Regulation of the Functional Expression of Hexose Transporter GLUT-1 by Glucose in Murine Fibroblasts: Role of Lysosomal Degradation†

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Received October 10, 1991; Revised Manuscript Received April 3, 1992

ABSTRACT: The nature of the membrane compartments involved in the regulation by glucose of hexose transport is not well defined. The effect of inhibitors of lysosomal protein degradation on hexose transport (i.e., uptake of [^{3}H]-2-deoxy-D-glucose) and hexose transporter protein GLUT-1 (i.e., immunoblotting with antipeptide serum) in glucose-fed and -deprived cultured murine fibroblasts (3T3-C2 cells) was studied. The acidotropic amines chloroquine (20 μM) and ammonium chloride (10 mM) cause accumulation (both ~4-fold) of GLUT-1 protein and a small increase (both ~25%) in hexose transport in glucose-fed fibroblasts (24 h). The endopeptidase inhibitor, leupeptin (100 μM) causes accumulation (~4-fold) of GLUT-1 protein in glucose-fed fibroblasts (24 h) without changing hexose transport (≤5%). These agents do not greatly alter the electrophoretic mobility of GLUT-1. Neither chloroquine nor leupeptin augment the glucose deprivation (24 h) induced increases in hexose transport (~4-fold) and GLUT-1 content (~7-fold). In contrast, chloroquine or leupeptin diminish the reversal by glucose refeeding of the glucose deprivation induced accumulation of GLUT-1 protein but fail to alter the return of hexose transport to control levels. These results with inhibitors of lysosomal function are consistent with a model in which for murine fibroblasts (i) degradation of GLUT-1 occurs by routing the carrier through acidified compartments to the lysosomes for proteolysis; (ii) inhibition of lysosomal degradation and buffering of acidified intracellular membrane compartments do not cause changes in the glycosylation of GLUT-1; (iii) degradation of GLUT-1 may be indirectly inhibited in glucose-deprived cells; (iv) degradation of GLUT-1 does not directly control its functional expression; and (v) the ability of glucose to downregulate the functional expression of GLUT-1 may involve "internalization" into compartments which are independent of the pathways involved in its lysosomal degradation.

Stereospecific glucose (Glc)[1] transport across the plasma membrane of mammalian cells is mediated by a family of related proteins (Bell et al., 1990; Thorens et al., 1990; Kasanicki & Pilch, 1990). These structurally and functionally homologous proteins, designated GLUT-*n* (nomenclature of Bell and associates (1990)), differ in their tissue distribution, affinity for Glc, and regulation (Bell et al., 1990; Thorens et al., 1990; Kasanicki & Pilch, 1990). GLUT-1, a M_r 55 000 transmembrane glycoprotein, was the first such transporter cloned and structurally identified (Mueckler et al., 1985; Birnbaum et al., 1986). It is found at high levels in brain microvessels, kidney, small intestine, placenta, mammalian fibroblasts, and human erythrocytes (Bell et al., 1990). It has been suggested that GLUT-1 is the "basal" Glc transporter in many cell types (Thorens et al., 1990).

The rate of hexose transport and the expression of GLUT-1 protein by mammalian fibroblasts can be acutely and/or chronically regulated by serum, growth factors, transformation, steroids, and nutrient deprivation (cf. Hiraki et al., 1988; Birnbaum et al., 1987; Horner et al., 1987; Rollins et al., 1988; Shawver et al., 1987; White & Weber, 1988; Haspel et al., 1986, 1991; Kalckar & Ullrey, 1984a,b; Flier et al., 1987; Yamada et al., 1983). In rodent fibroblasts Glc deprivation induced alterations in GLUT-1 expression can be resolved into two components: *accumulation* of *total* GLUT-1 polypeptides and the *appearance* of the *aglyco* carrier of M_r 38 000 (Haspel et al., 1986). Total accumulation is believed to, either directly or indirectly, result from decreased "carrier inactivation" (Yamada et al., 1983) or degradation of GLUT-1 in response to alterations in the levels of hexose phosphates (Kalckar & Ullrey, 1984b). Aglyco GLUT-1 results from the inhibition of oligosaccharide biosynthesis observed during chronic Glc deprivation (Gershman & Robbins, 1981; Rearick et al., 1981) and is derived from the same core polypeptide as the M_r 55 000 carrier (Haspel et al., 1986). This aglyco form is not observed when cells are fed alternative hexoses, e.g., fructose (Fru), which support oligosaccharide biosynthesis (Haspel et al., 1986) and are not substrates for GLUT-1 (Carruthers, 1990; Gould et al., 1991). The aglyco form is distinct from the core glycosylated M_r 42 000 polypeptide observed as a biosynthetic precursor or after treatment with inhibitors of oligosaccharide processing (Haspel et al., 1985, 1988a). In rodent fibroblasts, which express GLUT-1 protein but not GLUT-2 or GLUT-4 (Haspel et al., 1991; Bell et al., 1990), the Glc deprivation induced increases in hexose transport and content of GLUT-1 protein are not paralleled by GLUT-1 protein synthesis or mRNA content and are not attenuated by treatment with blockers of protein synthesis (Haspel et al., 1986; White & Weber, 1988). In contrast to this posttranslational effect, Glc deprivation has been shown to increase the amount of GLUT-1 mRNA in rat L6 skeletal muscle cells, primary cultures of rat brain glial cells, and chick embryo fibroblasts (Walker et al.,

† Supported by the Diabetes Research and Education Foundation, a Research and Development Award from the American Diabetes Association (H.C.H.), and a W. Burghardt Turner Dissertation Fellowship (P.A.O.).
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[1] Abbreviations: α-CT, antiserum specific to a carboxy-terminal synthetic peptide of type 1 hexose transporter; dGlc, 2-deoxy-D-glucose; DMEM, Dulbecco's-modified Eagle's medium; ER, endoplasmic reticulum; Fru, fructose; Glc, glucose; Glc-6-P_i, glucose 6-phosphate; GLUT-1, type 1 or erythrocyte/HepG2/brain hexose transporter; M_r, apparent molecular weight; PM, plasma membrane, SD, standard deviation; TGN, trans Golgi network.

