. The resulting pellet was resuspended and recentrifuged at 10000 ***g*** to remove mitochondrial contamination. The supernatant was centrifuged at 40000 ***g*** for 20 min and the final pellet was suspended in 20 mM Tris/HCl, pH 7.4, and was frozen until used.

Concanavalin A (con-A) fractionation of membranes solubilized in MEGA-10

Membrane fractions were applied to a column containing 2 ml of con A–Sepharose (Sigma) which was pre-equilibrated in 20 mM Tris/HCl containing 0.25% (w/v) MEGA-10, pH 7.4. The column was washed with the same medium until the A_{280} was zero, and was then eluted with 0.1 M mannose/0.1 M methyl-mannoside/20 mM Tris/HCl/0.25% (w/v) MEGA-10, pH 7.4 The eluted protein was monitored by measuring the extinction at A_{280}. Where appropriate the eluted protein was concentrated using Amicon microconcentrators (30000 cut-off).

Polyclonal antipeptide antibody production

The peptide VDRINFKTAGC was synthesized and coupled to keyhole-limpet haemocyanin using the cross-linker sulpho-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (obtained from Pierce). Rabbits were immunized with this conjugate at three-weekly intervals over three months. The resulting antiserum was purified by affinity chromatography using a column containing the peptide crosslinked to CNBr-activated Sepharose (Sigma). The bound antibody was eluted with 50 mM diethylamine, pH 11, and was immediately neutralized to pH 7.4 with HCl. Second antibodies were obtained from Sigma.

SDS-gels and Western blotting

SDS/8% (w/v) PAGE was performed as described by Laemmli [17]. Western blotting was performed by the method of Towbin et al. [18], except that 5% skimmed milk powder was used to block the nitrocellulose. Protein bands were visualized by reversible staining with Ponceau S to check satisfactory protein transfer before blocking. Peroxidase-conjugated anti-rabbit second antibodies were used and blots were routinely developed using enhanced chemiluminescent (ECL) reagents (Amersham International).

Electroelution of gels

Gels were stained with 0.3 M $CuCl_2$ in order to visualize the protein bands without fixing the protein. The gels were examined against a dark background and the appropriate band was excised, placed into dialysis tubing and electroeluted overnight (50 mA, 5–7 V) in a medium containing 20 mM Tris/HCl, 2 mM EDTA and 0.1% (w/v) SDS, pH 8.0.

RESULTS

Identification and N-terminal sequencing of an amino-acid-deprivation-induced protein from NBL-1 cells using conA chromatography

Results of previous work have shown an increase in the incorporation of [^{3}H]mannose into a protein in the range 110–130 kDa, on amino acid deprivation of NBL-1 cells [10]. In order to identify this glycoprotein, membranes from normally fed and amino-acid-starved cells were dissolved in the neutral detergent, MEGA-10, in 20 mM Tris/HCl, pH 7.4, and applied to a column of conA–Sepharose. The glycoprotein fraction was eluted with mannose containing methylmannoside. Figure 1 shows the protein patterns obtained from the total membrane extract and the conA-binding fraction. Little difference in the total protein pattern in membranes from fed and amino-acid-starved cells could be seen. However in the conA-binding fraction there was a specific increase in a protein of 110 kDa. The binding of this protein to conA was weak, possibly indicating a low degree of glycosylation. In this fractionation step, both the salt concentration and the choice of detergent were critical. Thus the 110 kDa protein did not bind to conA in the presence of 0.5 M NaCl, and similarly no binding was observed if the membranes were initially dissolved in the detergents Triton, laurylsarcosine or cholate. Chromatography using either wheat-germ lectin or peanut lectin linked to Sepharose did not bind this protein (results not shown).

