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Substrate-dependent adaptive regulation and trans-inhibition of System A-mediated amino acid transport. Studies using rat hepatoma plasma membrane vesicles

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Substrate-dependent regulation of amino acid transport by System A occurs by both direct action at the carrier (trans-inhibition) and transcriptional control (adaptive regulation). While experiments with intact cells have led to working models that describe these regulatory phenomena, the use of subcellular approaches will serve to refine the present hypotheses. Adaptive induction of System A transport following amino acid starvation of cells was shown to be dependent on de novo RNA and protein synthesis, and the stimulated activity was shown to be retained in isolated plasma membrane vesicles. This stimulated transport activity was tightly associated with the plasma membrane, but could be solubilized by 4 M urea and 2.5% cholate, and recovered following reconstitution of the protein into artificial proteoliposomes. These data support the working hypothesis that adaptive induction of transport is the result of de novo synthesis and insertion into the plasma membrane of System A carrier protein. In contrast, the activity of System ASC in the vesicles from the amino acid starved cells was actually reduced by 2–5-fold when compared to amino acid-fed cells. A more rapid form of regulation of System A activity is trans-inhibition. The use of isolated plasma membrane vesicles demonstrated that trans-inhibition in whole cells did not survive membrane isolation. However, substrate loading of isolated membrane vesicles containing high levels of System A activity, produced trans-inhibition in a very specific manner in that System A substrates resulted in decreased transport activity, while those amino acids which are poor substrates for the System A carrier did not. Thus, trans-inhibition is not the result of a recycling process involving an intracellular pool of carriers, but rather can be accounted for by differences in the kinetics for amino acid binding and/or translocation on the two sides of the membrane.

Introduction

Translocation of amino acids into the mammalian cell has been studied extensively and the general features reviewed [1–3]. Regulation of amino acid transport into animal tissues via the Na^+-dependent System A carrier is the result of a complex set of factors including hormones, growth factors, cell growth, and substrate availability [4–6]. The System A carrier shows the most reactivity with neutral amino acids having small, unbranched sidechains; the non-metabolizable alanine analogs 2-aminoisobutyrate (AIB) or its *N*-monomethylated derivative MeAIB are relatively specific substrates for the activity in a number of hepatoma cell lines including the one used in the studies described here [7]. In normal hepatocytes and hepatoma cells, as in many other mammalian cells and tissues, System A exhibits adaptive regulation, a RNA- and protein synthesis-dependent change in transport activity (either increase or decrease) in response to availability of substrate amino acids [8–10]. It is believed that adaptive induction of transport following amino acid starvation results from derepression of a gene encoding a plasma membrane glycoprotein [11]. The addition of a single substrate amino acid is sufficient to cause repression of the transport activity back to control levels. In general, addition of amino acids not transported by the carrier have no effect, although there are a few reported exceptions [10,12,13]. Decay of the stimulated transport following amino acid refeeding may result from both

Abbreviations: AIB; 2-aminoisobutyric acid; MeAIB, 2-(methylamino)isobutyric acid; PBS, phosphate-buffered saline; BCH, 2-amino[2.2.1]bicycloheptane-2-carboxylic acid.

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inactivation of the carrier itself and repression of the corresponding gene [14]. Unfortunately, in the absence of an anti-carrier antibody or a cDNA probe, definitive evidence for the mechanism of adaptive regulation is lacking.

In addition to the transcriptional control, substrate availability can produce rapid changes in System A transport activity via 'trans-inhibition'. In terms of metabolic control, one might consider trans-effects on transporters to be analogous to the allosteric control of an enzyme activity. Both of these processes provide rapid, protein synthesis-independent mechanisms for altering substrate flux. Neutral amino acid transport Systems ASC and L exhibit trans-stimulation, whereas System A is subject to trans-inhibition. If cells are placed in an amino acid-depleted medium, the rate of System A-mediated transport increases as the cytosolic pool of substrate amino acids decreases [15,16]. It is generally assumed that trans-inhibition is a kinetic phenomenon resulting from the fact that the carrier is linked to the sodium electrochemical gradient and therefore, essentially catalyzes a uni-directional reaction. When the carrier facing the cytoplasm is loaded with Na^+, amino acid, or both, it reorients towards the extracellular face much more slowly than unloaded carrier [16]. Kinetic analysis of the transporter in both the cytoplasmic or extracellular orientations supports this proposal. On the other hand, an alternative hypothesis involving amino acid-triggered recycling of carriers between a cytoplasmic pool and the plasma membrane, reminiscent of insulin action on glucose transport [17], has not been excluded. One could postulate that substrate deprivation would function as a signal to cause fusion of cytoplasmic transporter-rich vesicles with the plasma membrane, whereas elevated substrate concentrations would cause a reversal of this process. By analogy to the insulin-regulated glucose transport system, this process could function rapidly, and be readily reversible. Significant fluctuations in plasma membrane transporter activity as a result of recycling should be detectable by isolation of plasma membrane vesicles following alteration of the substrate amino acid levels in whole cells.

