Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 1126–1129, February 1984
Cell Biology

Further clues concerning the vectors essential to regulation of hexose transport, as studied in fibroblast cultures from a metabolic mutant

(phosphoglucose isomerase mutant/oxidative metabolism/glucose and mannose/UDP hexose/cycloheximide)

HERMAN M. KALCKAR AND DONNA B. ULLREY

Department of Chemistry, Boston University, CLA., Boston, MA 02215

Contributed by Herman M. Kalckar, October 24, 1983

ABSTRACT **A close study of the metabolic regulation of hexose transport in a hamster fibroblast mutant, highly defective in the enzyme phosphoglucose isomerase (PGI mutant), reveals the requirement for at least three vectors for transport regulation. The downward regulation of the hexose transport system, called the "transport curb," requires (*i*) a ligand for the transport system, (*ii*) oxidative energy metabolism, and (*iii*) some specific enzymes of the glucose-6-phosphate metabolism. Deprivation of glucose was shown to deprive the PGI mutant of UDP hexose, whereas the glucose-fed mutant contained high levels. The parental strain preserved the UDP hexose with or without glucose feeding. Cycloheximide added to the mutant showed two different types of effects. If added at the onset of glucose starvation, the up-regulation of the transport system was scarcely affected. If cycloheximide was added to the mutant at the onset of glucose refeeding, it prevented the development of the glucose-mediated transport curb. In the mutant, the glucose-mediated curb is not derived from energy metabolism but is solely dependent on certain enzymes of glucose-6-phosphate metabolism. The interference of this curb by cycloheximide requires evidently a reassessment, including that of the role of the UDP hexose pathway in regulation of the hexose transport system.**

From our previous studies on the mediated hexose transport down-regulation in cultured mammalian fibroblasts, we have been able to disclose a number of essential vectors for the onset of this type of regulation. This search has been greatly facilitated by maintaining the hamster fibroblast cultures for 24 hr in media (with or without serum) devoid of L-glutamine and pyruvate and varying the hexoses (1, 2). The mediated down-regulation of the hexose transport system shall be named the transport "curb." We have found the following requirements for mediating a transport curb of the hexose transport system.

(*i*) One ligand of the hexose transport system, be it glucose, galactose, mannose, or some of the nonmetabolizable analogues of glucose, must be present to initiate a transport curb. Fructose is not a ligand and is unable to initiate a curb (3).

(*ii*) Metabolites able to generate oxidative energy metabolism are needed. Inhibitors of oxidative energy metabolism (2,4-dinitrophenol, malonate) release the transport curb (3–5).

(*iii*) Glucose and glucose-6-phosphate metabolism, different from general energy metabolism [as disclosed from observations on a mutant DS7-defective phosphoglucose isomerase, PGI mutant (6)], generate vectors essential for the transport curb (2, 5).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

The release of the transport curb by glucose starvation of the PGI mutant seems not to be as consistently unaffected by the presence of cycloheximide (CHX) as described in the case of the parental line (7).

The restoration of the transport curb by refeeding glucose to starved cultures of the PGI mutant turns out to be abolished in the presence of CHX, reminiscent of the response observed in NIL fibroblasts (8, 9).

In this article we focus mainly on points *ii* and *iii* and on the restoration of the transport curb.

It seems well established that oxidative energy metabolism is needed for the development of the curb of the hexose transport system. This was observed not only for transport in 3T3 and NIL fibroblasts (4) but it also seems to apply to the lung fibroblast lines DS-7, the PGI mutant, and its parental strain 023 (2, 5).

If the PGI mutant was maintained in media deprived of L-glutamine as well as pyruvate, neither glucose nor mannose added separately was able to promote a curb. However, if the hexoses were added together, the mutant did respond with a marked curb (2).

The mutant was found to generate mere traces of lactate from glucose; in contrast, mannose permitted the generation of large amounts of lactate (2). Surprisingly enough, if the mutant was incubated with both of the hexoses, lactate generation from mannose was practically eliminated or at least severely curtailed by glucose or, more likely, by the accumulated glucose 6-phosphate. The phenomenon is reminiscent of related types of interferences between the two hexoses.* It is also reminiscent of the interference of hexokinase activity on glucose and 2-deoxyglucose observed in this mutant (6).