In order to obtain the N-terminal sequence of this protein, NBL-1 cells were cultured on a large scale. Cells from six 22-cm × 22-cm plates were cultured overnight in amino-acid-free medium. The cells were then osmotically lysed in 20 mM Tris/HCl, centrifuged and the pellet was dissolved in MEGA-10. Aliquots of the dissolved membranes were then separated on conA–Sepharose as desribed in the Materials and methods section. The conA-binding fractions were combined and concentrated and the proteins were separated by SDS/PAGE. The proteins were electroblotted on to ProBlot membrane and the 110 kDa band was excised and subjected to automated N-terminal sequence analysis (Applied BioSystems). The derived N-terminal sequence was VDRINFKT; this sequence was the

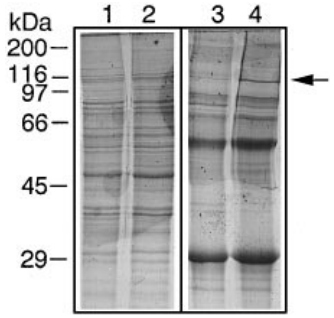


Figure 1 Coomassie-stained SDS gel of membranes from fed and amino-acid-starved NBL-1 cells before and after fractionation on conA–Sepharose

Membranes from NBL-1 cell monolayers, cultured either in normal medium or amino-acid-depleted medium for 18 h, were prepared and fractionated on conA–Sepharose as described in the Materials and methods section. Lane 1, 30 μg of total membrane from normally fed cells; lane 2, 30 μg of total membrane from amino-acid-starved cells; lane 3, 5 μg of a conA-binding membrane fraction from fed cells; lane 4, 5 μg of a conA-binding fraction from amino-acid-starved cells. In lane 4, the 110 kDa protein induced by amino acid depletion is indicated with an arrow. In lanes 3 and 4 the heavy, 29 kDa band is conA, which elutes from the column.

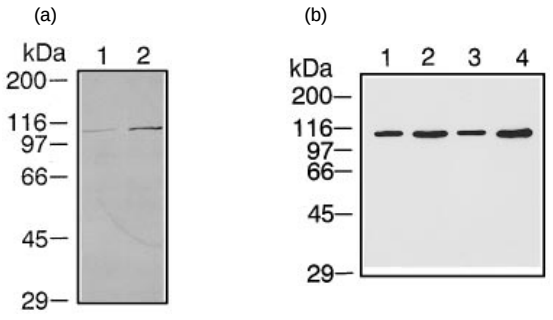


Figure 2 Western blots of the conA-binding fraction and of whole membranes from fed and amino-acid-starved cells probed with the anti-peptide antibody

(**a**) Blot of the conA-binding fraction of NBL-1 cell membranes probed with the antipeptide antibody. Lane 1, 5 μg of protein from normally fed cells; lane 2, 5 μg of protein from cells starved of amino acids for 18 h. The blot was developed with diaminobenzidine. (**b**) Separate blot of 30 μg of total membrane protein, probed with the antipeptide antibody and developed by ECL. Lanes 1 and 3, membranes from duplicate dishes of cells cultured in normal medium. Lanes 2 and 4, membranes from duplicate dishes of amino acid starved cells.

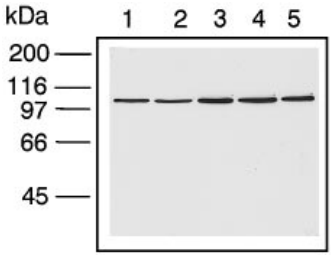


Figure 3 Western blot showing the time course of induction of the 110 kDa protein in NBL-1 cells

A representative Western blot of membranes from NBL-1 cells which had been cultured in amino acid-free medium for various times. The membranes were probed with the antipeptide antibody and the blot was developed using ECL, 30 μg of membrane protein were used in each case. The times of incubation in amino-acid-free medium were: lane 1, zero time; lane 2, 2 h; lane 3, 5 h; lane 4, 9 h; lane 5, 18 h.

same for three separate cell preparations. This amino acid sequence does not correspond to the N-terminal sequence of any protein in the SwissProt database, and hence represents a novel and hitherto uncharacterized protein.

Antipeptide antibodies to the 110 kDa protein

The peptide VDRINFKTAGC was synthesized and linked to keyhole-limpet haemocyanin via the sulphydryl group of the terminal cysteine. The conjugated peptide was injected into rabbits and the antiserum was purified on an affinity column of the peptide linked to CNBr-activated Sepharose. Figure 2(a) shows that this antibody recognized a single band of about 110 kDa in conA-binding protein fractions, and that relatively more of the epitope was present in the protein fractions of amino acid starved cells, indicating that the sequenced N-terminus of the protein was indeed recognized by the antipeptide antibody. In whole membranes from NBL-1 cells (Figure 2b), the antibody recognized a single protein of the same molecular mass, and again relatively more of this epitope was present per mg of protein in membranes from amino-acid-starved cells than from