A complication in studying the regulatory mechanisms for transport has been the uncertainty of differentiating between those requiring intracellular processes and those associated with the membrane-bound carrier itself. Isolated plasma membrane vesicles which retain the transport activity present in the whole cell at the time of isolation provide a simplified assay system to allow investigations aimed at distinguishing between these phenomena. For example, isolated rat liver plasma membrane vesicles retain the protein synthesis-dependent stimulation of transport activity in response to hormone treatment of liver tissue [18–20]. The goal of the present study was to determine if: (1) the increased System A activity in response to substrate starvation (adaptive induction) was retained in isolated plasma membrane vesicles; and (2) trans-inhibition of the carrier is dependent on a cytoplasmic component or maintenance of an intact cellular structure.

Experimental procedures

Cell culture. The rat Fao hepatoma cell line was grown as monolayer in 75-cm^2 culture flasks at 37 °C under a humidified atmosphere of 5% CO_2/95% air. The cells were maintained in sterile Eagle's minimum essential medium (MEM) (pH 7.4), supplemented with 2 mM glutamine, 24 mM $NaHCO_3$, 10 mg/l streptomycin, 100 mg/l penicillin, 28.4 mg/l gentamycin, 0.23 mg/l *n*-butyl *p*-hydroxybenzoate, 2 g/l bovine serum albumin, and 5% fetal bovine serum. 3–5 days prior to plasma membrane isolation, the cells were removed from culture flasks by trypsinization (0.05% trypsin and 0.02% EDTA in PBS (pH 7.4)) and transferred to 150 mm culture dishes (for membrane isolation) or 24-well Costar cluster dishes (for whole cell assays) at a ratio of four dishes per 75-cm^2 flask. Cells were used for experimentation upon attaining 80 to 90% confluence. To stimulate transport via adaptive regulation, the MEM was replaced with an amino acid-free Na^+-containing Krebs-Ringer bicarbonate (NaKRB) buffer with or without 20 mM L-asparagine. Although a portion of Na^+-dependent asparagine transport may be mediated by System N in liver tissue [2], asparagine, a good substrate for System A, is one of the most effective repressors of hepatic System A-mediated amino acid transport [10].

Transport measurements in cultured cells. Uptake measurements in cultured cells were performed by a modification [21] of the method of Gazzola et al. [22]. To initiate transport, 0.25 ml of radioactively-labeled amino acid in Na^+-containing or Na^+-free Krebs-Ringer phosphate buffer (37 °C) was added to each well of a 24-well cluster dish. The Na^+-free buffer was prepared by replacing the corresponding sodium salts with choline chloride and choline bicarbonate. The transport assay was terminated by rapidly washing the cells four times with 2 ml of ice-cold Na^+-free Krebs-Ringer phosphate buffer. The total cellular protein was precipitated with 220 µl of 10% trichloroacetic acid and the acid-soluble extract assayed for radioactivity. The precipitated protein was solubilized by the addition of 0.2 M NaOH containing 0.2% sodium dodecyl sulfate. The protein content was measured by a modification of the Lowry procedure [21]. The intracellular water content of the cells was measured by the 3-*O*-methylglucose equilibration method of Kletzien et al. [23].