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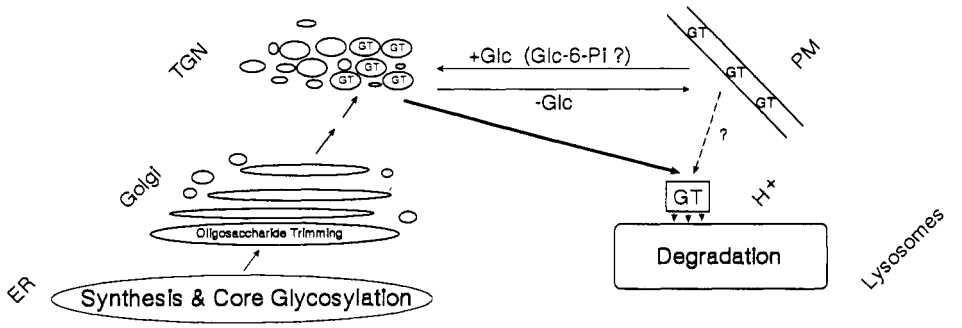


FIGURE 1: Model of regulation by glucose of the functional expression of GLUT-1 in murine fibroblasts. After synthesis and core glycosylation in the endoplasmic reticulum (ER), GLUT-1 (GT) undergoes oligosaccharide trimming in the Golgi. Most of the mature GLUT-1 of Glc-fed fibroblasts resides in unacidified intracellular membrane vesicles (small circles) of the trans Golgi network (TGN). From this site, it can cycle (double arrows) to and from the plasma membrane (PM). This process is regulated by Glc metabolism (+Glc (Glc-6-P_i?) and –Glc). The majority of GLUT-1 that is routed (thick arrow versus dashed arrow, ?) through acidified (H^+) intracellular compartments to (arrow heads) the lysosomes for degradation is derived from the intracellular pool of GLUT-1 present in the TGN. The model predicts that GLUT-1 "internalization and/or externalization", in contrast to GLUT-1 degradation, can be directly regulated by Glc metabolism.

1988, 1989, 1990; White & Weber, 1988; Koivisto et al., 1991; Shawver et al., 1987).

Unlike the well-characterized insulin-induced recruitment of both GLUT-4 and GLUT-1 to the plasma membrane observed in adipocytes and muscle (Simpson & Cushman, 1986; Kasinicki & Pilch, 1990), a role for the subcellular redistribution of GLUT-1 in the regulation of carrier degradation during Glc deprivation had not been examined in detail. Recently, we have characterized the subcellular localization of GLUT-1 during Glc deprivation induced accumulation of the carrier in rodent fibroblasts by using immunoblotting of subcellular fractions and immunofluorescence microscopy (Haspel et al., 1991). We demonstrated that most GLUT-1 protein is located in intracellular membranes in Glc-fed fibroblasts and that Glc deprivation induces the selective accumulation of GLUT-1 in the plasma membrane. On the basis of these findings, a working model for the regulation of GLUT-1 accumulation and localization during Glc deprivation was formulated (Haspel et al., 1991) and is elaborated on in Figure 1. Klip and associates have recently suggested a similar mechanism for these phenomena in L6 muscle cells (Koivisto et al., 1991). A related hypothesis had previously been proposed by Christopher (1984), but at that time the molecular tools were not available to examine its mechanistic characteristics. Our model predicts that in the Glc-fed state intracellular GLUT-1 protein is constitutively produced and stored in unacidified membrane vesicles of the trans Golgi network (TGN). In this fed state, only small amounts of GLUT-1 cycle to the plasma membrane. Changes in the glycemic state, e.g., Glc deprivation, can alter intracellular hexose phosphate levels (Kalckar & Ullrey, 1984b) and this may regulate the cycling of GLUT-1 to and/or from the cell surface. In order to be degraded, the carrier must return to this intracellular site before it is routed through acidified intracellular compartments to the lysosomes. The model proposes that Glc deprivation induces "externalization" of intracellular GLUT-1. This externalization may indirectly prevent GLUT-1 degradation by removing the transporter from prelysosomal pathways and therefore led to its overall accumulation in the plasma membrane. Direct determinations of GLUT-1 turnover are technically difficult (Haspel et al., 1985, 1986, 1991). This is because radiolabeling of relatively rare proteins, such as GLUT-1, to levels sufficient for detection requires amino acid deprivation, and this perturbation can itself alter GLUT-1 turnover (Haspel et al., 1985).

To examine the nature of the membrane compartments involved in the Glc deprivation effect, we now examine the effects of inhibitors of lysosomal protein degradation on hexose transport and GLUT-1 protein in murine fibroblasts. We contrast the effects of these agents with those of Glc deprivation and other agents which alter membrane protein sorting. The role of lysosomal degradation on Glc regulation of hexose transport has been studied previously (Christopher & Morgan, 1981; Christopher, 1984). In these studies, an acidotropic amine, NH_4^+, led to inhibition of the loss of hexose transport activity in hamster fibroblasts following blockage of protein synthesis. However, the results were complex and difficult to interpret, and effects on transporter protein were not examined. Previous reports (Suzuki & Kono, 1979; Hammons & Jarret, 1980; Ezaki et al., 1986; Oka et al., 1987) have not demonstrated an *acute* (≤2 h) effect of acidotropic amines on insulin-responsive hexose transport and hexose transporters (both GLUT-1 and GLUT-4) content/localization in rat adipocytes. These studies did not examine effects of these agents on the return of transport to basal levels following insulin removal and therefore did not preclude that recycling of hexose transporters involves an acidified compartment. Furthermore, chronic exposure to these agents was not examined. Recent ultrastructural studies on the immunolocalization of GLUT-4 in insulin-responsive tissues of the rat (Slot et al., 1991a,b) support the hypothesis that the "coated pit-endosome pathway" may be involved in the intracellular trafficking of some hexose transporters.

In this report we show that, in contrast to Glc deprivation, *chronic* (≥6 h) exposure to lysosomal inhibitors causes the accumulation of GLUT-1 protein without greatly altering hexose transport in murine fibroblasts. The roles of alterations in carrier degradation and localization in these phenomena are discussed.