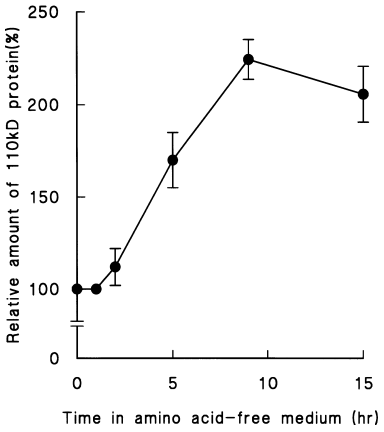


Figure 4 Quantitative analysis of blots showing the time course of protein induction

Three experiments similar to that shown in Figure 3 were performed. The ECL blots were scanned using a Chromoscan 3 (Joyce Loebl, Tyne and Wear, U.K.) and the intensity of the 110 kDa band was quantified. Values shown are means $\pm$ S.E.M. in each case, taking the intensity of the band from control cells as 100%.

normally fed cells. The presence of protease inhibitors during membrane solubilization was important; if these were omitted the antibody recognized a number of proteins of lower molecular mass.

The antipeptide antibody did not immunoprecipitate the 110 kDa protein from solubilized cell extracts, and immuno-affinity columns prepared by conjugation of the antibody to CNBr-activated Sepharose proved ineffective for purifying the protein in any quantity. Further, the antibody reacted with the 110 kDa protein on blots of SDS gels, but did not recognize the native protein in the absence of SDS. These findings suggest that the N-terminus of the native protein is not readily accessible to the antibody. This factor also limits the usefulness of the antibody for screening expression libraries to identify clones encoding the protein.

Characteristics of the induction of the 110 kDa protein by amino acid deprivation in NBL-1 cells

Confluent layers of NBL-1 cells were cultured for various times in normal Ham's F-12 medium or in an amino-acid-free medium. A crude cell membrane fraction was prepared from the cell cultures, separated on SDS/PAGE and blotted with the anti-peptide antibody. Figure 3 shows a blot of a representative experiment in which equal amounts of cell membrane protein were run on each track of the gel. Figure 4 shows a quantitative analysis of three such experiments over the same general time interval. The increase in the amount of protein, determined by Western blotting, was 2–3 fold and was maximal after 10–15 h. No increase in the amount of the 110 kDa protein was seen in the first 2–3 h. In control experiments, confluent monolayers of NBL-1 cells were continuously cultured over a period of several days in Ham's F-12 medium and no change in the amount of 110 kDa protein was observed.

The induction of the 110 kDa protein was largely abolished by the presence of either cycloheximide or tunicamycin in the culture medium (Table 1). In a further series of experiments, NBL-1 cells were cultured for 18 h in the amino acid free medium to a which a range of single amino acids (2 mM) were added. No

Table 1 Effect of cycloheximide and tunicamycin on induction of the 110 kDa protein

NBL-1 cells were incubated for 18 h in normal or amino-acid-free medium in the presence or absence of cycloheximide (10 μg/ml) or tunicamycin (0.1 μg/ml). Membranes were extracted and 30 μg of membrane protein was separated by SDS/PAGE, blotted and probed with the antibody as described in Figure 3. The ECL films were scanned and the area under the 110 kDa peak was quantified. For cells grown in control (normal) medium the area under the peak was normalized to 1.0. The results are the means $\pm$ S.E.M. of three different incubations in each case. $^*P < 0.005$ when compared with cells grown in control medium.

Culture conditions	Relative amount of 110 kDa protein
Control medium	1.0
Control medium + cycloheximide	0.8 $\pm$ 0.2
Control medium + tunicamycin	0.9 $\pm$ 0.2
Amino acid free medium	2.7 $\pm$ 0.4*
Amino acid free medium + cycloheximide	0.9 $\pm$ 0.1
Amino acid free medium + tunicamycin	1.1 $\pm$ 0.3