Plasma membrane isolation. A plasma membrane-enriched vesicle preparation was obtained as described by Dudeck et al. [24], with slight modifications. Fao

hepatoma cells from 16–20 culture dishes (150 mm) were recovered by removing residual NaKRB medium (± asparagine) with two rinses of ice-cold PBS, resuspending the cells in 30 ml SEB buffer (0.25 M sucrose, 1 mM EGTA, 10 mM Hepes-KOH (pH 7.5)) with a rubber policeman, and then pelleting by centrifugation at $3000 \times g$ for 5 min. The resulting pellet was resuspended in 10 ml SEB buffer and disrupted using 50 strokes of a Potter-Elvehjem homogenizer with a motor-driven, tight-fitting teflon pestle. The homogenate was spun at $500 \times g$ for 5 min to remove nuclei and unbroken cells. The supernatant was centrifuged for 30 min at $10000 \times g$ to recover the plasma membrane-enriched vesicles. The resulting membrane pellet was resuspended in SMB buffer (0.25 M sucrose, 1 mM $MgCl_2$, and 10 mM Hepes-KOH (pH 7.5)) at a concentration of approx. 10 mg/ml and stored in 200-µl aliquots at −70 °C. To insure minimal loss of amino acid transport activity in the plasma membrane vesicles, each aliquot was thawed only once.

Reconstitution. The solubilization and reconstitution of System A-mediated amino acid transport from plasma membrane was performed as described by Fafournoux et al. [25]. Briefly, plasma membrane proteins were solubilized using a cholate/urea buffer [26] and separated from the insoluble membrane remnants by centrifugation at $100000 \times g$ for 45 min at 4 °C. The soluble membrane proteins were removed from the cholate/urea solution using polyethylene glycol 8000 (PEG) precipitation, washed free of residual PEG, and then resuspended in KMB buffer (200 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes-KOH (pH 7.5)). Reconstitution was initiated by mixing the protein mixture with sonicated asolectin at a lipid to protein ratio of 20:1 [25,27]. This solution was frozen in liquid nitrogen, thawed at room temperature, diluted with 10 volumes of KMB buffer, and then sonicated for 20 s. Proteoliposomes were recovered by centrifugation at $45000 \times g$ for 30 min and resuspended in a minimal volume of KMB buffer. This freeze/thaw dilution procedure results in the formation of proteoliposomes which are competent in Na^+-dependent AIB uptake and which retain hormone-induced transport activity [25]. The system A activity present in the proteoliposomes has been shown to be equal to or slightly greater than that in the original plasma membrane vesicles (unpublished results).

Vesicle transport assay. Plasma membrane vesicles were diluted with SMB buffer to a final concentration of 1–2 mg protein per ml solution, aliquoted into 20 µl samples (20 to 40 µg protein), and incubated on ice. Each 20 µl aliquot of the vesicle suspension was incubated at 37 °C for 2 min prior to the addition of 20 µl uptake buffer (200 mM of either NaSCN or KSCN, 1 mM $MgCl_2$, and 10 mM Hepes-KOH (pH 7.5)) at 37 °C containing 0.4 mM radioactively-labelled amino acid. The membrane vesicles were incubated with the isotope solution for a specified period of time (10 s to 3 min) at 37 °C. Termination of amino acid uptake was performed by adding of 1 ml ice-cold PBS and immediately passing the mixture over a 0.45 µm nitrocellulose filter (Gelman, GN-6). The filter was washed twice with 4 ml of ice-cold PBS and then analyzed for trapped radioactivity by scintillation spectrometry. The Na^+-dependent transport activity was taken as the difference in the uptake rate observed in the presence of Na^+ (NaSCN buffer) and in the absence of Na^+ (KSCN buffer). The data are the averages ± S.D. of at least three individual assays and all experiments have been repeated using two different membrane preparations. Where not indicted, the standard deviation bars are contained within the symbol.

Results

Induction of System A-mediated amino acid transport by substrate starvation has been demonstrated in a large number of mammalian cells and tissues, including liver [8,9]. The data shown in Fig. 1 illustrates the stimulation of System A transport in Fao hepatoma

