Sugar Transport Regulation: Comparative Characterization of the Effect of NADH CoQ Reductase Deficiency in Two Cell Culture Systems (44559)

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Abstract. **In this report, we have characterized the upregulation of glucose transport in two different respiration-deficient fibroblast cell cultures. We have demonstrated that glucose transport increases in respiration-deficient cells as measured by 2 deoxy D-glucose transport and is readily observed in both the WG750 human and G14 Chinese hamster fibroblast respiration-deficient cell lines when compared with the MCH55 normal human and V79 parental Chinese hamster cell lines, respectively. Using subcellular fractionation techniques, the GLUT 1 glucose transporter was found located predominantly in the plasma membrane–enriched fraction of the human and hamster cell lines. In human cells, the expression of the GLUT 1 glucose transporter was elevated three-fold in the plasma membrane–enriched fraction of the WG750 respiration-deficient mutant cells. In the Chinese hamster cell lines, the respiration-deficient G14 cells exhibited no such GLUT 1 glucose transporter elevation in the plasma membrane–enriched fraction, yet expressed a >2-fold increase in glucose transport. Furthermore, the G14 cells had a similar content of GLUT 1 glucose transporter in the plasma membrane fraction when compared with the V79 parental cell line. Using Western blot analysis, the GLUT 1 glucose transporter in G14 cells exhibited a different mobility on a polyacrylamide gel when compared with the mobility of the GLUT 1 glucose transporter of the V79 cell line. This differential mobility of the glucose transporters in the hamster cells appeared to be related to glycosylation differences of the glucose transporters. Although normal human and hamster cell lines exhibited significant increases in insulin-stimulated sugar transport ($P < 0.05$), the two respective respiration-deficient cell lines exhibited no significant increases in insulin-stimulated sugar transport ($P > 0.05$). Additionally, the expression of the GLUT 1 mRNA in the human WG750 mutant cells was elevated when compared with GLUT 1 mRNA in normal cells. Insulin exposure significantly increased GLUT 1 mRNA in human cells ($P < 0.05$). No differences in the GLUT 1 mRNA were observed between both hamster cell lines. Thus, both respiration-deficient cell lines are insulin resistant (i.e., regarding their insulin-stimulated sugar transport). The respiration-deficient mutation results in an increased sugar transport in the human and hamster cells; however, the human cells adapt to the mutation by increasing their levels of GLUT 1 mRNA and eventually membrane-located glucose transporters. On the other hand, the hamster cells adapt by apparently modifying their glucose transporters' intrinsic activity *via* glycosylation. We feel that these cell systems can be effective models to study the multiple factors involved in sugar transport regulation in vertebrate cells.**

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In most animal cells, sugars are the primary fuels for the generation of energy and subsequent survival of the cell. In mammalian cells, sugar transport is mediated by a family of structurally related glucose transporter (GT) proteins (1) that are regulated by a number of physiological conditions (2). The mechanisms involved in sugar transport regulation include: (i) the translocation of GTs between internal membranes and the active plasma membrane site, (ii) altered synthesis and/or degradation of the GT, and (iii) modification and/or modulation of the intrinsic or catalytic activity of the GT (3–6).

We have reported earlier that the WG750 respiration-deficient (i.e., NADH CoQ reductase$^-$) human fibroblast cell line exhibited increased whole cell levels of GT (7) and sugar transport. Furthermore, our studies with the G14 mutant cell line derived from Chinese hamster lung fibroblasts (also NADH CoQ reductase$^-$) (7–11) produced the same enzyme defect and also exhibited increased sugar transport. Since early data indicated that sugar transport was increased in response to the mitochondrial defect, we sought to further characterize the mechanism(s) involved (7, 9, 11). In our continuing studies on the mechanism(s) involved in insulin resistance, elucidation of routes that cells employ in the control of glucose transport should shed some light on this problem. The studies reported herein will aid in our knowledge of transport control in the basal and hormonally stimulated state (e.g., in the presence of insulin). In this report, we have further characterized sugar transport in normal cells and the respective respiratory enzyme–deficient mutant cell lines indicating that the mechanism(s) employed by the different cell types to increase their transport of sugar is *via* transcription and/or glucose transporter synthesis in human cells and *via* post-translational modification of the glucose transporters in hamster cells.

Materials and Methods

Cell Culture. The WG750 (10-day-old donor) respiration-deficient and MCH55 (30-day-old donor) control human diploid fibroblast cell strains were obtained from Dr. P. Goodyer, Montreal Children's Hospital. The cells were grown in antibiotic-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 m*M* pyruvate and 10% (v/v) fetal calf serum (GIBCO). The cells were grown in an atmosphere of 7% CO_2/93% air. Cells were harvested from appropriate culture vessels after incubation for 5 min with 0.02% (wt/v) EDTA and 1–2 min with 0.04% (wt/v) trypsin (Difco Laboratories, VWR Canlab, Mississauga, Ontario). In all experiments with human fibroblasts, a donor age-matched cell strain was employed at similar passages (never exceeding 20 passages). For the Chinese hamster cell lines, the V79 (parental) and G14 (respiration-deficient) cells were grown in Dulbecco's modified Eagle's Medium supplemented with essential and nonessential amino acids at 37°C in 5% (v/v) fetal calf serum in a 7% CO_2/93% air environment. For clarity, Table I lists the characteristics of each of the cell types employed herein.

