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Catabolic control of the enhanced alanine-preferring system for amino acid transport in glucose-starved hamster cells requires protein synthesis

(α-aminoisobutyric acid/glucose shiftdown/membrane vesicles/cycloheximide)

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ABSTRACT **In cultured hamster cells starved for glucose for 24 hr there is an enhancement of the rate of α-aminoisobutyric acid transport ("shiftup"). When the starved cells are re-fed with glucose, the rate of transport shifts back down to the low, "regulated" rate typical of cells continuously fed with medium containing glucose ("shiftdown"). The high, deregulated rate of transport is maintained, however, when cycloheximide is present for 24 hr during the re-feeding with glucose. Maintenance of the high transport rate is evident only when the cells are incubated in amino acid-free medium just prior to the transport assay or when the assays are conducted with isolated membrane vesicles. A premature, pseudoshiftdown was observed in intact cells within as little as 2 hr after re-feeding when care was not taken to deplete the amino acid pool prior to the transport assay. In addition, a cycloheximide-insensitive increase in transport was observed when cultures were re-fed for 2 hr with amino acid-free medium containing fresh serum. These results emphasize the often overlooked precautions that should be taken to guard against artifacts that could mislead interpretations of amino acid transport data. More important, however, is the finding that Na^+-dependent amino acid transport in cultured animal cells is regulated in part by a factor (or factors) that becomes inactivated when the cells are maintained under nonglycolytic culture conditions. In order to reactivate the control mechanism, starved cells that have been re-fed with glucose must resynthesize the regulatory factor(s). Thus, in at least cultured hamster cells, Na^+-dependent amino acid transport regulation is much like the hexose transport regulation in that catabolic control (shiftdown) requires protein synthesis.**

The control of hexose and amino acid transport in cultured animal cells has been shown to be relaxed when D-glucose is either omitted from the culture medium or replaced by D-fructose—i.e., glucose starvation‡ (1–6). The transport rates for those nutrients are enhanced when cells are maintained in the absence of glucose for 18–24 hr. Moreover, because inhibitors of both RNA and protein synthesis prevent the full expression of enhancement in the more extensively studied hexose transport system, the cellular response to glucose starvation has been referred to as a derepression type of regulation (3, 4). However, because other physiologic factors contribute to the expression of the starvation-induced enhancement of both hexose and amino acid transport (1, 2, 6), we favor the less commital term "deregulation." In studies that extend the earlier findings, it was also found that the deregulated rate of hexose transport in cultured animal cells could be returned to the low, presumably controlled, rate simply by re-feeding the starved cells with medium containing glucose ("shiftdown") (1, 4, 6). Thus, glycolysis (or glucose catabolism) seems to be involved in the mechanism controlling hexose transport ("catabolic control"). We have demonstrated, however, that the catabolic control of the deregulated hexose transport system requires more than glycolysis (1, 2). Synthesis of a putative controlling factor is also required because no shiftdown occurs if inhibitors of protein synthesis are added to the culture medium at the time of the glucose feeding (1, 6).

By measuring Na^+-stimulated α-aminoisobutyric acid (AIB) transport by membrane vesicles isolated from glucose-fed and glucose-starved hamster cells, it has been shown that deregulation of an amino acid transport system is similar to the starvation-induced deregulation of the hexose transport system (2). The deregulation of transport (represented by starvation-induced increases in AIB transport) was thus shown to be expressed at the level of the membrane, uncomplicated by other intracellular functions (2). The data from that study further supported the interpretation that starvation-induced enhancements of transport most likely reflect changes in the number of membrane-associated, functional carriers (2).

Because it has been found that the control of the deregulated rate of hexose transport in cultured hamster cells depends on glucose catabolism (1, 4, 6) and protein synthesis (1, 6), it was of interest to determine if the deregulated Na^+-dependent, alanine-preferring system (A-system) for amino acid transport was similarly controlled. In this paper we present evidence suggesting that the catabolic control of the deregulated A-system in Nil cells does occur, is also dependent upon protein synthesis, and is therefore similar to the control of hexose transport.