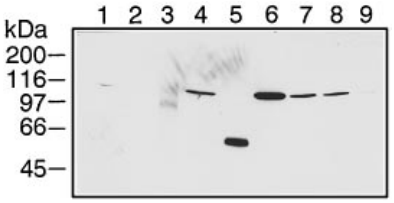


Figure 5 Tissue distribution of the 110 kDa protein

A Western blot of membranes from various rat tissues was probed with the antipeptide antibody and developed using ECL. Membranes from heart, brain, liver and leg skeletal muscle were prepared by centrifugation of the post-mitochondrial supernatant at 16000 ***g*** for 20 min. Brush border membranes from kidney and intestine were prepared by the Mg^{2+}-precipitation method. Total NBL-1 cell membranes were prepared as described in the Materials and methods section. Membrane protein (30 μg) was separated by SDS/PAGE in each case. Lane 1, rat kidney brush border membranes; lane 2, rat heart; lane 3, rat intestinal brush border membranes; lane 4, rat brain; lane 5, rat skeletal muscle; lane 6, rat liver; lane 7, 18 h amino acid starved NBL-1 cells; lane 8, normally fed NBL-1 cells; lane 9, bovine kidney brush border membranes.

consistent inhibition of the synthesis of the 110 kDa protein was observed with any single amino acid (results not shown).

Tissue distribution of the 110 kDa protein

Since very little of the 110 kDa protein can be obtained from NBL-1 cells, the antibody was used to determine its distribution in various rat tissues in the hope of identifying a better source of the protein for further studies. Tissues were homogenized and the high-speed post-mitochondrial membrane fractions were run on gels and blotted. Figure 5 shows that rat liver membranes contained the highest relative amount of the epitope per mg of protein. The protein was also found in rat brain, but little or none was detected in rat kidney, heart or intestine. In skeletal muscle, the antibody recognized a band at about 55 kDa. Although the epitope is present in the bovine renal cell line, NBL-1, very little reaction of the antibody with purified bovine brush border membranes was observed.

Purification of the protein from rat liver and determination of the internal sequence

In order to obtain more sequence information, the protein was partially purified from rat liver. High-speed post-mitochondrial

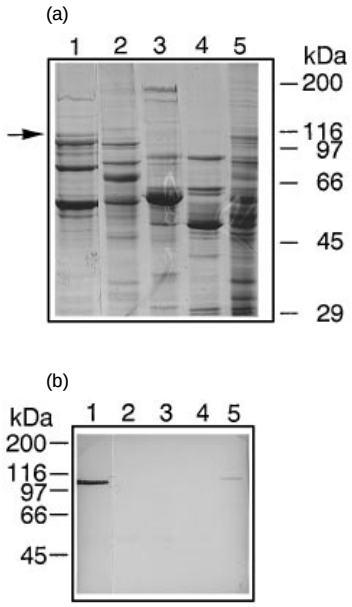


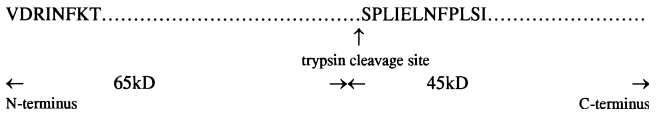
Figure 6 Partial purification of the 110 kDa protein from rat liver

(**a**) Coomassie-stained gel of protein fractions of MEGA-10 solubilized liver membranes separated on DEAE–cellulose by sequential elution at various salt concentrations. Lane 1, proteins eluted with 500 mM KCl; lane 2, proteins eluted with 200 mM KCl; lane 3, proteins eluted with 100 mM KCl; lane 4, proteins not binding to DEAE; lane 5, total membranes. The band recognized by the antipeptide antibody is indicated with an arrow. (**b**) Western blot of the gel shown in Figure 6 (**a**) probed with the antipeptide antibody showing that the protein recognized by the antipeptide antibody was eluted only at high salt concentrations.

fractions from liver were dissolved in MEGA-10 in the presence of protease inhibitors, were separated in various ways and the 110 kDa protein was identified by reaction with the antibody on Western blots. The liver protein bound very poorly to conA–Sepharose, and this method was not a useful purification step. The protein proved to bind very strongly to DEAE–cellulose and to hydroxyapatite, and optimum separation was obtained on DEAE–cellulose at high salt concentrations. Subsequent fractionation on hydroxyapatite provided no further purification.

Figure 6(a) shows the partial purification of a liver plasma membrane fraction dissolved in MEGA-10 using DEAE–cellulose and Figure 6(b) shows the corresponding blot. The 110 kDa protein bound tightly to the column and was not eluted with 200 mM salt. The protein was eluted when the salt concentration was increased to 500 mM. The 110 kDa protein was reasonably well separated from other proteins in the preparation and the protein band was cut out of the gel and electroeluted. After electroelution a single protein band was obtained and this was recognized by the antibody (not shown).