The case of the severe interference of lactate generation from mannose by the simultaneous presence of glucose, eliminating >97% of the lactate production (2) and yet preserving the transport curb, might pose another question. Does the transport curb invariably depend on energy generation? This in turn raises a second question. Is lactate production really a reliable barometer for assessing the extent of curtailment of hexose phosphorylation and the further oxidative energy generation? It is probably not, because lactate production seems to be a function of overproduction of fructose mono- and diphosphate. If oxidative metabolism is essential for the curb, addition of malonate or other inhibitors of oxidative energy metabolism should "release" the curb

Abbreviations: CHX, cycloheximide; PEI, phosphoglucose isomerase; 3-*O*-MeGlc, 3-*O*-methylglucose.

*Sols *et al.* (10) have reported that honey bees possess PGI but only traces of phosphomannose isomerase. In this organism, feeding mannose brings about an accumulation of mannose 6-phosphate that apparently interferes with the phosphorylation of glucose, a crucial step in the nutrition of honey bees. This may be the basis for the toxicity of mannose in honey bees (cf. ref. 10).

(3). The case under scrutiny has been examined in the present article.

One of the most intriguing questions that has arisen from our studies on the metabolically mediated transport curb in the PGI mutant is the specific requirement for glucose over and above mannose (2). Both are ligands of the hexose transport system; hence, one would expect that mannose, which is able to replace L-glutamine as an energy resource (2), would qualify as a metabolite in the mediated curb. Yet, the mutant needs glucose as well, either via the specialized catabolism, the glucose-6-phosphate pentose shunt, or the anabolic pathway, yielding UDP-glucose and UDP-galactose. In which one of the two pathways and at which step is the crucial enzyme involved? Is the crucial enzyme dependent on protein synthesis? In the present paper, the effect of CHX on the development of the glucose-mediated transport curb in the mutant is addressed.

MATERIALS AND METHODS

Cell Cultures. Chinese hamster lung fibroblasts of the following lines were used. DS-7, a cell line with a marked defect in the enzyme PGI (PGI^-), also referred to as "the PGI mutant,"† was compared with its parental line, called 023 (PGI^+) (for references to DS-7 and 023, see refs. 5, 6). The fibroblast lines were grown and maintained on modified Dulbecco's modified Eagle's medium (DME medium) as described (2, 5). Prior to the transport tests, the cultures were maintained with or without glucose or with different hexoses, as stated. The maintenance period lasted from 16 to 20 hr, in some cases followed by a second incubation period, as stated. In some samples, L-glutamine was omitted.

Galactose Uptake Test. The galactose uptake test was performed as follows. Cultures were rinsed three times with 37°C phosphate-buffered saline (pH 7.2) (P_i/NaCl). They were then incubated with [^{14}C(U)]galactose (0.1 mM; 0.2–0.5 μCi/ml; 1 Ci = 37 GBq; New England Nuclear, NEC 520) for 10 min at 37°C. L-[^{3}H]glucose (NET 456) was added to the labeled galactose as usual to test for completeness of washing. After the uptake test, cultures were rinsed three times with chilled P_i/NaCl and then extracted with 70% ethanol for 10 min or more. An aliquot of the ethanol extract was assayed for radioactivity in a scintillation counter.

3-*O*-Methylglucose Transport. (3-*O*-MeGlc). Before the transport test, the cells were preloaded with nonradioactive 3-*O*-MeGlc (5). Cells were rinsed three times with sugar-free and serum-free DME medium. They were then incubated for 30 min at 37°C with serum-free and glucose-free DME medium containing 50 mM 3-*O*-MeGlc. The cultures were then rinsed rapidly, at least three times at 22°C with P_i/NaCl and then given 3-*O*-[^{14}C]MeGlc (1 μCi/ml; NEC 647), 3 μM, usually for 20 sec at 22°C. [^{3}H]Glucose (NET 456) was also present to check the rinsing of the cultures, which were finally rinsed rapidly with ice-cold P_i/NaCl, then extracted with 70% ethanol, and assayed for radioactivity in the scintillation counter.

The use of labeled 3-*O*-MGlc is preferable to the use of labeled galactose as a transport test, especially when dealing with cell cultures in which the carbohydrate source is galactose.

Lactic Acid Determination. Lactic acid determination was carried out by the lactic acid dehydrogenase method (Sigma, no. 826 UV). Because the fibroblast cultures were well anchored, it is expressed as μmol/mg of cell protein.

