Differential control of the functional cell surface expression and content of hexose transporter GLUT-1 by glucose and glucose metabolism in murine fibroblasts

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The present paper evaluates the contributions of glucose and its metabolites to the post-translational regulation of hexose transport and GLUT-1 content in murine fibroblasts. The effects of 3-*O*-methylglucose, a nearly non-metabolizable glucose analogue, on 2-deoxyglucose-uptake, cell-surface expression and content of GLUT-1, glucose 6-phosphate levels, and phosphoglucose isomerase (PGI) and hexokinase activities of murine fibroblasts were compared with those of glucose and fructose. Glucose ($EC_{50} \sim 6$ mM) or 3-*O*-methylglucose ($EC_{50} \sim 12$ mM), which are substrates of GLUT-1, but not fructose, which is not transported by GLUT-1, are able to prevent the glucose-deprivation-induced increases in both hexose transport and cell-surface expression of GLUT-1. In contrast, glucose ($EC_{50} \sim 6$ mM), but not 3-*O*-

methylglucose or fructose, prevents the glucose-deprivation-induced accumulation of total GLUT-1 polypeptides. Glucose (≥ 5 mM), but not fructose or 3-*O*-methylglucose, leads to significant glucose 6-phosphate accumulation. Although 3-*O*-methylglucose is weakly phosphorylated by fibroblasts, accumulation of phosphorylated product does not correlate with hexose-transport regulation. The activities of hexokinase and PGI are not altered by glucose, fructose or 3-*O*-methylglucose. We suggest that, in murine fibroblasts: (i) hexose transport and GLUT-1 content are differentially regulated; (ii) substrates of GLUT-1 and/or their immediate metabolites regulate the cell-surface expression of functional GLUT-1; and (iii) glucose metabolism is required for the regulation of GLUT-1 content.

INTRODUCTION

The adaptive regulation of hexose transport by substrate in mammalian cells is a well-studied phenomenon [1–11]. Control of this process has been shown to involve the availability of free glucose [1,12,13], glucose metabolism [1,12–15] and overall energy metabolism [8,15–17]. The relative roles of these individual components have, however, not been thoroughly elucidated. Mechanistically, the long-term regulation of hexose transport by glucose has been reported to alter the synthesis [5,6,8–10,18] and/or degradation [1,4–6,10,11,19–21] of some glucose transporter isoforms. The molecular basis of this chronic regulation varies with species and tissue of origin [1–11]. Regulation of the localization [2,5,21–23] and/or intrinsic activity [5,20,24] of hexose transporters in these phenomena has also been reported.

We have focused on the regulation of hexose transport by glucose deprivation in rodent fibroblasts [1,2,20]. These fibroblasts express only hexose transporter isoform GLUT-1 (brain/human erythrocyte type). These cells do not express GLUT-2 (liver/pancreatic β -cell type), GLUT-4 (muscle/adipose type), GLUT-3 (neuronal type), or GLUT-5 (gut/testes type) [2,20,25; P. A. Ortiz and H. C. Haspel, unpublished work]. In these cells, the increase in hexose transport and accumulation of hexose transporter GLUT-1 induced by glucose deprivation are post-translationally regulated at the level of GLUT-1 turnover [1,2,20] and subcellular localization [2]. The drastic alterations in protein glycosylation observed in glucose-starved (i.e. ‘stressed’) fibroblasts [26,27,28], which result in the appearance and accumulation of ‘aglyco’ GLUT-1 and this more subtle regulation of GLUT-1 by glucose deprivation have been dissected