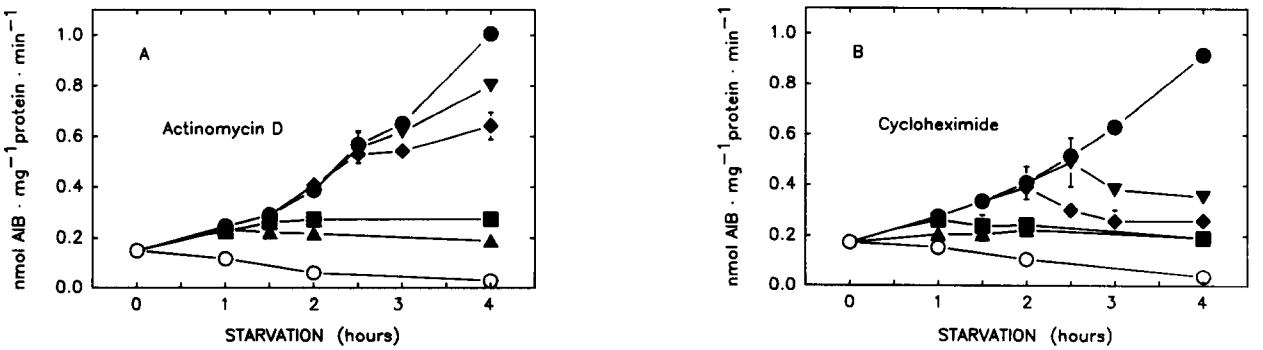

Fig. 1. Adaptive induction of System A activity in Fao hepatoma cells. Cells were transferred to either amino acid-free medium (●, NaKRP alone) or amino acid-supplemented medium (○, NaKRP containing 20 mM asparagine) and System A transport activity was measured at the indicated times. The Na^+-dependent uptake of 0.05 mM AIB was measured for 1 min at 37 °C. At 0 (▲), 30 (■), 90 (◆), or 120 (▼) min either actinomycin D (Part A) or cycloheximide (Part B) was added to amino acid starved cultures at final concentrations of 0.025 mM and 0.1 mM, respectively.

cells following amino acid deprivation, and the ability to distinguish between adaptive induction and release from trans-inhibition. The distinguishing feature between these two phenomena is the dependence of adaptive regulation on de novo RNA and protein synthesis. Transfer of hepatoma cells to amino acid-free medium in the presence of actinomycin D or cycloheximide ($t = 0$) resulted in a rapid (0–60 min), but slight increase in transport activity. This increase, not prevented by inhibition of protein synthesis, is magnified when compared to the transport rate of cells incubated in the presence of 20 mM asparagine which exhibit uptake well below that measured at the start of the experiment. Presumably, the cells ($t = 0$) have become partially depleted of intracellular amino acids following culture of 24 h without a media change and subsequent placement into medium containing the high asparagine content causes further suppression of transporter activity (Fig. 1). This decrease in activity was not prevented by cycloheximide and thus, represents trans-inhibition. Most of the increase in transport activity observed during the 4 h of amino acid deprivation was blocked by inhibition of transcription or translation. Addition of actinomycin D 30 min after amino acid removal also prevented nearly all of the adaptive response (Fig. 1A). However, when the cells were substrate starved for 90 or 120 min prior to addition of the RNA synthesis inhibitor, the transport activity increased for the next 60 min at a rate comparable to the starved cells lacking inhibitor. The decline in the degree of stimulation after 1 h of actinomycin exposure suggests a decrease in the carrier mRNA levels and is indicative of a relatively short-lived mRNA. Gazzola et al. [28] have reported similar findings for human fibroblasts, although the half-life of the carrier mRNA may be somewhat longer in that cell type.

Parallel experiments using the protein synthesis inhibitor cycloheximide illustrated that starvation for 0–30 min prior to inhibitor addition resulted in nearly complete prevention of the adaptive response (Fig. 1B). Once again allowing 90 or 120 min of starvation prior to addition of the inhibitor resulted in an increased transport rate for the following 30 min and then a decrease thereafter. Maintenance of the activity in the presence of cycloheximide is consistent with other observations indicating a relatively short carrier protein half-life (< 2 h) in the presence of repressor amino acids, but considerably longer in their absence [9,10,28]. Collectively, these data support the working hypothesis that adaptive induction of hepatic System A activity is the result of de novo synthesis of a mRNA and a corresponding protein, and indicate that within 60–90 min after substrate removal near maximal levels of both molecules have been synthesized. Previous studies in normal rat hepatocytes have demonstrated that specific inhibition of asparagine-linked glycoprotein biosynthesis by tunicamycin also blocks adaptive induction [11].

TABLE I

Retention of adaptive induction of System A activity in isolated membrane vesicles

Rat Fao hepatoma cells were incubated in either amino acid-free (NaKRB) or amino acid-supplemented media (NaKRB containing 20 mM asparagine) to alter System A activity via adaptive regulation. The intact cells and reconstituted proteoliposomes were assayed for Na^+-dependent AIB (0.2 mM) transport for 1 min at 37 °C as described in the text. The plasma membrane-enriched vesicle preparations were assayed for System A activity by measuring Na^+-dependent AIB (0.2 mM) uptake for 30 s at 37 °C.