UDP Hexose. UDP hexose was determined by an enzymatic method previously described (12); the spectrofluorometer used was Perkin Elmer model MPF 44 with PE recorder 56.

Protein Determination. Protein determination was carried out by a modified Lowry technique (3).

CHX. CHX was used in two concentrations (7 and 35 μM) according to previous observations (8, 9), as specified in the legend to Table 3.

RESULTS

We shall first address the question regarding the restored transport curb observed in the PGI mutant, maintained on curtailed medium (i.e., devoid of L-glutamine and pyruvate) by the combination of glucose and mannose (2). As mentioned in the Introduction, the simultaneous presence of glucose renders the lactate production from mannose much lower, in some cases bringing it to a virtual standstill (2). To ascertain that this type of transport curb is indeed an energy-dependent curb and not merely an unspecific inhibition of the transport system by mannose, we examined the effect of malonate on the combined transport curb and on lactate production. Because the PGI mutant has a very low consumption of glucose (6), 2 mM was considered a sufficient excess over consumption. In contrast, the mutant offered 20 mM mannose consumed large amounts, generating a large proportion as lactic acid. In this case, 2 mM glucose decreased the amount of lactate formed from 20 mM mannose down to 20% of the amount generated by mannose alone (see Table 1).

The transport curb that ensued from addition of 2 mM glucose to 20 mM mannose seemed substantial; the transport went down to 40% of that which was recorded with mannose or glucose separately. This curtailment in the transport rate is not due to a simple inhibition of the hexose transport system, because the further presence of malonate abolished most of the curtailment of this system, partly restoring uncontrolled transport (see Table 1). The lactate formation in the presence of malonate increased, as would be expected if the tricarboxylate cycle is inhibited. The data indicate that lactate generation is not involved with the transport curb but that the tricarboxylate cycle is a likely source in this case as well.

Interference with lactate generation from mannose in the PGI mutant can be avoided if galactose is replacing glucose during the feeding period. In this case, the transport test has to be conducted with labeled 3-*O*-MeGlc because the transport test with labeled galactose is apt to be affected by the possible accumulation of galactose 1-phosphate during the feeding period. From Table 2, it can be seen that galactose was able to supplement mannose in promoting a transport curb. Because galactose is phosphorylated through the action of galactokinase, one is exempt from the competition of

Table 1. Transport curb (galactose) and lactate formation in the PGI mutant, maintained in medium devoid of L-glutamine and pyruvate

Substrate*	[^{14}C]Galactose uptake, nmol/mg of protein†	Lactate formed, μmol/mg of cell protein‡
Mannose	3.45	33.70
Glucose	3.39	1.60
Mannose and glucose	1.38	6.83
Mannose, glucose, and malonate	2.68	11.84

The concentrations of substrates were: 20 mM mannose, 2 mM glucose, and 25 mM malonate.
*Over 20 hr at 37°C.
†10 min at 37°C.
‡20 hr at 37°C.

†A variant of DS-7, called DS7-T (11), T referring to higher tumorigenicity compared with DS-7 (11), seemed to manifest the same type of PGI defect as DS-7 in culture (ref. 11 and unpublished data). Neither DS-7 nor DS7-T proliferated as rapidly as the parental 023 strain (unpublished data).

Table 2. Transport curb (3-*O*-MeGlc) and lactate formation by the PGI mutant, maintained in medium devoid of L-glutamine and pyruvate

Substrate*	3-*O*-[^{14}C]MeGlc transport, pmol/mg of protein†	Lactate formed, μmol/mg of cell protein‡
Galactose	9.62	0.46
Mannose	6.15	29.79
Galactose and mannose	3.12	24.42

The concentrations of substrates were: 20 mM galactose and 20 mM mannose.
*Over 20 hr at 37°C.
†20 sec at 23°C.
‡20 hr at 37°C.

the two hexoses for the same kinase. This is probably the reason why the joint presence of mannose and galactose did not interfere with lactate generation from mannose (Table 2). Yet, galactose was almost as competent as glucose in the test for the transport curb.

As mentioned in the Introduction, the fact that the establishment of a transport curb in the PGI mutant maintained in the absence of L-glutamine requires, besides mannose, glucose or galactose raises some new problems that probably could only have been disclosed through the observations on the mutant.