[1,2,20,29]. We have compared the effects of metabolizable (i.e. mannose, galactose and 2-deoxyglucose) and nearly non-metabolizable (i.e. 3-*O*-methylglucose) glucose analogues on the accumulation of GLUT-1 in glucose-deprived murine fibroblasts. Our findings suggested that the transported sugar itself, as well as early steps in glucose metabolism, are involved in this post-translational regulation of GLUT-1 [1]. In a conceptually similar manner, the oxohexose fructose, which is not transported by GLUT-1 [30] and enters the glycolytic pathway at a step subsequent to aldohexoses such as glucose [31], does not prevent the glucose-deprivation-induced increase in hexose transport and accumulation of GLUT-1 [1,2]. Using phosphoglucose isomerase (PGI)-deficient hamster fibroblasts, Kalckar and co-workers [3,4,15,32] reported that changes in early steps in glucose metabolism can ‘curb’ the glucose-deprivation-induced increase in hexose transport. In contrast, Germinario et al. [13] used various glucose analogues to suggest that control of hexose transport in human fibroblasts is independent of both glucose metabolism and carrier–substrate interaction. More recently Maher and Harrison [18], using cultured L6 myocytes, in which a component of GLUT-1 regulation is at the transcriptional level, have suggested that GLUT-1 gene expression and hexose transport are regulated by independent pathways. In preliminary studies of the effects of various sugars and their analogues on the regulation of GLUT-1 by glucose deprivation, we also found indirect evidence in support of different mechanisms regulating hexose transport and GLUT-1 content in murine fibroblasts (P. Ortiz and H. C. Haspel, unpublished work). All previous studies on glucose regulation of GLUT-1 did not, however, differentiate between the effects of carrier–substrate interaction

Abbreviations used: ATB-BMPA, 2-*N*-[4-(1-*az*i-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(*D*-mannos-4-*yl*oxy)propyl-2-amine; α -CT, C-terminal antiserum to GLUT-1; PGI, phosphoglucose isomerase.

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and glucose metabolism on transporter content and activity because the cellular content of glucose metabolites was not examined directly. This is important, because conditions which effect glucose metabolism, such as altered glycemic state, may change the low intracellular concentrations of glucose by altering the steady-state levels of its immediate metabolites (e.g. glucose 6-phosphate). Here we compare the effects of glucose, fructose and 3-*O*-methylglucose on the regulation of hexose transport, cell-surface expression and content of GLUT-1, glucose 6-phosphate levels, and hexokinase and PGI activities in murine fibroblasts. We demonstrate that hexose transport and GLUT-1 content are differentially regulated by glucose metabolism in murine fibroblasts.

EXPERIMENTAL

Materials

Radioisotopes were purchased from Amersham (Arlington Heights, IL, U.S.A.). 2-N-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(*D*-mannos-4-yloxy)propyl-2-amine (ATB-BMPA) and ATB-[2-³H]BMPA (~ 10 Ci/mmol) were kindly provided by Dr. Geoffrey Holman (University of Bath, Bath, U.K.) and were synthesized as described previously [47]. Glucose 6-phosphate dehydrogenase, yeast hexokinase, NADP, 3-*O*-methylglucose, glucose, glucose 6-phosphate, 2-deoxyglucose, fructose, phloretin, and dialysed calf serum were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Calf intestinal alkaline phosphatase was from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The 3-*O*-methylglucose is certified to be glucose-free by the manufacturer, and our glucose-free medium (see under 'Cell culture and treatments' below) containing 25 mM-3-*O*-methylglucose was found to contain ≤ 0.5 mg-glucose/l when analysed on a Beckman ASTRA analyser [1]. The antiserum, α -CT (C-terminal antiserum to GLUT-1), was elicited to a synthetic peptide corresponding to the 13 C-terminal amino acid residues of rat GLUT-1 [33]. The preparation, properties and specificity of this antiserum have been described in detail elsewhere [33].

Cell culture and treatments

Murine 3T3-C2 fibroblasts were cultured as previously described [1]. Cells were grown to confluence in high glucose (4.5 g/l) Dulbecco's modified Eagle's medium containing non-essential amino acids, 2 mM-glutamine and 9% (v/v) heat-inactivated calf serum with a humidified CO₂/air (1:19) atmosphere at 37 °C. After a 12–24 h refeeding, confluent cells were rinsed and again fed for 24 h in similar medium containing no glucose and dialysed calf serum (i.e. ≤ 0.5 mg-glucose/l) [1]. This medium was supplemented with various sugars when indicated. After the indicated time, membranes were isolated for immunoblotting, appropriate extracts prepared for glucose-6-phosphate/hexokinase/PGI determinations, photolabelling with ATB-BMPA performed, or hexose transport was assayed. Care was taken to use only quiescent (i.e., 1-day post-confluence monolayers) cells for these studies because GLUT-1 expression is very tightly regulated by growth [34,35]. Care was also taken to maintain the concentration of glutamine in the culture medium because hexose transport in cultured cells has been reported to be regulated by overall energy metabolism [3,4]. It has been demonstrated that glutamine can support overall energy metabolism [i.e., no drastic alteration of ATP/ADP and NAD(P)/NAD(P)H ratios] in cultured cell lines (i.e., HeLa cells and mouse L-cells), even in the absence of glucose [36–38].