MATERIALS AND METHODS

α-Amino[^{14}C]isobutyric acid, α-[*methyl*-^{3}H]aminoisobutyric acid, and L-[^{3}H]glucose were purchased from New England Nuclear. Culture media and fetal calf sera were obtained from GIBCO and Microbiological Associates. Cycloheximide was purchased from Sigma.

Hamster fibroblasts (Nil strain) were grown to confluence in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal calf serum (modified medium). Cells, in 490-cm^2 roller bottles, were starved of glucose for 24 hr by changing the culture medium to modified medium containing 10% dialyzed fetal calf serum and 22 mM D-fructose in place of D-glucose (starvation medium‡) as described (2). The cultures

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Abbreviations: AIB, α-aminoisobutyric acid; A-system, alanine-preferring system.

‡ When Nil hamster cells are maintained in medium containing fructose instead of glucose, their hexose and amino acid transport rates increase as if the cells are starved of glucose and the enhancements of transport that develop with fructose feeding are often higher and more sustained than when the cells are totally deprived of glucose (1, 2).

with enhanced AIB transport were divided into four groups of 10 roller bottles each, and the old medium was removed and replaced with one of the following media: (*i*) fresh starvation medium (i.e., containing 22 mM fructose); (*ii*) starvation medium containing 2 μg of cycloheximide per ml of medium; (*iii*) medium containing 22 mM glucose (shiftdown medium); or (*iv*) shiftdown medium containing 2 μg of cycloheximide per ml. After 18–24 hr, the cells were harvested by scraping and aliquots, representing about 10% of the total cells from each group, were removed for whole cell AIB transport assays, the details of which have been described (2). Membrane vesicles were prepared from the remainder of the suspended cells as described (2).

Before the whole cells that were scraped from the roller bottles were assayed for AIB transport, they were collected by centrifugation (900 × *g* for 10 min) and resuspended in amino acid-free, sugar-free modified medium containing 10% heat-inactivated (65°C for 10 min) dialyzed fetal calf serum. The resuspended cells were then incubated (with occasional stirring) in a CO_2 incubator (10% CO_2/90% air) for 60 min to deplete the intracellular amino acid pool (7). The preincubated cells were then collected by centrifugation, washed twice with ice-cold Dulbecco's phosphate-buffered saline and assayed at 25°C for the transport of 0.1 mM AIB as described (2). L-Glucose (0.1 mM) was included in the assay medium to correct for non-carrier-mediated absorption or influx of substrate (2). For selected experiments, small-scale cultures (maintained as monolayers in 35-mm culture dishes) were re-fed, washed, and, while the cells remained attached to the dish, assayed for AIB transport as described (2). The preparation of membrane vesicles and the method of assaying the transport of AIB by the membrane vesicles have been described in detail (2).

RESULTS

Effect of Amino Acids on Apparent Shiftdown. A spurious shiftdown of AIB transport was observed when starved cells were re-fed with medium containing D-glucose (or glucose plus cycloheximide) (Table 1). When the starved cultures were washed and re-fed with fresh glucose or glucose plus cycloheximide medium (complete with the usual, full complement of essential amino acids and fresh serum), a marked cycloheximide-insensitive shiftdown of AIB transport became evident by 2 hr. However, this shiftdown appeared to be a result of a sudden increase in intracellular amino acids because preincubation of parallel cultures for 2 hr in amino acid-free medium did not reveal the decrease in AIB transport. In fact, instead of a shiftdown, preincubation of the cultures in the amino acid-free medium (with or without cycloheximide) resulted in variable 40–70% increases in the AIB transport rate. This increase in transport after the 2-hr preincubation in fresh amino acid-free medium appeared to be due mainly to serum because serum preheated at 65°C for 10 or 15 min did not cause an increase in the transport. In order to observe a catabolically controlled shiftdown of AIB transport from the deregulated state in whole cells, it was important to follow the extended shiftdown culture period with a preincubation period in amino acid-free medium containing heat-inactivated serum. Disregard of this prerequisite was found to lead to ambiguous results.