Experimental Procedures

Reagents. Leupeptin was purchased from Transformation Research (Boston, MA), and concentrated (100×) stocks were prepared in Glc-free Dulbecco's-modified Eagle's medium (DMEM) and stored at 4 °C for <1 week. Chloroquine was purchased from Sigma Chemical Co. (St. Louis, MO), and concentrated (100×) stocks were prepared in 0.9% (w/v) NaCl and stored protected from light at 4 °C ($\epsilon_{342.5} = 1.93 \times 10^4$ $M^{-1}\cdot cm^{-1}$; this value was determined from absorbance spectra of solutions, in 0.9% NaCl, of chloroquine diphosphate made anhydrous by vacuum desiccation over calcium chloride). Ammonium chloride was purchased from Sigma Chemical Co., and concentrated stocks (100×) were prepared in 0.9% NaCl and stored at 4 °C. Monensin was purchased from

Sigma Chemical Co., and concentrated stocks (100×) were prepared in ethanol and stored at −20 °C. The calcium ionophore A23187 was purchased from Calbiochem (San Diego, CA), and concentrated stocks (100×) were prepared in dimethyl sulfoxide and stored at −20 °C. Radioisotopes were purchased from Amersham (Arlington Heights, IL). The antiserum, α-CT, used in this work was prepared previously (Haspel et al., 1988b). α-CT was elicited to a synthetic peptide corresponding to the 13 carboxy-terminal amino acid residues of rat GLUT-1. The properties and specificity of this antiserum have been described in detail (Haspel et al., 1988b, 1991).

General Methods. Membrane isolation, protein assays, and immunoblotting of GLUT-1 were performed as previously described (Haspel et al., 1985, 1986, 1988b). Briefly, cell monolayers (60-mm plates) are rinsed with phosphate-buffered saline, collected by scraping into sonication buffer (1 mL), and sonically lysed (2 × 30 s at 75 W). The lysates are centrifuged at 550*g* for 5 min at 4 °C, and the supernatant fluid is centrifuged at 200000*g* for 22 min at 4 °C. The crude postnuclear membrane pellets are resuspended in iced membrane buffer (50–100 μL), and equivalent amounts of membrane protein (35 μg) are subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (11% gels). The resolved proteins are electrophoretically transferred to nitrocellulose, the blots are probed with α-CT (1:100 dilution), and bound immunoglobulin is detected with ^{125}I-protein A. Dried blots are subjected to autoradiography with an intensifying screen at −80 °C.

Hexose transport by fibroblast monolayers was assayed as [^{3}H]-2-deoxy-D-glucose (dGlc) uptake [see Silverman et al. (1989) for details]. Briefly, cell monolayers are rinsed and preincubated (15 min at 23 °C) in assay buffer (140 mM NaCl, 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid, 1 mM $CaCl_2$, 5 mM KCl, 2.5 mM $MgSO_4$, pH 7.4 (Walker et al., 1988)). Uptake is initiated by addition of [^{3}H]-dGlc (final concentration: 0.2 mM, 0.5 μCi/mL) and terminated after 10 min at 23 °C by washing in iced phosphate-buffered saline, and cell-associated radioactivity and protein are determined. Under these conditions, dGlc uptake is linear under all conditions examined for ≥20 min.

Quantitation of Results and Statistical Analysis. GLUT-1 levels are determined from exposure intensities on the autoradiograms of the immunoblots. Multiple exposures are quantified on a Biomed (Fullerton, CA) scanning laser densitometer using a computer program employing two-dimensional analysis and area-normalized background subtraction. Transport results are normalized to control values, set to 100%, for each experiment. The percent change from control values for each condition from multiple experiments is averaged and the mean ± standard deviation (SD) is reported. The variation in hexose transport for controls was <10% [i.e., 3.15 ± 0.27 nmol of [^{3}H]-dGlc/(mg of cellular protein·10 min) N = 29]. Data were statistically analyzed with a one-way analysis of variance using the InStat program by GraphPAD (San Diego, CA).

Cell Culture and Treatments. Murine 3T3-C2 fibroblasts were cultured as previously described (Haspel et al., 1986). Briefly, cells were grown to confluence in high Glc (4.5 g/L) DMEM containing nonessential amino acids, 2 mM glutamine, and 9% (v/v) heat-inactivated calf serum with an atmosphere of 5% humidified CO_2 at 37 °C. Following a 12–24 h refeeding, confluent cells were washed and refed for 24–48 h in similar media containing no Glc and dialyzed calf serum (this medium contained ≤0.5 mg of Glc/L) (Haspel et al., 1986, 1991). This medium was supplemented with either Glc (4.5 g/L) (i.e., Glc-fed or control), Fru (1 g/L) (i.e., Glc-deprived but Fru-fed), or no hexose (i.e., Glc-deprived, or "starved"). Agents which alter lysosomal degradation and/or membrane protein sorting or the appropriate vehicle were also added at this time. After the indicated time, membranes were isolated for immunoblotting or hexose transport was assayed.

Results

Chloroquine Causes Accumulation of GLUT-1 Protein and a Small Increase in Hexose Transport in Glucose-Fed Fibroblasts. The role of acidified membrane compartments in the content and function of the hexose transporter isoform GLUT-1 was studied in confluent monolayers of murine 3T3-C2 fibroblasts by using the acidotropic amine chloroquine (deDuve, 1983) as a probe. Chloroquine is a weak base that accumulates in, and buffers, acidic compartments such as lysosomes and late endosomes (Poole & Ohkuma, 1981). It has classically been used to impair lysosomal protein degradation and endosomal dissociation of internalized ligand–receptor complexes. At high concentrations (>100 μM), chloroquine has been shown to inhibit endocytosis and have generalized cytotoxicity (Seglen et al., 1979).

Total membranes were isolated from Glc-fed fibroblasts that had been incubated with different concentrations of chloroquine (0–100 μM) for 24 h. These membranes were immunoblotted with antisera specific to GLUT-1 (Haspel et al., 1986, 1988b, 1991) (Figure 2A, top). Glc-fed cells have a broadly migrating M_r 55 000 polypeptide, characteristic of GLUT-1 (Simpson & Cushman, 1986), which accumulates with increasing concentrations of chloroquine. The EC_{50} for chloroquine was ~20 μM and a maximal accumulation of ~7-fold is observed at ~50 μM. At all concentrations tested, chloroquine did not greatly alter the electrophoretic mobility of GLUT-1. Total membranes were isolated from Glc-fed fibroblasts that had been incubated with chloroquine (20 μM) for different times (0–48 h) and immunoblotted for GLUT-1 (Figure 2A, bottom). The chloroquine-induced accumulation of GLUT-1 approaches a maximum of ~7-fold at ~24 h and is half-maximal at ~9 h. The electrophoretic mobility of GLUT-1 is unchanged at all time points examined.