Hexose transport

[³H]2-deoxyglucose uptake by fibroblast monolayers was assayed as previously described [20]. Cell monolayers were rinsed and preincubated (15 min at 23 °C) in assay buffer (140 mM NaCl/20 mM Hepes/1 mM CaCl₂/5 mM KCl/2.5 mM MgSO₄, pH 7.4). Uptake is initiated by addition of [³H]2-deoxyglucose (final concn. 0.2 mM; 0.5 μ Ci/ml) and terminated after 10 min at 23 °C by washing in iced PBS. Cells were solubilized in 1% (w/v) SDS, and cell-associated radioactivity and protein were determined. 2-deoxyglucose-uptake was linear for ≥ 20 min.

Cell-surface expression of GLUT-1

Cell-surface expression of GLUT-1 in washed cell monolayers was determined by photolabelling with ATB-[2-³H]BMPA, immunoprecipitation of GLUT-1 from detergent extracts of the cells, SDS/PAGE of the immunoprecipitates, and determination of radioactivity in gel slices by liquid-scintillation counting as described previously [48]. Values were normalized to the protein content of cell extracts.

GLUT-1 content

Membrane isolation, protein assays, and immunoblotting of GLUT-1 were performed as previously described [1]. Cell monolayers were rinsed with iced PBS, collected by scraping into iced sonication buffer (5 mM EDTA/250 mM sucrose/50 mM NaF/1 mM phenylmethane sulphonyl fluoride/100 mM sodium phosphate, pH 7.4) (1 ml), and sonically lysed (30 s at 75 W) at 4 °C. The lysates were centrifuged at 550 *g* for 5 min at 4° and the post-nuclear supernatant centrifuged at 200 000 *g* for 22 min at 4 °C. The crude post-nuclear membrane pellets were resuspended in iced membrane buffer (1 mM EDTA/250 mM sucrose/1 mM phenylmethane sulphonyl fluoride/5 mM sodium phosphate, pH 7.4) (50–100 μ l), and 35 μ g samples of membrane protein were subjected to SDS/11%-PAGE. The resolved proteins were electrophoretically transferred to nitrocellulose, the blots probed with α -CT (1:100 dilution), and bound IgG was detected with ¹²⁵I-protein A (~ 5 $\times 10^5$ c.p.m./ml). Dried blots were subjected to autoradiography with an intensifying screen at –80 °C.

Glucose 6-phosphate determinations

Glucose 6-phosphate content was assayed fluorometrically [39]. Perchloric acid-treated extracts of cells were clarified by centrifugation and neutralized with K₂CO₃. The glucose 6-phosphate content in aliquots of these samples was assayed in NADP⁺-linked reactions employing glucose 6-phosphate dehydrogenase. The assay was standardized with known amounts of glucose 6-phosphate and the values were normalized to total cellular protein. The assay has a detection limit of ~25 pmol of glucose 6-phosphate.

Enzyme activities

Hexokinase and PGI activities were assayed spectrophotometrically [40]. Aliquots of cytosol were assayed for their ability to phosphorylate glucose or catalyse the conversion of fructose 6-phosphate into glucose 6-phosphate, for hexokinase or PGI respectively. The values were normalized to the protein content of the cytosol to calculate the specific activities.

Phosphorylation of 3-*O*-methylglucose

The phosphorylation of 3-*O*-methylglucose by murine fibroblasts was determined by the method of Klienzzler and McAvoy [46] as detailed by Gould et al. [30]. Murine fibroblasts were incubated

Table 1 Phosphorylation of 3-*O*-methylglucose by murine fibroblasts and 3-*O*-methylglucose content of murine fibroblasts after washing and preincubation for assay of hexose transport

Confluent fibroblasts were cultured, fed, and refed different concentrations of [^3H]3-*O*-methylglucose (10 $\mu\text{Ci/ml}$) for 24 h as indicated. Cells were either washed with iced PBS containing 0.2 mM-phloretin and extracts prepared immediately ('Total 3-*O*-methylglucose uptake') or after rinsing and preincubation in hexose-transport assay buffer (15 min at 23 °C) ('3-*O*-methylglucose remaining'). 'Phosphorylated 3-*O*-methylglucose' was calculated after subtraction of the $\text{ZnSO}_4/\text{Ba}(\text{OH})_2$ -soluble fraction, non-phosphorylated, from total 3-*O*-methylglucose uptake. Individual determinations from two similar experiments are presented.