AIB Transport Shiftdown, Resulting from Extended Glucose Re-feeding, Is Blocked by Cycloheximide. After cultures in roller bottles were starved for glucose for 24 hr, the starvation medium was removed and the cells were re-fed for 24 hr with fresh culture medium containing D-glucose (with or without cycloheximide) or were continued under starvation conditions by changing the medium to fresh starvation medium (with or without cycloheximide). At the end of this second 24-hr culture period, the cells from each group were scraped from the surface of the roller bottles and aliquots of the suspended cells were removed for the whole cell transport assay. The remainder of the cells in each group were used to prepare membrane vesicles. The whole cells, assayed immediately after being removed from the culture environment, had AIB transport characteristics similar to those shown in the second part of Table 1: cells that were continued under starvation conditions had the highest rate of transport whereas cells cultured in the presence of glucose (or glucose plus cycloheximide) and cells cultured in the presence of cycloheximide under starvation conditions had low rates of transport (data not shown). However, when the suspended cells were preincubated for 60 min in amino acid-free and sugar-free medium containing heat-inactivated serum, the relative differences in the rate of transport by the representative cells changed in one important respect. Even though the cells re-fed with glucose medium for 24 hr did shift from the high rate of AIB transport (typical of starved cells) to a low rate (typical of glucose-fed cells), cells cultured in the presence of glucose plus cycloheximide did not revert to a low rate of AIB transport (Fig. 1). Also, cells that were continued for 24 hr under starvation conditions in the presence of cycloheximide maintained a relatively high transport rate. This rate of transport by the cycloheximide-treated starved cells was similar to the rate in cycloheximide-treated, glucose-fed cells. However, in both cycloheximide-treated groups the AIB transport rate was substantially lower than the rate in cells that were simply continued under starvation conditions (without cycloheximide).

Table 1. Pseudoshiftdown of AIB transport within 2 hr of glucose re-feeding

Culture conditions*		AIB transport, nmol/mg protein/min†	Relative transport
At 24 hr	At 2 hr		
Zero time			
Glc	No change	0.42 ± 0.03	0.41
Fru	No change	1.03 ± 0.07	1.00
Complete medium			
Glc	Glc	0.39 ± 0.02	0.38
Fru	Fru	0.79 ± 0.05	0.77
	Glc	0.54 ± 0.0	0.52
	Glc and cycloheximide	0.53 ± 0.03	0.51
Amino acid-free			
Fru	Fru	1.62 ± 0.07	1.57
	Glc	1.75 ± 0.15	1.70
	Glc and cycloheximide	1.47 ± 0.47	1.43

* Confluent Nil cultures were washed with sterile Dulbecco's phosphate-buffered saline and re-fed with Dulbecco's medium containing 10% dialyzed fetal calf serum and 22 mM D-glucose (Glc) or 22 mM D-fructose (Fru). After 24 hr, the cultures were washed and either assayed immediately (zero time) or incubated for an additional 2 hr in complete or amino acid-free Dulbecco's medium containing 10% dialyzed fetal calf serum. When present, cycloheximide was 2 μg/ml medium.

† Shown as mean ± SD.

Membrane Vesicles Corroborate Cycloheximide Block of the AIB Transport Rate Shiftdown That Results from Extended Glucose Re-feeding. Fig. 2 shows the results of AIB transport studies using membrane vesicles isolated from the cells whose whole cell transport rates are shown in Fig. 1. In the presence of Na^+, the membrane vesicles from the starved cells that were re-fed with glucose had the slowest rate of transport and the lowest overshoot profile (Fig. 2*A*). The rates of AIB