The effects of chloroquine on hexose transport were compared to its effects on GLUT-1 protein. The uptake of [^{3}H]-deoxy-D-Glc (dGlc) by Glc-fed fibroblasts that had been incubated with chloroquine (0–100 μM) for 24 h was determined (Figure 2B). In contrast to its effect on GLUT-1 content, the increase in hexose transport caused by each concentration of chloroquine is small (≤30% at 24 h) and not statistically significant. In a similar manner, the uptake of [^{3}H]-dGlc by Glc-fed fibroblasts that had been incubated with chloroquine (20 μM) for different times (0–48 h) was determined (Figure 2C). At 24 h, chloroquine increases hexose transport by ≤30%. At 48 h, chloroquine increases transport only slightly (<10%, relative to 48 h controls) and the cells show gross morphologic changes indicative of cytotoxicity.

Leupeptin Causes Accumulation of GLUT-1 Protein Glucose-Fed Fibroblasts without Changing Hexose Transport. The role of lysosomal proteolysis in the content and function of GLUT-1 was studied in murine fibroblasts by using the endopeptidase inhibitor leupeptin (Seglen et al., 1979) as a probe. Leupeptin is a competitive inhibitor of serine proteases such as lysosomal cathepsins (Kirschke et al., 1977) and has classically been used to inhibit lysosomal protein degradation (cf. Seglen et al., 1979; Croze et al., 1989).

Total membranes were isolated from Glc-fed fibroblasts that had been incubated with different concentrations of leupeptin

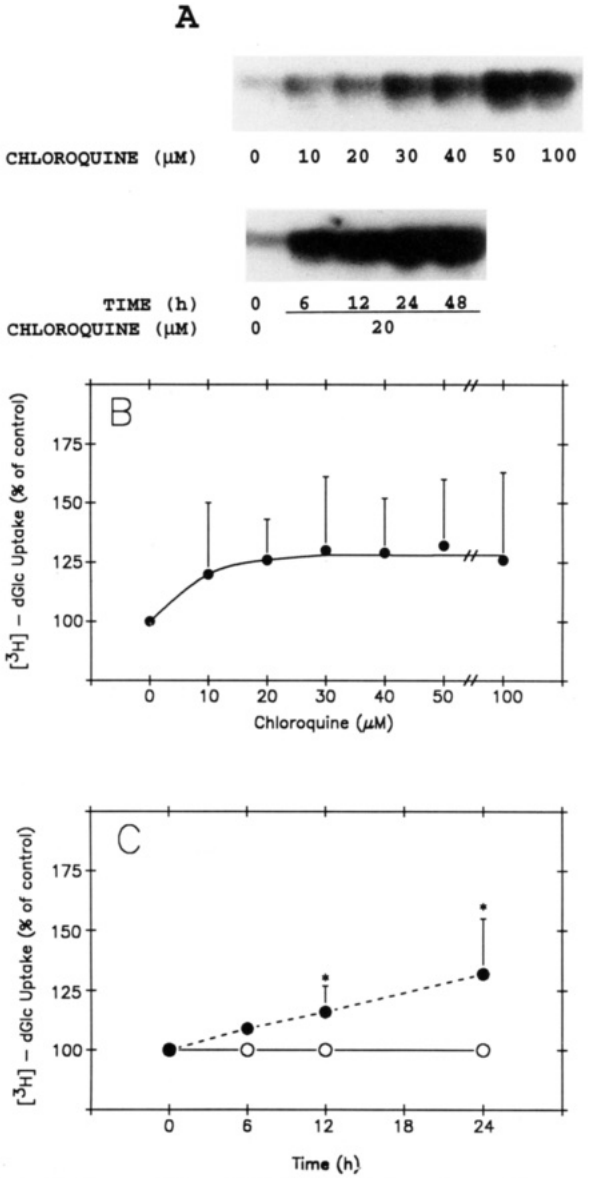


FIGURE 2: Accumulation of GLUT-1 protein and a small increase in hexose transport in glucose-fed fibroblasts caused by chloroquine. 3T3-C2 fibroblasts were grown to confluence in 35-mm six-well dishes for hexose transport (panels B and C) or 60-mm dishes for immunoblotting (panel A) and fed 12–24 h before use. Cells were then refed and treated with chloroquine (0–100 μM) for 24 h (panels A (top) and B) or with (closed symbols) or without (open symbols) chloroquine (20 μM) for 0–48 h (panels A (bottom) and C) as indicated. Cells treated with chloroquine (≥40 μM) for 24 h or with 20 μM chloroquine for 48 h show gross morphologic changes due to cytotoxicity. Membranes were prepared and 35 μg of membrane protein was immunoblotted for GLUT-1 protein with α-CT (1:100 dilution) (panel A). Autoradiograms representative of ≥3 similar experiments are depicted, and the M_r 55 000 GLUT-1 polypeptide is observed. Hexose transport was assayed as [^{3}H]-dGlc uptake as described in Experimental Procedures (panels B and C). Error bars are ±SD; N = 3; an asterisk indicates $p < 0.05$ versus control.

(0–100 μM) for 24 h. These membranes were immunoblotted with antisera specific to GLUT-1 (Figure 3, top). Glc-fed cells have a M_r 55 000 GLUT-1 polypeptide which accumulates with increasing concentrations of leupeptin. The EC_{50} for leupeptin is ~35 μM, and a maximal accumulation of ~5-fold is observed at 50 μM. At all concentrations tested, leupeptin does not alter the electrophoretic mobility of GLUT-1. Total membranes were isolated from Glc-fed fibroblasts that had been incubated with leupeptin (100 μM) for different times (0–48 h) and immunoblotted for GLUT-1 (Figure 3, bottom).