The electroeluted protein was subjected to N-terminal sequencing and proved to have the same sequence, VDRINFKT, as the protein obtained from NBL-1 cells. The protein was then subjected to limited digestion by modified trypsin. Two major bands of approximately equal intensity were obtained at 65 kDa and 45 kDa. The 65 kDa band reacted with the antibody (results not shown) which suggested that it was the N-terminal fragment of the protein. The 45 kDa fragment did not react with the antibody, and its N-terminus was sequenced. The information is shown in Scheme 1. Neither the N-terminus of the protein nor the internal sequence corresponded to any protein in the data-



Scheme 1 Partial sequence of the 110 kDa protein

The N-terminal sequence was obtained from the NBL-1 cell protein; the liver sequence is identical. The internal sequence was obtained from the rat liver protein. See text for details.

bases. The protein proved to be unstable on electroelution and no further sequence could be determined by this method.

Extensive attempts were made to use PCR to obtain a product from either rat liver or NBL-1 cells encoding the protein sequence between the N-terminus and the internal sequence. cDNA was synthesized by standard protocols from rat liver and from amino acid starved NBL-1 cells. The primers used were as follows: forward primer, 5′-GTG/A/T GAT A/CGG/A/T ATT/A AAT TTT AAG/A AC (corresponding to the amino acid sequence VDRINFK); reverse primer, 5′-A/T/CGG AAA ATT A/T/CAA/G C/TTC T/AAT C/T/A/AG (the reverse complement of the coding sequence for LIELNFP). No conditions were found which led to the synthesis of an authentic product of the appropriate size. More internal sequence is required which will require better methods of purification of the protein.

DISCUSSION

The results presented above identify a novel 110 kDa protein in NBL-1 cell membranes which is induced by amino acid deprivation. Since the protein can be purified on conA, it is presumed to be a glycoprotein. This protein represents another of the growing family of proteins which is induced when these cells are stressed by amino acid depletion. A protein with an identical N-terminal sequence to that of the 110 kDa protein from NBL-1 cells occurs in rat liver plasma membranes, and some internal sequence of this protein has been determined. The N-terminal and internal amino acid sequences of the protein do not correspond to any sequences in the SwissProt database. In particular, the sequences do not correspond to sequences of either of two other 110 kDa proteins which occur in liver plasma membranes, dipeptidylpeptidase [19] or gp110 [20]. There is also no similarity between the seqeunce of this novel protein and that of a recently characterized 110 kDa heat shock protein [21].

In NBL-1 cells there are parallels between the induction of the 110 kDa protein by amino acid deprivation and induction of the activity of amino acid transport System A. The 110 kDa protein is one which we had previously observed to be glycosylated in parallel with System A induction [10]. Tunicamycin inhibits both the induction of System A activity and the glycosylation of the 110 kDa protein. The time course of the induction of the protein is similar to the induction of System A activity and is considerably slower than the induction of stress proteins, such as grp75. The novel 110 kDa protein occurs in membranes from liver and brain where System A activity is high. Specifically, although the antibody used was raised to a sequence from bovine renal cells, it did not react with brush border membranes from bovine kidney. We have shown that System A activity is essentially absent in bovine brush border membranes [22].

There is considerable indirect evidence in the literature that induction of System A by amino acid deprivation involves induction of a hypothetical transport activating protein rather than induction of the transporter itself. It is clear from the above results that the 110 kDa protein identified in this study is a candidate for such an activating protein. It should, however, be noted that inclusion of single amino acids in the otherwise amino-acid-free medium did not suppress the induction of the 110 kDa protein in NBL-1 cells, whereas induction of System A activity in these cells is inhibited. The mechanism by which amino acids repress the induction of System A activity is not understood. If the 110 kDa protein is indeed related to the activation of System A activity, then amino acids must exert their suppressive effect at a locus other than that of synthesis of the activator protein.

Definitive evidence regarding the involvement or otherwise of this protein in the stimulation of amino acid transport will require the molecular cloning of cDNA for the protein and its expression in a suitable cell system. The very small amount of this protein in NBL-1 cells, the lack of a method for the complete purification of the protein from liver, the low efficiency of electroelution of the liver protein from gels and its instability on electroelution constitute particular difficulties in achieving this at present.

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