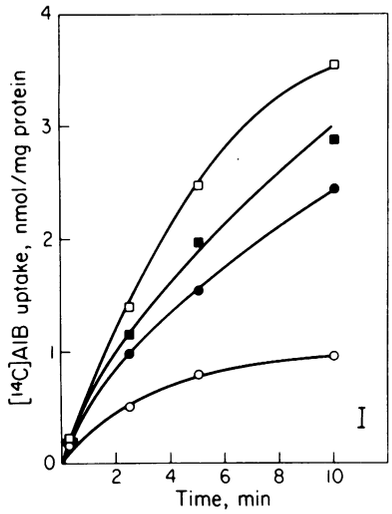

FIG. 1. Effect of re-feeding starved cells with glucose for 24 hr on AIB transport rate by whole cells is blocked by cycloheximide. Cells were maintained in roller bottles. Cells were changed from starvation medium to fresh medium as follows: ○, to glucose medium; ●, to glucose medium plus cycloheximide; □, to starvation medium; and ■, to starvation medium plus cycloheximide (when present, cycloheximide was at 2 μg/ml of culture medium). Representative aliquots of the starved/re-fed cells were suspended in preincubation medium (amino acid-free, hexose-free Dulbecco's medium containing 10% heat-inactivated, dialyzed, fetal calf serum) for 1 hr to deplete the amino acid pool. Then, the cells were collected by centrifugation and resuspended in 1.5 ml of Dulbecco's phosphate-buffered saline containing 0.1 mM [^{14}C]AIB (0.5 μCi/ml) and 0.1 mM L-[^{3}H]glucose (2 μCi/ml) (1 Ci = 3.7 × 10^{10} becquerels). At appropriate times, duplicate 100-μl aliquots were removed and the cells were separated from the assay solution by centrifugation through an oil mixture that had a density intermediate between that of the cells and that of the assay solution (2). Corrections for trapped assay solution and simple diffusion were made by subtracting the amount of L-glucose from the amount of AIB in each sample. Protein content in representative aliquots, taken at the beginning and at the end of each assay, was determined as described (2). The amount of AIB calculated to be in a volume equivalent to the water volume estimated to be in cells containing 1 mg of protein (3–5 μl; see ref. 7) is indicated by the vertical bar.

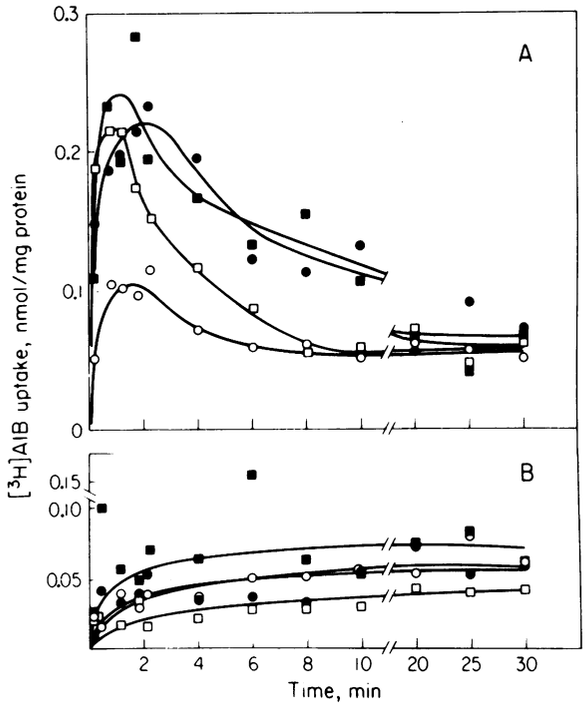

FIG. 2. Differences in the rate of Na^+-stimulated AIB transport by membrane vesicles isolated from glucose-starved and glucose-re-fed cells reflect the differences in whole cell AIB transport. Membrane vesicles were prepared from the majority of the cells that were used for the whole cell AIB transport study shown in Fig. 1. Vesicles (approximately 600 μg of membrane protein) from each group of cells were suspended in 200 μl of 1 mM Tris·Hepes buffer (pH 7.5) containing 100 mM sorbitol and either 100 mM NaSCN (*A*) or 100 mM choline chloride (*B*). The assay was initiated by the addition of [^{3}H]AIB (0.1 mM; 100 μCi/ml), and 15-μl aliquots were removed at the times indicated. The vesicles were diluted in ice-cold, Na^+-free buffer and filtered; the radioactivity results were corrected for background as described (2). Symbols are as in Fig. 1.

transport and overshoot profiles of vesicles from the continuously starved cells or the cells treated with cycloheximide (with or without glucose) were practically indistinguishable and were at least 4 times higher than those of vesicles from the cells re-fed with glucose (Fig. 2*A*). In the absence of sodium (i.e., Na^+ replaced by choline), only small differences in the rate of transport were apparent, no overshoot of AIB transport occurred, and the steady state of intravesicular AIB (30-min assay time) was approximately the same with all preparations. In addition, the steady state (30 min) in the absence of Na^+ was the same as the steady state (30 min) in the presence of Na^+, indicating that the intravesicular volumes were approximately the same in all the vesicle preparations regardless of the presence or absence of Na^+. On the basis of the 15- or 30-sec assay points as being indicative of approximate initial rates of AIB transport and correction for the influx of AIB in the absence of Na^+ (Fig. 2*B*), the order of transport velocities is essentially the same as was with the whole cells (Fig. 1).