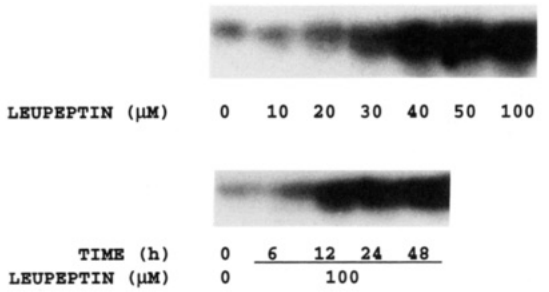


FIGURE 3: Accumulation of GLUT-1 protein in glucose-fed fibroblasts caused by leupeptin. Confluent fibroblasts, cultured and fed before use as in Figure 2, were refed and treated with leupeptin (0–100 μM) for 24 h (top) or with leupeptin (20 μM) for 0–48 h (bottom) as indicated. Membranes were isolated and immunoblotted as in Figure 2. Autoradiograms representative of ≥3 similar experiments are depicted, and the M_r 55 000 GLUT-1 polypeptide is observed.

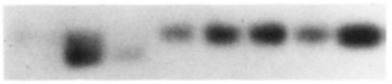
The leupeptin-induced accumulation of GLUT-1 approaches a maximum of ~6-fold at ~24 h and is half-maximal at ~12 h. Even at 48 h leupeptin does not greatly alter the electrophoretic mobility of GLUT-1.

The effects of leupeptin on hexose transport were compared to its effects on GLUT-1 protein. The uptake of [^{3}H]-dGlc by Glc-fed fibroblasts that had been incubated with leupeptin (0–100 μM) for 24 h was determined (not shown). In contrast to its effect on GLUT-1 content, leupeptin, at all concentrations tested, does not significantly alter hexose transport (≤5%). In a similar manner, the uptake of [^{3}H]-dGlc by Glc-fed fibroblasts that had been incubated with leupeptin (100 μM) for different times (0–48 h) was determiend (not shown). At all times examined, leupeptin fails to significantly alter hexose transport (≤5%).

Taken in concert, the effects of the lysosomal inhibitors chloroquine and leupeptin suggest that, although inhibition of lysosomal function causes GLUT-1 protein to accumulate, most of the accumulated carrier is not functionally expressed.[2] Furthermore, neither chloroquine nor leupeptin alters the glycosylation of GLUT-1.

Effects of Other Agents Which Alter Membrane Protein Sorting on Hexose Transport and GLUT-1 Protein. The effect of some other agents which alter membrane protein sorting on hexose transport and GLUT-1 protein were also examined and compared to the effects of the lysosomal inhibitors chloroquine and leupeptin. Total membranes were isolated from Glc-fed fibroblasts which had been incubated with or without these agents for 24 h and immunoblotted for GLUT-1 (Figure 4). Like chloroquine and leupeptin, ammonium chloride (5 and 10 mM), an acidotropic amine (Poole & Ohkuma, 1981), causes accumulation (~4-fold) of GLUT-1 without altering the electrophoretic mobility of the carrier protein. The divalent cationophore, A23187 (Pressman et al., 1976), increases cytoplasmic Ca^{2+} concentrations, disrupts glycoprotein processing, and alters intracellular membrane sorting (Sambrook, 1990). A23187 (10 μM) does not cause GLUT-1 to accumulate (<30%) but does alter its electrophoretic mobility (appearance of M_r 42 000 GLUT-1 polypeptide). The monovalent cationophore (Tartakoff, 1983), monensin (10 μM), which has been shown to have multiple effects on membrane protein sorting and glycoprotein processing (Tartakoff, 1983; Elbien, 1987; Kornfeld & Kornfeld, 1985), causes a ~6-fold

[2] Chloroquine (20 μM) and leupeptin (100 μM) have no effect (data not shown) on total protein synthesis by Glc-fed fibroblasts (24 h) as determined from the incorporation of [^{3}H]lysine (100 μCi/mL, 100 μM, 1-h pulse) into trichloroacetic acid-insoluble radioactivity (Haspel et al., 1985).



CONCENTRATION (mM)	0	.01	.01	0.1	5	10	.01	.02
AGENT	C	M	A	L	NH_4^+		C	
	O	O	2	E			H	
	N	N	3	U			L	
	T		1	P			O	
	R		8				R	
	O		7				O	
	L							

FIGURE 4: Effects of other agents that alter membrane protein sorting on GLUT-1 protein. Confluent fibroblasts, cultured and fed before use as in Figure 2, were refed and treated with or without (control) chloroquine (10 and 20 μM, chloro), ammonium chloride (5 and 10 mM, NH_4^+), leupeptin (100 μM, leup), monensin (10 μM, mon), or calcium ionophore (10 μM, A23187) for 24 h as indicated. Cells treated with monensin or A23187 show gross morphologic changes indicative of cytotoxicity. Membranes were isolated and immunoblotted as in Figure 2. An autoradiogram representative of $\geq$3 similar experiments is depicted, and the M_r 55 000 and M_r 42 000 GLUT-1 polypeptides are observed as the upper and lower bands, respectively.

Table I: Effects of Other Agents That Alter Membrane Protein Sorting on Hexose Transport[a]

agent	concn	[^{3}H]-dGlc uptake (% of control $\pm$ SD)
none (control)		100
monensin	10 μM	60 $\pm$ 20[b]
A23187	10 μM	52 $\pm$ 0[b]
leupeptin	100 μM	103 $\pm$ 8
ammonium chloride	5 mM	107 $\pm$ 25
	10 mM	124 $\pm$ 8[b]
chloroquine	10 μM	122 $\pm$ 31
	20 μM	129 $\pm$ 20[b]

[a] Confluent fibroblasts, cultured and fed before use as in Figure 2, were refed and treated with or without (control) the indicated agents for 24 h. Cells treated with monensin or A23187 show gross morphologic changes indicative of cytotoxicity. Hexose transport was assayed as in Figure 2. $N = 3$. [b] Indicates $p < 0.05$ versus control.

accumulation in total GLUT-1 protein and leads to the appearance and accumulation of a M_r 42 000 GLUT-1 polypeptide ($\sim$60% of total). The uptake of [^{3}H]-dGLC by Glc-fed fibroblasts that had been incubated with ammonium chloride (5 and 10 mM), leupeptin (100 μM), chloroquine (10 and 20 μM), A23187 (10 μM), or monensin (10 μM) for 24 h was also examined (Table I). The acidotropic amines increase hexose transport slightly ($\leq$30%). Leupeptin has no effect ($\leq$5%) on hexose transport, while A23187 and monensin inhibit hexose transport by 45% and 40%, respectively. Exposure of fibroblasts to the vehicles, ethanol (1% v/v) or dimethyl sulfoxide (1% v/v), has no effect (5%) on hexose transport, GLUT-1 content, or GLUT-1 glycosylation (data not shown).