[3- <i>O</i> -methylglucose] (mM)	Total 3- <i>O</i> -methylglucose uptake (nmol/mg of protein)	Phosphorylated 3- <i>O</i> -methylglucose (% precipitable)	3- <i>O</i> -methylglucose remaining (% of total uptake)
3	34	12	< 2
10	118, 115	13, 18	< 2
25	266, 296	9, 16	< 2

with 3–25 mM [^3H]3-*O*-methylglucose (10 $\mu\text{Ci/ml}$) for 24 h and either immediately washed with iced PBS containing 0.2 mM phloretin, or rinsed, preincubated in hexose-transport assay buffer (15 min at 23 °C), and then washed. Cells were harvested by trituration with 90 °C water and aliquots were removed for protein, total cell-associated 3-*O*-methylglucose and non-phosphorylated 3-*O*-methylglucose determinations. Non-phosphorylated 3-*O*-methylglucose was determined in aliquots of supernatants from $\text{ZnSO}_4/\text{Ba}(\text{OH})_2$ precipitations [30]. Less than 2% of the [^3H]3-*O*-methylglucose (1 mM; 1 $\mu\text{Ci/ml}$) not incubated with cells is precipitated in this assay (results not shown). Alkaline phosphatase (10 units/ml) treatment (24 h at 37 °C) of cell extracts [diluted 1:3 with 50 mM glycine (pH 10.5)/1 mM MgCl_2 /1 mM ZnCl_2] from cells incubated (24 h) with 25 mM [^3H]3-*O*-methylglucose (10 $\mu\text{Ci/ml}$) made soluble the small fraction (~10%; see Table 1 above) of precipitable radioactivity (results not shown). Incubation (24 h at 37 °C) of 1 mM [^3H]3-*O*-methylglucose (1 $\mu\text{Ci/ml}$) with yeast hexokinase (4 units/ml) in the presence of 2 mM ATP, 4 mM MgCl_2 , 2 mM dithiothreitol, 0.1 mM EDTA and 0.2 M Tes, pH 7, generated precipitable radioactivity (> 85%) which was made soluble by treatment with alkaline phosphatase (results not shown).

Quantification of results

GLUT-1 levels were determined from exposure intensities on the autoradiograms of the immunoblots. Multiple exposures were quantified on a Biomed (Fullerton, CA, U.S.A.) scanning laser densitometer using a program employing two-dimensional analysis and area-normalized background subtraction. Transport results were normalized to control values, set to 100%, for each experiment. The percentage changes from control values for each condition are reported. The variation in hexose transport for controls is < 10% [20]. Glucose 6-phosphate levels were determined in duplicate and are reported as nmol/mg of cellular protein for individual experiments. Individual values for the cell surface expression of GLUT-1 are reported as percentage change in total radioactivity associated with photolabelled GLUT-1 (c.p.m./mg of cellular protein) relative to control values set to 100% for each experiment.

RESULTS

Hexose transport and glucose metabolism

The effects of either glucose, fructose, 3-*O*-methylglucose, or

fructose and 3-*O*-methylglucose on hexose transport by murine fibroblasts were examined (Figure 1). 3T3-C2 cells were incubated with the concentrations of sugars as indicated for 24 h and [^3H]2-deoxyglucose uptake was determined. Glucose or 3-*O*-methylglucose, but not fructose, prevent the increase (~3.5-fold) in hexose transport observed with glucose deprivation. The EC_{50} values for glucose and 3-*O*-methylglucose were 6 mM and 12 mM respectively. Fructose does not alter the ability of 3-*O*-methylglucose to down-regulate hexose transport.

In agreement with previous studies in human fibroblasts [13], these changes are not due to competitive inhibition of transport by residual 3-*O*-methylglucose. First, residual 3-*O*-methylglucose measured in cells incubated for 24 h with 3–25 mM [^3H]3-*O*-methylglucose (10 $\mu\text{Ci/ml}$) and processed as described for hexose-transport determinations amounted to < 2% of total 3-*O*-methylglucose uptake (Table 1). If complete equilibration with the hexose-transport assay buffer (1 ml) is assumed, then the residual 3-*O*-methylglucose would result in an effective extracellular concentration of < 6 μM . This concentration would not compete significantly for 2-deoxyglucose uptake. Secondly, although 3-*O*-methylglucose equilibrates rapidly at 37 °C [42], no changes in hexose transport are observed when cells are incubated for < 6 h with 25 mM 3-*O*-methylglucose (results not shown). The lack of a rapid effect is not consistent with the interpretation that the effects of 3-*O*-methylglucose on hexose transport are simply due to temporary alterations. These results demonstrate that 3-*O*-methylglucose can replace glucose in preventing glucose deprivation enhanced hexose transport.