Incubation condition	Retention (pmol/mg protein per min)		
	intact cells	membrane vesicles	proteo-liposomes
Amino acid-starved	3550 ± 600	1790 ± 60	1820 ± 20
Amino acid-fed	280 ± 40	530 ± 80	820 ± 80

If adaptive induction of System A transport activity is the result of de novo synthesis of the carrier itself, the stimulated transport activity should be detectable in isolated plasma membrane vesicles from amino acid-starved cells. Hepatoma cells were incubated in amino acid-free or amino acid-supplemented media for 3–4 h, the transport activity in the intact cells was measured, and then plasma membrane vesicles were prepared from the remaining cells (Table I). Amino acid deprivation resulted in a significant increase in transport activity regardless of whether the activity was measured in whole cells or plasma membrane vesicles. The difference in the magnitude of the stimulated transport between whole cells and membrane vesicles is probably due to differences in the assay methodology, although carrier inactivation may also contribute. Nonetheless, these results demonstrate that the induced transport activity is associated with the plasma membrane. When the starvation-induced Fao hepatoma System A carrier was solubilized in a mixture of detergent/urea and then subjected to reconstitution to generate proteoliposomes, the elevated transport rates observed in the intact cells and freshly isolated membrane vesicles were also reflected in the transport by the proteoliposomes (Table I).

A time-course of the Na^+-dependent AIB uptake by Fao membrane vesicles illustrated that the transport activity in membranes from substrate-deprived cells was significantly greater (Fig. 2). AIB accumulation reached a peak at approx. 1 min and then declined. In contrast, AIB uptake by vesicles from the amino acid-fed cells was considerably less and did not exhibit a rapid overshoot response, but instead the activity remained relatively constant between 1 and 3 min (Fig. 2). This 'overshoot' phenomenon that has been ascribed to dissipation of the Na^+ gradient as a result of rapid influx

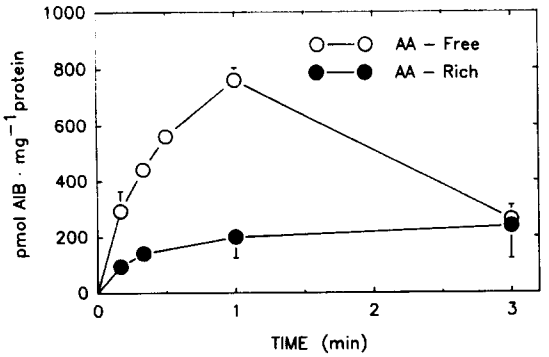

Fig. 2. Retention of adaptive induction for System A transport activity in plasma membrane vesicles from Fao hepatoma cells. Hepatoma cells were incubated in either amino acid-rich (NaKRP containing 20 mM asparagine) or amino acid-free (NaKRP alone) medium for 4 h to induce System A transport activity by adaptive regulation. The cells were rinsed free of media with ice-cold PBS and a plasma membrane-enriched vesicle preparation isolated as described in the text. System A transport activity in the two vesicle populations was measured by assaying the Na^+-dependent uptake of 0.2 mM AIB at 37 °C at the times indicated.

via the Na^+-dependent carrier as well as non-saturable ion permeability. The meaning of the difference in the shape of the two curves is unclear at present given the wide variety of factors which can contribute to the magnitude and time at which the overshoot occurs [29]. Measurement of System ASC-mediated Na^+-dependent threonine transport [15] in vesicles from amino acid-starved or amino acid-fed cells indicated that plasma membrane-associated System ASC activity was significantly reduced by amino acid deprivation of the cells (Fig. 3). It has been demonstrated by numerous laboratories, that System ASC activity is not induced by amino acid deprivation of intact cells [1–3], and previous reports have suggested that the decreased transport rate observed in amino acid-starved cells results from a