These results, taken in concert with our earlier reports on the role of glycosylation in GLUT-1 structure and function (Haspel et al., 1985, 1986, 1988a, 1991), suggest that GLUT-1 cycles through an acidified compartment during membrane protein sorting and/or degradation. However, inhibition of lysosomal function, while causing GLUT-1 accumulation, fails to greatly alter hexose transport. Therefore, GLUT-1 may occupy or transit acidified vesicles during its intracellular trafficking. It may accumulate in this compartment or lysosomes if degradation is impaired. The ionophores monensin and A23187 caused alterations in glycosylation which are presumably mediated by changes in protein sorting/processing in the endoplasmic reticulum and Golgi. The main focus of this study is the role of lysosomal degradation in GLUT-1 regulation. In this context, a clear interpretation of the ionophore results is difficult because these agents alter the bio-

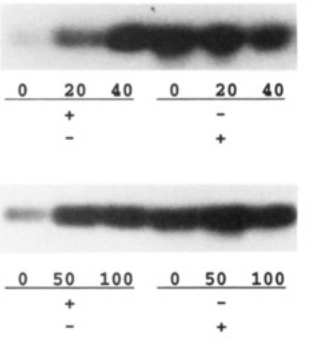


FIGURE 5: Lack of augmentation of GLUT-1 accumulation in glucose-deprived fibroblasts treated with either chloroquine or leupeptin. Confluent fibroblasts, cultured and fed before use as in Figure 2, were fed with (+) or without (–) Glc (4.5 g/L) or Fru (1 g/L) and treated with chloroquine (0–40 μM) (top) or leupeptin (0–100 μM) (bottom) for 24 h as indicated. Glc-deprived cells treated with chloroquine (40 μM) show gross morphologic changes indicative of cytotoxicity. Membranes were isolated and immunoblotted as in Figure 2. Autoradiograms representative of $\geq$3 similar experiments are depicted and the M_r 55 000 polypeptide is observed.

synthesis, i.e., glycosylation, of GLUT-1. It is unclear what general effects these agents have on lysosomal function. However, comparing the different effects of monensin and A23187 suggests, as we have previously proposed (Haspel et al., 1986, 1988a, 1991), that altering glycoprotein processing is not sufficient to cause transporter accumulation.

Neither Chloroquine nor Leupeptin Augments the Glucose Deprivation Induced Increase in Hexose Transport and Accumulation of GLUT-1. We have previously demonstrated (Haspel et al., 1986, 1991) that Glc deprivation of rodent fibroblasts causes a 5–20-fold increase in GLUT-1 levels and a 2–5-fold increase in hexose transport. This is strikingly different than the effects of chloroquine and leupeptin on Glc-fed cells. In both cases, the accumulation of GLUT-1 protein is likely due to a decrease in the degradation of the carrier.[2] However, the mechanisms by which these alterations in the turnover of GLUT-1 are mediated may involve somewhat different pathways of membrane protein sorting. To address this question, we examined the effects of chloroquine and leupeptin on the Glc deprivation induced accumulation of GLUT-1 and increase in hexose transport.

Total membranes were isolated from fibroblasts which had been Glc-fed, Glc-deprived but Fru-fed, or Glc-starved (data not shown) in the presence or absence of chloroquine (0–40 μM) (Figure 5, top) or leupeptin (0–100 μM) (Figure 5, bottom) for 24 h (see Experimental Procedures for details of culture conditions) and immunoblotted for GLUT-1. As previously reported (Haspel et al., 1986), Glc-deprived cells show a $\sim$7-fold increase in GLUT-1 polypeptides and, when starved for all carbohydrates, the aglyco carrier (M_r 38 000) is detected (data not shown). In Glc-fed fibroblasts, we again observe chloroquine- and leupeptin-induced increases (both $\sim$4-fold) in GLUT-1. In contrast, the presence of chloroquine or leupeptin during Glc deprivation *does not increase* the content of accumulated GLUT-1 polypeptides (Figure 5) and does not alter the ratio of glycosylated to aglyco GLUT-1 in the Glc-starved cells (data not shown). The slight decrease in GLUT-1 content observed in the presence of 40 μM chloroquine for Glc-deprived cells is attributed to cytotoxicity.

We also examined the effect (Table II) of chloroquine and leupeptin on the Glc deprivation induced increase in hexose transport. The uptake of [^{3}H]-dGlc by fibroblasts which had been Glc-fed, Glc-deprived but Fru-fed, or Glc-starved (data not shown) in the presence or absence of chloroquine (0–40 μM) or leupeptin (0–100 μM) for 24 h was determined.

Table II: Chloroquine and Leupeptin Do Not Augment the Glucose Deprivation Induced Increase in Hexose Transport[a]

agent	concn	[^{3}H]-Glc uptake (% of control ± SD) Glc-fed	Glc-deprived and Fru-fed
chloroquine	0 μM	100	395 ± 45[b]
	20 μM	126 ± 17[b]	376 ± 102[b]
	40 μM	129 ± 33[b]	195 ± 75[b,c]
leupeptin	0 μM	100	360 ± 45[b]
	50 μM	106 ± 3	373 ± 81[b]
	100 μM	103 ± 4	349 ± 34[b]

[a] Confluent fibroblasts, cultured and fed before use as in Figure 2, were fed and treated with the agents as indicated for 24 h. Glc-deprived and Fru-fed cells treated with chloroquine (40 μM) show gross morphologic changes indicative of cytotoxicity. Hexose transport was assayed as in Figure 2. $N = 3$. [b] Indicates $p < 0.05$ versus Glc-fed control (no agent added). [c] Indicates $p < 0.05$ versus similarly fed controls.