Cell-surface expression of GLUT-1 and glucose metabolism

The effects of glucose, fructose and 3-*O*-methylglucose on cell surface expression of GLUT-1 were examined (Table 2). 3T3-C2 cells were incubated with the indicated concentrations of sugars for 24 h and the photolabelling of cell surface GLUT-1 by ATB-BMPA was determined. Confirming our immunofluorescence studies in normal rat kidney cells [2], glucose deprivation (i.e., fructose-fed or fed no sugar) of murine fibroblasts induces an accumulation (~3-fold) of GLUT-1 on the cell surface. These

Figure 1 Effects of glucose, fructose, and 3-*O*-methylglucose on the increase in hexose transport induced by glucose deprivation of murine fibroblasts

3T3-C2 fibroblasts were grown to confluence and fed for 12–24 h before use. Cells were then refed glucose-free medium containing 0–25 mM glucose (○), 3-*O*-methylglucose (●), fructose (□), or 3-*O*-methylglucose plus 5 mM fructose (△) for 24 h. Hexose transport was then assayed, in triplicate, as [^3H]2-deoxyglucose uptake. The Figure shown is a composite of seven experiments, and individual determinations from each experiment are presented. For clarity, only four of seven points at 0 mM sugar are shown. The abscissa is a logarithmic scale.

Table 2 Effects of glucose, fructose, and 3-*O*-methylglucose on the cell surface appearance of functional GLUT-1 induced by glucose deprivation of murine fibroblasts

Confluent fibroblasts were cultured, fed and refed glucose-free medium containing 25 mM glucose, 5 mM fructose, 25 mM 3-*O*-methylglucose, or no sugars, for 24 h. Cell-surface expression of functional GLUT-1 was assessed by photolabelling with ATB-[2-³H]BMPA. Cell-surface expression of GLUT-1 was determined in duplicate experiments. Individual values are presented as a percentage change in total radioactivity associated with photolabelled GLUT-1 (c.p.m./mg of cellular protein) relative to control values set to 100% for each experiment. Control photolabelling for the two experiments presented is 2700 and 1624 c.p.m./mg of cellular protein respectively.

Sugar	Cell-surface GLUT-1 (% of control)
Glucose	100, 100
None	270, 260
Fructose	380
3- <i>O</i> -methylglucose	150, 130

Figure 2 Effects of glucose, fructose and 3-*O*-methylglucose on the accumulation of GLUT-1 polypeptides induced by glucose deprivation of murine fibroblasts

Confluent fibroblasts were cultured, fed and refed the indicated concentrations of sugars for 24 h as in Figure 1. Membranes were prepared and GLUT-1 polypeptides were detected by immunoblotting with α -CT (1:100 dilution). Autoradiograms representative of $\geq$ three similar experiments are depicted. The native (M_r 55 000) and algyco (M_r 38 000) GLUT-1 polypeptides are the upper and lower bands respectively.

results correlate well with the changes in hexose transport (Figure 1) and are consistent with translocation of GLUT-1 in response to glucose deprivation. Only one ATB-BMPA-labelled band of M_r 55 000 is observed under all conditions examined (results not shown). This result implies that the 'algyco' M_r 38 000 GLUT-1 polypeptide, which has been observed in glucose-starved fibroblasts [1,2,20] (see Figure 2), is either not translocated in response to glucose deprivation or is not efficiently photolabelled by ATB-BMPA. The resolution of GLUT-1 polypeptides with different M_r values by this methodology is, however, difficult. Significantly, 3-*O*-methylglucose and glucose both prevent the glucose-deprivation-induced increase in cell-surface GLUT-1 as measured by ATB-BMPA photolabelling. These results demonstrate that 3-*O*-methylglucose can replace glucose in preventing the cell-surface expression of functional GLUT-1.