The following experiments on the PGI mutant were designed. The mutant was maintained in minimal essential medium (ME medium) without glucose; the medium contained 4 mM L-glutamine. The effects of CHX on the regulation of the hexose transport system at the onset of glucose starvation as well as at the refeeding period were ascertained.

The course of the deregulation was reminiscent of the starvation response of hamster fibroblast cultures (7–9). CHX was permitting some deregulation of the transport system (data not shown). Conversely, CHX was markedly counteracting the curb mediated by refeeding glucose to the mutant (see Table 3, two-phase incubation).

The transition of the state of glucose starvation and the deregulated state of the transport system to the state of refeeding and the glucose-mediated transport curb was very well expressed (Table 3). It should be remembered that in the PGI mutant, the role of glucose is different from that of energy supply. Yet, much like the response by NIL fibroblast cultures (9), the addition of CHX at this stage counteracted the glucose-mediated transport curb.

In the PGI mutant, glucose feeding is still able to set in motion the first steps of the glucose-6-phosphate dehydrogenase pentose shunt (the later steps might also be sustained by fructose). Of no less importance are the anabolic pathways of glucose 6-phosphate—i.e., mainly the enzymatic synthesis of UDP-glucose and UDP-galactose. In this mutant, these important glucosyl and galactosyl donors have to

Table 3. Effect of CHX on the glucose-mediated transport curb in the PGI mutant, maintained in a medium containing L-glutamine

One-phase incubation, 16 hr		Two-phase incubation, 16 hr/8 hr	
Medium	3-*O*-[^{14}C]MeGlc transport, pmol/mg of cell protein*	Medium	3-*O*-[^{14}C]MeGlc transport, pmol/mg of cell protein*
Glucose	8.86	0/Glucose	8.10
0	25.43	0/Glucose and CHX	17.41
		0/0	25.46

ME medium with 4 mM L-glutamine, with or without glucose (termed "glucose" or "0," respectively), incubated 16 hr at 37°C. In the two-phase incubation, glucose-starved cultures (for 16 hr) were fed 8 hr: glucose, 0/glucose; if CHX (35 μM) was also present (cf. ref. 9), 0/glucose and CHX; if starvation was continued 16 + 8 hr, 0/0. See text for discussion of the transport test.
*3 μM 3-*O*-[^{14}C]MeGlc; 20 sec at 23°C.

be synthesized exclusively from glucose. However, if the mutant should contain traces of PGI activity, it would show up if a sensitive test for UDP-glucose were used. According to Pouyssegur *et al.* (6), the parental fibroblast line 023 contains 40 times higher PGI activity than that of hexokinase. The authors list the PGI activity in the mutant as less than a factor of 0.05 (i.e., <5 nmol/min per mg) compared with the parental type (6). A leakiness of 1–2 nmol/min per mg might still show up if the mutant is incubated several days with fructose instead of glucose. Using the sensitive histochemical test for glucose-6-phosphate dehydrogenase, Franchi *et al.* (11) were unable to find traces of staining in the mutant if incubated 24 hr with fructose 6-phosphate, but they found rapid and marked staining if glucose 6-phosphate was used. We have confirmed their observations (unpublished data).

We next decided to try to detect traces of leakiness in PGI activity by comparing UDP-glucose and UDP-galactose levels in the mutant fed fructose and glucose over 72 hr and to examine the levels of the same nucleotides in the parental strain fed the two different hexoses. The fluorometric analyses performed by means of two highly specific enzymes, UDP-glucose dehydrogenase and UDP-glucose epimerase, are summarized in Table 4. Fructose-fed PGI$^-$ cells contained only traces of UDP hexose.

DISCUSSION

In the present paper, we have shown that glucose, in spite of its confined role in the uptake regulation in the PGI mutant, is able to initiate a delayed transport or uptake curb. Moreover, this transport curb was annulled by the addition of CHX (Table 3). However, the present design does not permit us to make any specific statements about the nature of the constituents that need to be "rekindled" by protein synthesis.