Strikingly, chloroquine (≤20 μM) and leupeptin (≤100 μM) do not prevent the Glc deprivation induced increase (~4-fold) in hexose transport. The decrease in hexose transport observed in the presence of 40 μM chloroquine for Glc-deprived cells is attributed to cytotoxicity.

Neither chloroquine nor leupeptin augment the glucose deprivation induced increase in hexose transport and accumulation of GLUT-1 (Figure 5 and Table II). Yet, lysosomal degradation can alter the cellular content of GLUT-1 but not functional expression (Figures 2 and 3). These results suggest that the accumulation of GLUT-1 protein and functional expression of the carrier can be mechanistically dissected. Glc deprivation might regulate the accumulation of GLUT-1 by *indirect or independent* mechanisms. In the Glc-deprived state, the functional expression of GLUT-1 is increased. In our simple model (Figure 1), this might occur by removing carriers from prelysosomal compartments to the plasma membrane and thereby *indirectly* inhibiting the degradation of GLUT-1. In contrast, Glc deprivation could inhibit the degradation of GLUT-1 directly but this may be *independent* of the regulation of its functional expression. It should also be noted that functional expression, i.e., hexose transport, is a combined measure of *both* GLUT-1 localization and the intrinsic activity of the carrier.

Chloroquine or Leupeptin Prevents the Reversal by Glucose Refeeding of the Glucose Deprivation Induced Accumulation of GLUT-1 but Fails to Alter the Return of Hexose Transport to Glucose-Fed Levels. The effect of chloroquine and leupeptin on the reversal by Glc refeeding of the Glc deprivation effect was determined. Fibroblasts were Glc-fed or Glc-deprived but Fru-fed for 24 h. The cells were then refed Glc in the presence or absence of chloroquine (20 μM) or leupeptin (100 μM). Total membranes were isolated from parallel cultures after the initial Glc deprivation and subsequent refeedings (6 and 24 h) and immunoblotted for GLUT-1 (Figure 6A). In this experiment Glc deprivation causes a ~6-fold accumulation of GLUT-1 protein. Glc refeeding of Glc-deprived cells for 6 and 24 h causes a ~20% and ~60%, respectively, decrease in GLUT-1 protein. Refeeding in the presence of leupeptin or chloroquine prevents this loss of accumulated GLUT-1 protein. In the presence of chloroquine, GLUT-1 protein content is increased by ~30% and ~10% of the initial (i.e., at 0 h) Glc-deprived content after 6 and 24 h of refeeding, respectively. In the presence of leupeptin, GLUT-1 protein content is increased by ~10% and decreases by ~25% of the initial (i.e., at 0 h) Glc-deprived content after 6 and 24 h of refeeding, respectively. It should be noted that the apparent

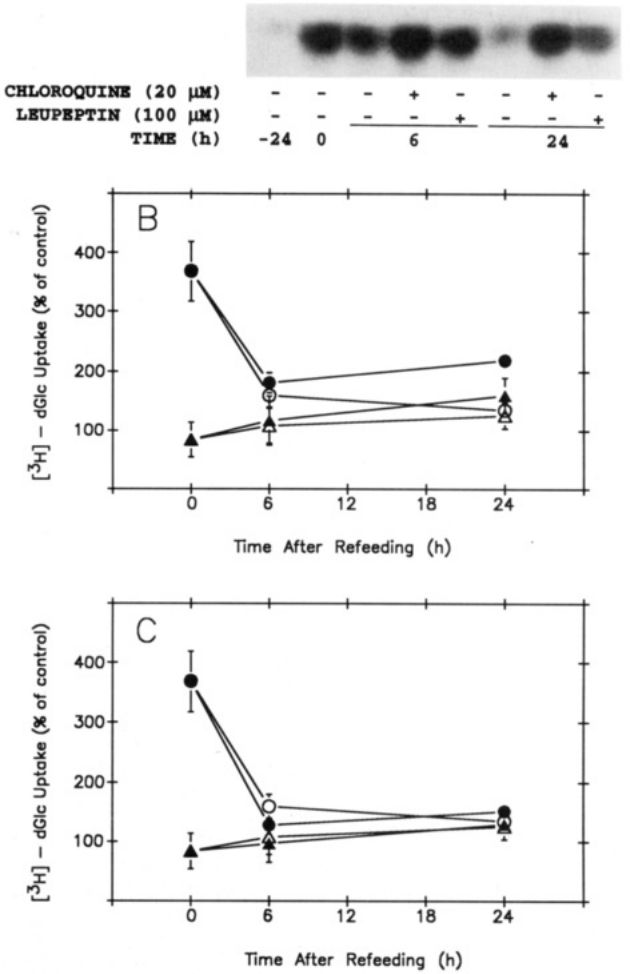


FIGURE 6: Effects of chloroquine or leupeptin treatment on reversal of the glucose deprivation effect. Confluent fibroblasts, cultured and fed before use as in Figure 2, were fed Glc (4.5 g/L) (circles) or Fru (1 g/L) (triangles) for 24 h (0 h after refeeding). The cells were then refed Glc (4.5 g/L) with (+, closed symbols) or without (–, open symbols) chloroquine (20 mM) (panels A and B) or leupeptin (100 μM) (panels A and C) for 6 or 24 h. Membranes were isolated and immunoblotted as in Figure 2 (panel A). An autoradiogram representative of ≥3 similar experiments is depicted and the M_r 55 000 GLUT-1 polypeptide is observed. The immunoblotting results for the glucose controls are not shown for clarity; these results were similar to those presented in Figures 2, 3, and 4. Hexose transport was assayed (panels B and C) as in Figure 2. Error bars are ±SD; $N = 3$; an asterisk indicates $p < 0.05$ versus control.

decrease in GLUT-1 degradation[2] upon Glc refeeding may be due in part to lysosomal inhibitors directly causing transporter accumulation (see Figures 2, 3, and 4).