GLUT-1 content and glucose metabolism

The effects of either glucose, fructose, 3-*O*-methylglucose, or fructose and 3-*O*-methylglucose, on the content of hexose transporter GLUT-1 of murine fibroblasts were examined (Figure 2). 3T3-C2 cells were incubated with the concentrations of sugars as

Table 3 Effects of glucose, fructose and 3-*O*-methylglucose on glucose 6-phosphate content of murine fibroblasts

Confluent fibroblasts were cultured, fed and refed the indicated concentrations of sugars for 24 h, as in Figure 1. Extracts were prepared and glucose 6-phosphate content was determined. The values are normalized to total cellular protein. Individual determinations from three similar experiments are presented.

Sugar	Concn. (mM)	Glucose 6-phosphate (nmol/mg of cellular protein)
None	—	< 0.13*
Glucose	25	1.89, 1.78, 2.11
	10	2.11, 2.31
	5	3.21, 2.64, 2.17
	2.5	< 0.13*
	1.0	< 0.13*
Fructose	25	0.16
	5	< 0.13*
3- <i>O</i> -Methylglucose	25	< 0.13*
	15	< 0.13*
	10	< 0.13*
	5	< 0.13*
3- <i>O</i> -Methylglucose/Fructose	25/5	< 0.13*

* These values are below the detection limits of the assay, and this was demonstrated at least twice for each condition.

indicated for 24 h, membranes were isolated, and GLUT-1 content was determined by immunoblotting with α -CT. As observed previously [1,2,20,29], lowering glucose concentration induces accumulation of total GLUT-1 polypeptides ($\sim$ 8-fold). This includes both 'native' (M_r 55 000) and 'algyco' (M_r 38 000) GLUT-1 polypeptides. Accumulation of total GLUT-1 polypeptides is half-maximal at $\sim$ 6 mM-glucose and the algyco-polypeptide is not observed at $>$ 5 mM-glucose. Fructose does not prevent the accumulation of native GLUT-1, although it does prevent the appearance of the algyco-polypeptide because this sugar can support oligosaccharide synthesis and GLUT-1 glycosylation [1,26].

Substituting 3-*O*-methylglucose for glucose does not prevent accumulation of both GLUT-1 polypeptides (Figure 2). This contrasts with the ability of 3-*O*-methylglucose to prevent the increases in hexose transport (Figure 1) and cell-surface expression of GLUT-1 (Table 2). The content of GLUT-1 increased but the algyco-polypeptide was not observed in cells fed fructose and 3-*O*-methylglucose. This is similar to that observed for fructose alone. These results demonstrate that 3-*O*-methylglucose, which is a substrate for the transporter, cannot prevent glucose-deprivation-induced accumulation of GLUT-1.

Effect of 3-*O*-methylglucose on glucose-6-phosphate levels and hexokinase and PGI activities

The effects of glucose, fructose, or 3-*O*-methylglucose on glucose 6-phosphate levels and hexokinase and PGI activities of murine fibroblasts were examined. 3T3-C2 cells were incubated with the concentrations of sugars as indicated for 24 h, appropriate cell extracts prepared, and the glucose 6-phosphate content determined (Table 3). In cells fed $<$ 5 mM glucose, the glucose 6-phosphate content was below the detection limits of the assay (i.e. $<$ 0.13 nmol/mg of cellular protein). In cells fed $\geq$ 5 mM glucose, the content of glucose 6-phosphate is 2.28 ± 0.43 nmol/mg of cellular protein (mean $\pm$ S.D., $n = 8$). In cells fed 25 mM fructose, the content of glucose 6-phosphate is $<$ 10% of

that observed in cells fed ≥ 5 mM-glucose. In cells fed 3-*O*-methylglucose with or without 5 mM fructose, the content of glucose 6-phosphate remained below detectable levels. The effect of 3-*O*-methylglucose on the glucose 6-phosphate content of cells fed glucose was not examined, because it would be difficult to correct for the competitive inhibition of glucose uptake by 3-*O*-methylglucose. The activities of hexokinase and PGI were not altered by glucose, fructose and/or 3-*O*-methylglucose feeding (results not shown). These results (Table 3 and results not shown) are in agreement with early work in HeLa and mouse L-cells [37]. Furthermore, 3-*O*-methylglucose does not inhibit hexokinase activity of control extracts *in vitro* (results not shown). These results are consistent with 3-*O*-methylglucose being unable to alter early steps in glucose metabolism.