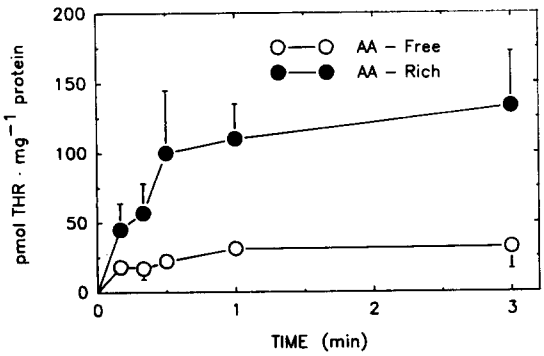

Fig. 3. Effect of amino acid starvation of rat hepatoma cells on System ASC activity in isolated plasma membrane vesicles. Fao cells were incubated in either amino acid-free or amino acid-supplemented NaKRP as described in the legend to Fig. 2. After isolation of membrane vesicles, the System ASC activity was determined by monitoring the Na^+-dependent uptake of 0.2 mM [^{3}H]threonine in the presence of unlabelled 10 mM MeAIB at 37 °C for the indicated times. The inclusion of MeAIB in the transport assay eliminated possible mediation of threonine transport by System A.

decline in trans-stimulation usually maintained by the presence of a sufficient level of cytoplasmic substrates [28]. However, a direct action by free amino acids seems to be an inadequate explanation for the data shown in Fig. 3. In this case, the influence of cellular amino acid deprivation survived isolation of plasma membrane vesicles which have been shown to be depleted of amino acids (see the legend to Fig. 5). Others have reported evidence that the System ASC activity in isolated plasma membrane vesicles does not always reflect the activity observed in whole cells [30]. Irregardless, the lack of System ASC induction by amino acid starvation contrasts with System A and provides a negative control. We have also measured Na^+-dependent System Gly [31] activity in the two membrane preparations and observed no difference in transport rates (uptake in vesicles from amino acid-fed or amino acid-starved cells was 176 ± 30 and 192 ± 25 pmol Gly/mg protein per min, respectively).

As mentioned above, two different mechanisms can be proposed to explain the phenomenon of trans-inhibition of System A. (1) Amino acid may function as a signal to cause rapid translocation of pre-synthesized carriers between the plasma membrane and the cytoplasmic compartment in a manner analogous to insulin action on the glucose carrier [17]. If this model were correct, a difference in transport activity should be observed in isolated plasma membrane vesicles prepared from amino acid-loaded and amino acid-depleted cells. (2) Trans-inhibition may be the result of a difference in the kinetics for substrate binding and/or translocation (either amino acid or Na^+), depending on carrier orientation toward the cis- or trans-face of the membrane relative to the Na^+ gradient. If this hypothesis were correct, trans-inhibition of intact cells would not be observable in freshly isolated membrane vesicles from those same cells given the loss of amino acids during the membrane isolation procedure. However, trans-inhibition should be demonstrable by loading isolated plasma membrane vesicles with trans-inhibitory substrates. Experiments utilizing isolated plasma membrane vesicles were performed to test these hypotheses.

To establish the appropriate concentration of MeAIB required to completely trans-inhibit System A transport activity, hepatoma cells were incubated in media containing different concentrations of ^{14}C-MeAIB for 60 min prior to assay of System A activity by measuring Na^+-dependent ^{3}H-AIB transport (Fig. 4). As the intracellular concentration of MeAIB increased, there was a corresponding decrease in the System A transport activity. The concentration of intracellular MeAIB required to produce 50% inhibition was approx. 4 mM. As the result of Na^+ gradient-driven MeAIB accumulation, this intracellular concentration was achieved at an extracellular concentration of approx. 0.34 mM. The suppression of System A activity by MeAIB was com-

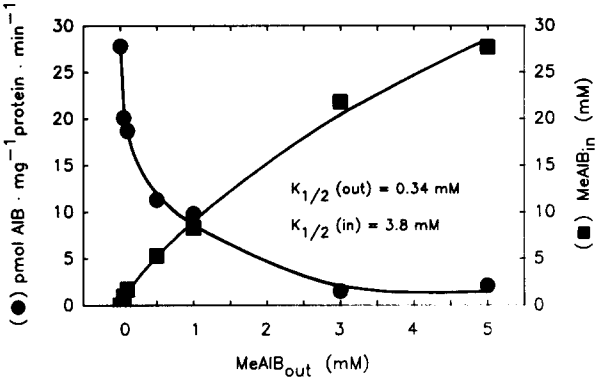

Fig. 4. Concentration-dependent trans-inhibition of System A activity by MeAIB in rat hepatoma cells. Cells were incubated in the presence of unlabelled MeAIB at concentrations ranging from 0.1 to 5 mM for 60 min at 37 °C. After rapidly rinsing with Na^+-free KRP buffer, the System A activity was measured by monitoring the Na^+-dependent uptake of 0.05 mM ^{3}H-AIB for 1 min 37 °C. Parallel incubations contained ^{14}C-MeAIB to establish the intracellular concentration of the trans-inhibitory amino acid. The intravesicular water space was measured by the 3-*O*-methyl glucose procedure of Kletzien et al. [23]. The Na^+-free KRP was prepared by substituting the corresponding choline salts for sodium phosphate and sodium chloride. The $K_{1/2}$ values for MeAIB trans-inhibition were estimated by non-linear regression computer analysis.