In contrast, reversal of the Glc deprivation induced increase in hexose transport upon Glc refeeding is *not* altered by chloroquine (Figure 6B) or leupeptin (Figure 6C). It should be noted, as previously observed for L6 muscle cells (Koivisto et al., 1991), that the return of hexose transport to Glc-fed levels is more rapid than the loss of GLUT-1 protein. The Glc deprivation induced increase of transport is reversed by ~75% and ~95% at 6 and 24 h, respectively, of refeeding. While even at 24 h of refeeding, GLUT-1 protein has diminished by only ~60%.

These results suggest that upon refeeding Glc-deprived fibroblasts, the functional expression of GLUT-1 decreases by a mechanism which is not altered by buffering of acidified

intracellular compartments or inhibition of lysosomal degradation. The effects of lysomotropic agents on the loss of GLUT-1 during Glc refeeding of Glc-deprived fibroblasts probably involve inhibition of the degradation of *both* newly synthesized GLUT-1 protein and those carriers which accumulated during Glc deprivation. Importantly, the apparent decrease in carrier protein degradation we observe does not alter the return of hexose transport to Glc-fed levels. Thus, Glc appears to regulate the level of GLUT-1 protein by either an *indirect* mechanism involving translocation of intracellularly localized carriers to the cell surface (Figure 1) or an *independent* mechanism which is not *directly* linked to functional expression.

Discussion

We have demonstrated that in murine fibroblasts inhibition of lysosomal degradation and buffering of acidified intracellular compartments (i) cause GLUT-1 accumulation but do not increase hexose transport greatly (Figures 2, 3, and 4 and Table I); (ii) do not alter the electrophoretic mobility of GLUT-1 (Figures 2, 3, and 4); (iii) do not augment the Glc deprivation induced increase in hexose transport or accumulation of GLUT-1 (Figure 5 and Table II); and (iv) prevent the reversal by Glc refeeding of the Glc deprivation induced accumulation of GLUT-1 but do not alter the return of hexose transport to Glc-fed levels (Figure 6).

Several studies have used indirect approaches, e.g., inhibition of protein synthesis, to propose that Glc deprivation diminishes the degradation of hexose transporters of mammalian fibroblasts (Yamada et al., 1983; Haspel et al., 1985; Shawver et al., 1987; Christopher, 1984). Consistent with this proposal are the reported decreases in lysosomal protease activities, e.g., cathepsins, observed during Glc deprivation (Christopher & Morgan, 1981). Our studies suggest that although impairment of lysosomal proteolysis is able to greatly increase the cellular content of GLUT-1 there is only a small effect on its functional expression (Figures 2, 3, and 4 and Table I). This result differs somewhat from that of Christopher (1984). However, in the studies of Christopher, the effect of inhibition of lysosomal function on the decrease in hexose transport observed upon refeeding Glc-starved hamster fibroblasts was examined in the presence of cycloheximide. The complexity, i.e., biphasic concentration dependence, of cycloheximide effects are well established (Yamada et al., 1983: Haspel et al., 1985; Christopher, 1984) in this context. This makes a direct comparison of our work with that of Christopher difficult. The involvement of proteins with relatively short half-lives in the regulation of hexose transport has been proposed (Yamada et al., 1983; Haspel et al., 1985; Shawver et al., 1987; Christopher, 1984). If such proteins are involved in regulating functional expression and other such proteins control the degradation of GLUT-1, then these differences might be partially explained. We believe that other cellular factors, in addition to lysosomal degradation, are involved in the regulation by glucose of GLUT-1.

Our recent demonstration that Glc deprivation induces the selective accumulation, i.e., recruitment or translocation, of GLUT-1 in the plasma membrane (Haspel et al., 1991), when taken in concert with the present findings, gives further support to the simple model depicted in Figure 1. Several conclusions and speculations are implicit in this model and are consistent with our findings. GLUT-1 is degraded in lysosomes. En route from the plasma membrane to the lysosomes, GLUT-1 traverses both unacidified and acidified compartments. The acidified compartments may be affected by acidotropic amines, and a block at this step may account for the small increase in the functional expression of GLUT-1 (Figures 2 and 4). On the other hand, inhibition of lysosomal proteolysis with leupeptin probably has no direct effect on these acidified compartments and therefore the functional expression of GLUT-1 remains unchanged (Figures 3 and 4). Lysosomal inhibitors are not able to further *increase* GLUT-1 content or hexose transport in Glc-deprived fibroblasts (Figure 5 and Table II). This is consistent with the idea that in Glc-deprived cells little or no GLUT-1 is entering the degradative pathway. Furthermore, in Glc-deprived cells, GLUT-1 degradation may already, albeit *indirectly*, be inhibited. Alternatively, GLUT-1 degradation and functional expression may be *independently* regulated. In this case, functional expression might be controlled by translocation and/or intrinsic activation. Changes in hexose transport occur more rapidly than changes in GLUT-1 levels (Figure 6). This suggest that *direct* alterations in functional expression are probably the more physiologically relevant issue.

During reversal of the Glc-deprivation effect, loss of functional expression of GLUT-1 is observed despite inhibition of GLUT-1 degradation (Figure 6). This implies that the critical step for regulation by Glc availability of the functional expression of GLUT-1 is not carrier degradation but is either translocation and/or intrinsic activation. This allows us to predict that unacidified intracellular compartments may be involved in the regulation by Glc metabolism of hexose transport. This unacidified compartment is probably proximal to acidified vesicles and lysosomes and may represent an intracellular population of membrane vesicles enriched in GLUT-1. Membrane vesicles enriched in insulin-regulated hexose transporters have been isolated from adipocytes and predicted to be involved in the acute stimulation of hexose transport by insulin (cf. Biber & Lienhard, 1986; James et al., 1987; Zorzano et al., 1989). The GLUT-1 enriched vesicle population proposed in our model (Figure 1) may be a critical site in the regulation of hexose transport by Glc metabolism. It will be of interest to isolate and biochemically characterize these subcellular membrane vesicles from rodent fibroblasts.

Acknowledgments

The participation of Dr. Dennis C. Mynarcik in the early phases of this work is recognized. The expert technical assistance of Ms. Laura Cipp is greatly appreciated. Helpful discussions with Drs. Mario J. Rebecchi, Ira S. Cohen, and Ian A. Simpson are acknowledged.

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