Phosphorylation of 3-*O*-methylglucose by murine fibroblasts

Although levels of phosphorylated sugar were undetectable in fibroblasts incubated with 3-*O*-methylglucose (Table 3), we were still concerned the 3-*O*-methylglucose might be phosphorylated to a significant extent. Our glucose 6-phosphate assay might not be able to detect this putative product. The ability of murine fibroblasts to phosphorylate 3-*O*-methylglucose was, therefore, examined directly. 3T3-C2 cells were incubated with 3–25 mM [3 H]3-*O*-methylglucose (10 μ Ci/ml) for 24 h, appropriate cell extracts prepared, and $\text{ZnSO}_4/\text{Ba}(\text{OH})_2$ -precipitable radioactivity was determined (Table 1). $\text{ZnSO}_4/\text{Ba}(\text{OH})_2$ precipitation has been used to separate sugar phosphates in cell extracts [46,30]. The fraction of total cell-associated [3 H]3-*O*-methylglucose precipitated by the $\text{ZnSO}_4/\text{Ba}(\text{OH})_2$ treatment (i.e., phosphorylated 3-*O*-methylglucose) is $\sim 14\%$ and is independent of 3-*O*-methylglucose concentration. This is consistent with previous studies which indicate 3-*O*-methylglucose is a poor substrate of hexokinase both *in vitro* and *in vivo* [43–45]. These results demonstrate that long-term incubation (24 h) of cells with 25 mM 3-*O*-methylglucose results in a significant intracellular content of 3-*O*-methylglucose 6-phosphate (~ 35 nmol/mg of cellular protein). This level of sugar phosphate appears to greatly exceed the steady-state levels of glucose 6-phosphate (< 3 nmol/mg of cellular protein) found in cells incubated with ≥ 5 mM glucose. This demonstrates that although 3-*O*-methylglucose is generally considered to be nearly non-metabolizable, a relatively large amount of 3-*O*-methylglucose 6-phosphate accumulates under the conditions of our experiments.

DISCUSSION

Differential regulation of the cell-surface expression of functional GLUT-1 and GLUT-1 content

The EC_{50} values for the regulation of both hexose transport (Figure 1) and GLUT-1 content (Figure 2) by glucose are similar and correlate with the observed increases in the steady-state levels of glucose 6-phosphate (Table 3). Initially, we believed that this was consistent with GLUT-1 being regulated, at all levels, by a unitary mechanism. The results presented herein demonstrate, however, that the cell-surface expression of functional GLUT-1 and GLUT-1 content are differentially regulated by 3-*O*-methylglucose in murine fibroblasts. 3-*O*-methylglucose, a nearly non-metabolizable glucose analogue, prevents the enhancement of hexose transport and cell-surface expression of GLUT-1 which are induced by glucose deprivation (Figure 1 and Table 2), but does not prevent accumulation of GLUT-1 polypeptides (Figure 2). The inability of 3-*O*-methylglucose to alter GLUT-1 content is in agreement with our previous findings [1,2,20] and is

consistent with the hypothesis that glucose metabolism is involved in this aspect of GLUT-1 regulation.

3-*O*-methylglucose does not alter the electrophoretic mobility of GLUT-1 polypeptides observed in cells deprived of glucose, and it has no effect on the accumulation of GLUT-1 polypeptides, even in the presence of fructose (Figure 2). Fructose prevents the appearance of aglyco-GLUT-1 because it can support protein glycosylation [1]. The electrophoretic mobility of glycoproteins such as GLUT-1 is very sensitive to alterations in glucose metabolism [1,26–29]. The results suggest that 3-*O*-methylglucose, unlike 2-deoxyglucose [1,26–29], is unable to directly perturb the use of sugars and their metabolites for oligosaccharide synthesis.