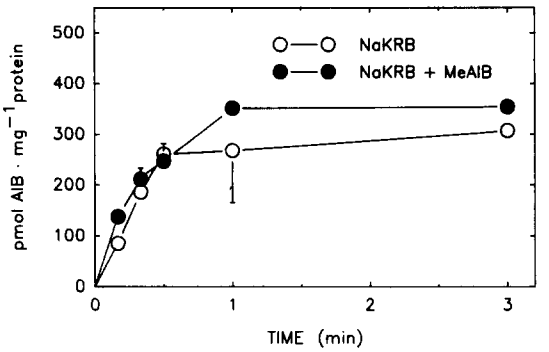

Fig. 5. Trans-inhibited System A activity in whole cells is not retained in isolated plasma membrane vesicles. Fao hepatoma cells were incubated for 60 min at 37 °C in the absence or presence of 10 mM MeAIB to produce trans-inhibition of the System A transport activity [10]. The cells were rapidly rinsed in ice-cold PBS and plasma membrane vesicles were prepared as described in the text. The System A transport activity in the vesicles was assayed by monitoring the Na^+-dependent uptake of 0.2 mM AIB at 37 °C. Parallel incubations with ^{14}C-MeAIB included during the incubation with whole cells and subsequent determination of radioactivity associated with the isolated plasma membranes demonstrated that amino acid accumulated intracellularly was not trapped to a significant degree in the membrane vesicles.

pletely insensitive to protection by RNA or protein synthesis inhibitors pointing towards trans-inhibition rather than repression as the mechanism [9].

To determine whether the trans-inhibition in whole cells would survive isolation of plasma membrane vesicles, Fao hepatoma cells were incubated in the presence or absence of 10 mM MeAIB for 60 min and then used to prepare plasma membrane vesicles. The vesicles exhibited similar System A activity regardless of whether the cells had been incubated in the presence or absence of trans-inhibitory amino acids (Fig. 5). These results argue against the carrier translocation mechanism, but are consistent with the hypothesis that trans-inhibition results from substrate binding kinetics. To test further the latter hypothesis, isolated plasma membrane vesicles were incubated in the presence of 10, 25, or 50 mM MeAIB to load the intravesicular compartment in a manner analogous to elevated levels of cytoplasmic amino acids for intact cells. Amino acids which are clearly not substrates of System A, such as BCH or D-leucine, were used as negative controls. The intravesicular water space was calculated by the 3-*O*-methylglucose method of Kletzien et al. [23] and was approx. 2.5 μl/mg. Loading the vesicles with MeAIB caused a concentration-dependent decrease in measurable System A activity (Fig. 6). In contrast, equal or greater concentrations of intravesicular D-leucine or BCH had little or no effect on transport activity. For example, loading the vesicles in the presence of 50 mM BCH resulted in an intravesicular concentration of 23.7 mM which caused no inhibition of transport activity (99% of control), whereas loading with 50 mM MeAIB yielded an intravesicular level of 11.7 mM and 66% inhibition. Similar results have been obtained using normal rat liver plasma membrane vesicles. For those membranes,