DISCUSSION

As a result of glucose starvation, cultured Nil hamster cells deregulate their Na^+-dependent amino acid transport. This starvation-induced loss of control manifests itself as an enhancement in the rate of transmembrane transport and the corresponding increases in V_{max} values suggest that the increases are due to increases in the number of functional carriers (2). In view of similar glucose starvation-induced changes in hexose transport in cultures of both mammalian (5, 6) and avian (3, 4, 8) cells, it was of interest to determine if exposing the starved cells to medium containing glucose would result in the same kind of enhancement reversal (shiftdown) previously observed for the hexose transport system (1, 4, 6). Moreover, because inhibition of protein synthesis during the re-feeding period completely blocked the shiftdown of the hexose transport activity (1, 6), it was of further interest to determine if the regulation of the Na^+-dependent amino acid transport system could be blocked by reagents such as cycloheximide.

Previous studies of Na^+-dependent transport of AIB by isolated membrane vesicles (2) provided a convenient means to observe changes in transport at the level of the membrane uncomplicated by potential distortions resulting from intracellular (e.g., soluble cytoplasmic) factors. In addition, the Na^+-dependent, "active" transport of amino acids by vesicles allows for the accumulation (albeit transient) of substrate over several minutes (2), making the quantitative analysis of transport considerably easier than the transport of hexoses (or nucleosides) which are taken up by way of a rapidly equilibrating, facilitated diffusion mechanism (8, 9).

In this study, the membrane vesicles provided the most convincing evidence that the control of Na^+-dependent amino acid transport is in fact much like the control these cells exert over the hexose transport system. That is, cells starved for glucose lose the activity of presumptive regulatory factor in such

a way that, in order to reinstate a controlled low level of transport, the regulatory factor(s) must be resynthesized by the cell (1, 6). From the results shown in Table 1, however, it can be appreciated that the expression of the control of amino acid uptake at the level of transport in whole cells can easily be masked by other overriding factors. Stimulatory effects of serum on transport systems are already well documented (10, 11) but it is interesting that the stimulatory effect of serum on AIB transport can be eliminated by preheating the serum. We have recently found that the cycloheximide-insensitive, serum-induced stimulation of hexose transport described by Kletzien and Perdue (10) is also eliminated when "heat-inactivated" serum is used during the re-feeding of cells with fresh medium (unpublished data). At the other extreme, direct inhibition of AIB transport can result in spurious shiftdown effects, presumably as a result of "transinhibition" from accumulated amino acids that were transported into the cells from the culture medium (12). In the starved cells, the high rate of amino acid transport probably provides an exaggerated intracellular accumulation and this is probably further exaggerated by inhibition of protein synthesis by cycloheximide. Thus, the premature, cycloheximide-insensitive "pseudoshiftdown" of AIB transport shown in Table 1 could, relative to the long-term shiftdown, be considered an artifact. These data also reemphasize obvious precautionary procedures that should be used during studies of amino acid transport by intact cells (7, 13).

As with the hexose transport system, the control of Na^+-dependent amino acid transport is a control effect that appears to be mediated through a labile macromolecular factor (1, 6). The activity of this presumptive factor appears, moreover, to be connected in some manner to glucose catabolism. Catabolism alone is insufficient to promote the shiftdown of either the hexose (1, 6) or the Na^+-dependent amino acid transport system. This is evident from experiments in which starved cells were re-fed in the presence of cycloheximide. Under these conditions glucose catabolism resumes (6) but enhanced transport remains unperturbed. It has been proposed that turnover of membrane-associated carriers may be the regulatory mechanism for the control of hexose transport (14) and this may also be true for the control of amino acid transport systems. However, at present there is no direct proof of turnover of hexose or amino acid carriers. Nevertheless, it is striking that the regulation of two independent transport systems is so similar and therefore, whatever the mechanism, it may be more general than previously was anticipated (1, 2, 6).

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