Glucose metabolism and GLUT-1 regulation

The finding that 3-*O*-methylglucose can prevent the glucose-deprivation-induced increases in hexose transport and cell-surface expression of GLUT-1 (Figure 1 and Table 2) suggests that glucose metabolites are not required for the regulation by glucose of the functional expression of GLUT-1. Essential to this conclusion is the demonstration that 3-*O*-methylglucose is not metabolized and does not alter glucose metabolism. It has been reported that 3-*O*-methylglucose is phosphorylated to a very limited extent in rat heart [43] and not at all in fibrosarcoma cells [44]. 3-*O*-methylglucose has also been shown to be weakly (~ 1000 -fold lower V_{max} and ~ 100 -fold higher K_m) phosphorylated *in vitro* by yeast and bovine hexokinase [45]. In fibroblasts incubated with 3–25 mM 3-*O*-methylglucose for 24 h we found that $\sim 14\%$ of the intracellular 3-*O*-methylglucose is phosphorylated (Table 1). Unlike the glucose-concentration-dependence for cellular glucose 6-phosphate content (Table 3), the 3-*O*-methylglucose-concentration-dependence of 3-*O*-methylglucose 6-phosphate content (Table 1) does not exhibit an inflection with correlates with the EC_{50} for suppression of hexose transport (Figure 1). This accumulation of 3-*O*-methylglucose 6-phosphate also suggests that this sugar phosphate is not readily metabolized and may be handled, albeit less efficiently, like 2-deoxyglucose [1]. Furthermore, unlike 2-deoxyglucose, 3-*O*-methylglucose does not alter protein glycosylation (Figure 2) and does not cause ATP depletion [1]. The inability of 3-*O*-methylglucose to change glucose 6-phosphate levels (Table 3), hexokinase and PGI activities (result not shown) in glucose-deprived cells also suggests that this glucose analogue may be acting independently of metabolism. However, the possibility that 3-*O*-methylglucose 6-phosphate acts as an analogue of glucose 6-phosphate (or other metabolite) which has a much lower affinity for the glucose-deprivation-regulated target(s) cannot be excluded.

Carrier–substrate-interaction and GLUT-1 regulation

The cellular content of glucose 6-phosphate does not increase above 5 mM glucose (Table 3). Therefore, when GLUT-1 is saturated, it appears that hexose transport is not limiting for glucose metabolism. The best estimates of the K_m for glucose entry via GLUT-1 indicate a value of < 5 mM (see Carruthers [41], for a review). Below 5 mM glucose, where transport is not saturated, glucose 6-phosphate levels are very low. This is consistent with rapid metabolism of glucose and glucose 6-phosphate through glycolysis and, to a lesser extent, the hexose monophosphate shunt [31,36–38]. The culture medium employed contains glutamine, which supports overall energy metabolism even in the absence of glucose [36–38]. Therefore, under these conditions, the established role of overall energy metabolism in

the regulation of hexose transport [3,4] is not relevant. Regulation of the functional expression of GLUT-1 correlates with *both* saturation of the carrier (Figure 1 and Table 2) and the generation of glucose 6-phosphate (Table 3). The effects of 3-*O*-methylglucose (Figure 1 and Table 2), a nearly non-metabolizable glucose analogue, allows us to partially resolve these two parameters and suggests that substrate for the carrier may directly regulate the functional expression of GLUT-1. However the significant accumulation of 3-*O*-methylglucose 6-phosphate (Table 1) precludes a definitive separation of these issues.

Thus, in murine fibroblasts, functional expression of GLUT-1 can apparently be regulated by transported substrate and/or an immediate metabolite; i.e., glucose and/or glucose 6-phosphate. This could involve the transport process itself (c.f. [1,13]). The correlation of the EC_{50} for 3-*O*-methylglucose on hexose-transport regulation (12 mM) (Figure 1) and the various values of the K_m of GLUT-1 for this glucose analogue (2–20 mM depending on assay conditions, cell system and kinetic models [24,41,42]) prevent one from distinguishing between this model and one which involves a 'specialized glucose sensor'. The potential for direct interactions of hexose phosphates like glucose 6-phosphate and 3-*O*-methylglucose 6-phosphate with GLUT-1 is presently unclear.

Conclusions

We have demonstrated that downstream glucose metabolism is required for the post-translational regulation by glucose of the content, but not the functional expression (i.e. cell-surface localization and/or intrinsic activity), of GLUT-1 in murine fibroblasts. Our findings may explain some conflicting aspects of previous reports [1,13,18] and further our understanding of the regulation of hexose transport by glucose. The demonstration that substrates of the carrier themselves or immediate metabolites of these substrates can regulate the functional expression of hexose transporters is intriguing. The lack of a unifying mechanism for the glucose-deprivation effect is supported by our finding of differential metabolic control mechanisms for hexose transport and GLUT-1 content. The molecular nature of the 'sensor(s)' and 'signal(s)' for this regulation of membrane transport by substrate remains an open question.

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