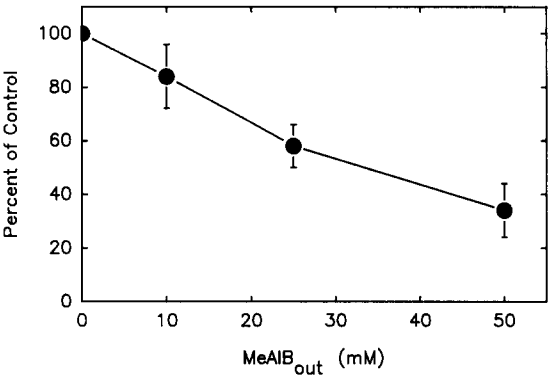

Fig. 6. System A transport activity in isolated plasma membrane vesicles is subject to trans-inhibition. Fao hepatoma cells were incubated for 3 h in NaKRP to induce System A transport activity by adaptive induction. Plasma membrane vesicles were then prepared as described in the text and used to test for trans-inhibition by incubation for 1 h at 4 °C in KMB buffer containing 10–50 mM of either MeAIB, D-leucine, or BCH (see text for D-leucine and BCH results). After the incubation period, the vesicles were pelleted by centrifugation in a Beckman Airfuge at 150000 × *g* for 5 min, and then resuspended in KMB buffer at a final protein concentration of 1–2 mg/ml. System A activity was measured for 1 min at 37 °C as Na^+-dependent AIB (0.2 mM) uptake. In parallel incubations, radioactively-labelled MeAIB, D-leucine, or BCH was used to calculate the intravesicular concentration at the time of the transport assays. The intravesicular water space was measured by 3-*O*-methyl glucose distribution [23]. The data are presented as percent of control transport in the absence of amino acid loading. For two different experiments, the control velocities were 433 ± 120 and 487 ± 113 pmol AIB/mg protein per min.

loading with 25 mM MeAIB resulted in a 60% reduction in transport activity.

Discussion

Metabolite-dependent regulation represents a fundamental concept in intermediary metabolism, and it is quite natural that such processes would impinge on the trans-membrane transport of nutrients as well as on enzymes within the metabolic pathways. Amino acid-dependent regulation of gene transcription has been well studied in bacterial systems [32] and to some extent in yeast [33]. However, with the exception of a limited number of observations, little information is available at the molecular level concerning metabolite control of gene expression in mammalian systems. Substrate-dependent control of the System A carrier has been recognized for nearly two decades [34,35]. Based on the effect of inhibitors of both RNA and protein synthesis, it is generally believed that both the adaptive induction, observed when cells are starved for amino acids, and the adaptive repression upon refeeding of substrates are dependent on active transcription and translation. Several working models have been proposed to explain adaptive regulation of the System A carrier [12,28] and most have the following common features: (1) amino acids maintain the System A gene in a repressed transcriptional state, (2) removal of amino acids from the incubation medium results in release from this repression allowing increased transcription of the gene; and (3) amino acid refeeding of starved cells results in an increased rate of carrier inactivation at the membrane and repression of the carrier gene expression. Evidence from a number of laboratories supports each of these general concepts, yet in the absence of molecular tools such as antibodies or cDNA probes additional indirect evidence is needed to support and refine the working models. The results presented here extend our knowledge of adaptive regulation by demonstrating that the induced transport activity is retained in plasma membrane vesicles isolated from starved cells. Furthermore, the data indicate that the protein responsible for the adaptive induction is tightly bound to the plasma membrane, yet the stimulated System A activity can be solubilized by exposure of membranes to 2.5% cholate/4 M urea and reconstituted into artificial proteoliposomes. The System A activity in vesicles or proteoliposomes generated from membranes of amino acid-starved versus amino acid-fed cells reflects the difference in transport observed in whole cells. Although not conclusive evidence, these data argue that the starvation-induced protein, which is known to be a glycoprotein [11], is the carrier itself, or a tightly membrane-associated subunit. The maintenance of the induction in isolated membranes, in conjunction with the reconstitution assay, will provide an excellent opportunity for attempts at purification of the substrate-regulated System A carrier.

The results reported also illustrate that trans-inhibition of System A in whole cells does not survive plasma membrane isolation, but can be demonstrated by raising the intravesicular concentration of substrate once membrane vesicles have been prepared. These data argue against recycling of transporters, and for the kinetic mechanism as an explanation for trans-inhibition. The ability to utilize plasma membrane vesicles to study the phenomena of trans-inhibition will eliminate a number of the complications that occur with intact cells resulting from metabolism or compartmentalization. For example, although estimates for the kinetic constants of the System A carrier oriented toward the cytoplasm have been obtained in whole cells [16], the true concentration of the amino acid in the cytoplasm cannot be determined due to possible sequestration in the various compartments and organelles within the cell. Likewise, such kinetic analysis is impossible for System A substrates which are rapidly metabolized. The application of the isolated membrane methodology should benefit the study of transport systems subject to either trans-inhibition or trans-stimulation.

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