Table 4. Effect of glucose or fructose feeding on cellular UDP hexose levels and on galactose uptake regulation in PGI$^-$ and PGI$^+$ fibroblast cultures

		UDP hexose†		[^{14}C]Galactose uptake‡	
Strain	Medium*	nmol/mg of cell protein	Relative level	nmol/mg of cell protein	Relative level
DS-7, PGI$^-$	Glucose	1.63	1.00	1.25	1.00
DS-7, PGI$^-$	Fructose	0.24	0.15	4.55	3.64
023, PGI$^+$	Glucose	2.28	1.00	1.27	1.00
023, PGI$^+$	Fructose	1.82	0.80	2.86	2.25

*ME medium (4 mM L-glutamine) with glucose or fructose for 72 hr.
†UDP hexose determination according to ref. 12, using UDP-glucose dehydrogenase and NAD and subsequently UDP-glucose epimerase.
‡10 min at 37°C.

In the design, the first 16 hr of incubation were performed in the presence of L-glutamine, a good generator of energy metabolism (13, 14, 15), but left the hexose carriers without ligands and the enzymes of the glucose-6-phosphate pathways without the corresponding substrates. The biosynthesis of the hexose carriers seems to develop even faster in the absence of glucose (5). However, the lack of substrates for the glucose-6-phosphate pathways and their respective enzymes may create a demand for resynthesis of some of the enzymes of the catabolic or anabolic pathways.

In trying to explore the requirements for restoring the biosynthesis of the enzymes of the UDP hexose system, one might proceed according to the findings illustrated in Table 4. Such a program would involve assays of the UDP hexose levels in the mutant, 4–5 hr after re-initiation of the glucose-mediated transport curb. This should be matched with corresponding UDP hexose assays in the incubates containing glucose + CHX (cf. Table 3).

Additional steps in the catabolic or anabolic pathways of glucose-6-phosphate metabolism will also have to be assessed (including glycogen levels) as to their importance in the development of the glucose-mediated transport curb.

Addendum. The experiment illustrated in Table 3 presents similar features, if conducted in the absence of L-glutamine but with preincubation with mannose, succeeded by addition of glucose. A transport curb also ensued if the opposite sequence was followed—i.e., preincubation with glucose, followed by addition of mannose. CHX counteracted either type of curb. It remains an intriguing question which condition represents the "fed" state of the plasma membrane of the PGI mutant.

Our thanks are due to Dr. Jacques Pouyssegur and his staff for sending us the variant fibroblast culture DS7-T in addition to the DS-7 and 023 Chinese hamster lung fibroblast cultures, all isolated at Centre de Biochimie, Université de Nice, France. This work was supported by National Science Foundation Research Grant PCM-8302034.

1. Kalckar, H. M., Ullrey, D. B. & Laursen, R. A. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5958–5961.
2. Ullrey, D. B. & Kalckar, H. M. (1982) *Biochem. Biophys. Res. Commun.* **107,** 1532–1538.
3. Ullrey, D. B. & Kalckar, H. M. (1981) *Arch. Biochem. Biophys.* **209,** 168–174.
4. Kalckar, H. M., Christopher, C. W. & Ullrey, D. B. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 6453–6455.
5. Ullrey, D. B., Franchi, A., Pouyssegur, J. & Kalckar, H. M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3777–3779.
6. Pouyssegur, J., Franchi, A., Salomon, M. C. & Silvestre, P. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2698–2701.
7. Franchi, A., Silvestre, P. & Pouyssegur, J. (1978) *Biochem. Biophys. Res. Commun.* **85,** 1526–1534.
8. Christopher, C. W., Ullrey, D. B., Colby, W. & Kalckar, H. M. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2429–2433.
9. Christopher, C. W., Colby, W. & Ullrey, D. B. (1976) *J. Cell. Physiol.* **89,** 683–692.
10. Sols, A., Cadenas, E. & Alvarado, F. (1960) *Science* **131,** 297–298.
11. Franchi, A., Silvestre, P. & Pouyssegur, J. (1981) *Int. J. Cancer* **27,** 819–827.
12. Ullrey, D. B., Kalckar, H. M. & Christopher, C. W. (1978) *J. Cell. Physiol.* **96,** 23–30.
13. Donnely, M. & Scheffler, I. E. (1976) *J. Cell Physiol.* **89,** 39–52.
14. Reitzer, L. J., Wice, B. M. & Kennell, D. (1980) *J. Biol. Chem.* **255,** 5616–5626.
15. Sumbilla, C. M., Zielke, G. L., Reed, W. D., Ozand, P. T. & Zielke, H. R. (1980) *Biochim. Biophys. Acta* **675,** 301–304.