Table I. Cell Types Employed in this Study

Cell type	Designation	Gene defect	Reference no.
Human fibroblast	Normal MCH 55	NONE	8
Human fibroblast	Mutant[a] WG 750	NADH CoQ reductase deficiency	8
Hamster fibroblast	Parental V 79	NONE	9
Hamster fibroblast	Mutant G 14[b]	NADH CoQ reductase deficiency	9

[a] Insulin resistant, sugar transport modulated by elevated GLUT1 transporter protein in plasma membrane.
[b] Insulin resistant, sugar transport modulated by modification of GLUT1 transporter protein by glycosylation.

Sugar Transport Procedures. Details of the experimental protocols can be found elsewhere (7, 10). Briefly, cells were plated at 10^4 cells/cm^2 on plastic Petri dishes (35 mm diameter, Falcon, BD Biosciences, Oakville, Ontario) and grown to confluence. For all cell types, the cell monolayers were rinsed once in serum-free DMEM (containing 1 mg/ml bovine serum albumin (BSA) (0% DMEM) and incubated serum-free 24 hr before exposure to experimental conditions. Hexose transport was assessed by measuring the uptake of 2-(^{3}H)- deoxy-D-glucose (2-DG) in PBS (pH 7.4) containing 0.05 m*M* 2-DG (specific activity: 1.1μ Ci/mmole) in hamster cells or 1 m*M* 2-DG (specific activity 11.2 μCi/mmol) in human cells at 37°C. Zero-time controls were subtracted, the radioactive medium was removed, and the monolayers were rinsed 4× with 4°C PBS. The monolayers were dissolved in 1*N* NaOH, and aliquots were taken for liquid scintillation counting and protein determination (12).

Cell Fractionation and Membrane Isolation. Fibroblasts from confluent monolayers of 850-cm^2 roller bottles (Falcon) were treated, and plasma membranes were isolated according to a modified method of Buchanan (6, 13, 14). In short, after hypotonic lysis, a 27,000*g* spin yielded a crude plasma membrane–enriched pellet (i.e., P1 fraction) from six roller bottles of human fibroblasts or from one roller bottle of hamster fibroblasts. The 5′-nucleotidase content of the different fractions in the human cells was determined as described elsewhere (15). In Chinese hamster cells, the 5′-nucleotidase activity is very low (Amira Klip, personal communication) and is inadequate as a marker enzyme. Instead, a protocol was developed and used to evaluate another plasma membrane marker, ouabain-sensitive Na^+-ATPase (16).

Western Analysis Procedure. Whole cell monolayers or membrane fractions were suspended in 0.5 ml PBS with proteinase inhibitor (phenylmethylsulfonyl fluoride) at a final concentration of 1 m*M*. Prior to electrophoresis, samples were solubilized in 2% sodium dodecyl sulfate (SDS) (Pierce Chemical Co., Rockford, IL), 10% glycerol,

1 m*M* EDTA, 0.002% bromphenol blue, and 100 m*M* TrisHCl (pH 6.8). Electrophoresis was performed as described previously (6, 7, 14). Purified human erythrocyte glucose transporter was prepared as described by others (17) and used as a control GLUT 1 in Western analysis. Also, it was employed to prepare a rabbit polyclonal antibody that specifically recognizes the human and hamster GLUT 1 transporters (18). The monoclonal antibody to this transporter (G3) (19) was a gift from Dr. Gus Lienhard (Dartmouth Medical School, Hanover, NH). Proteins were blotted onto nitrocellulose paper treated with BSA, then the monoclonal antibody and subsequently ^{125}I-labeled sheep anti-mouse Fab at 1.2×10^6 DPM/ml were added. Autoradiography was on KODAK XAR film exposed at 85°C overnight. Spots were localized on the nitrocellulose, cut out, and counted in an LKB Gamma Counter (Uppsala, Sweden).

RNA Isolation. Cell pellets were snap-frozen in liquid nitrogen, and total cellular RNA was obtained by the acid/phenol extraction method of Chomczynski and Sacchi (20). RNA was quantitated spectrophotometrically by absorbance at 260 nm and stored as an ethanol precipitate at −85°C.

Northern Blot Analysis. Aliquots of 10 μ*g* of total RNA were fractionated by 1% agarose-formaldehyde gel electrophoresis. The procedures employed have been described elsewhere (21). The riboprobe employed was produced from a human GLUT 1 cDNA in a pGEM vector (a gift from Dr. J. Flier, Beth Israel Hospital and Harvard Medical School, Boston, MA). The human β-actin probe was a gift from Dr. Barry E. Knox (Syracuse University, Syracuse, NY). The hybridization was carried out overnight at 60°C. Two high-stringency washes were carried out at 60°C, and the washed blots were exposed to KODAK XAR-5 film at −70°C. Densitometric analysis was performed using a Model 2202 Ultrascan Laser densitometer.

Materials. 2-(^{3}H)-deoxy-D-glucose and(^{32}P)-α rATP were purchased from ICN Radio-Chemical Division (Mississauga, Ontario). ^{125}I-labeled sheep anti-mouse Fab was purchased from Amersham Pharmacia Biotech (Baie D'Urfe, Quebec). 2-DG was purchased from Calbiochem (San Diego, CA); BSA from Nutritional Biochemicals (Burlington, Ontario) and phenylmethylsulfonylfluoride from Sigma (Oakville, Ontario).

Statistics. The level of significance chosen for any statistical analysis employed herein was 5%.

Results

Glucose Transporter Content of Membrane Fractions Derived from Normal and Respiratory-Deficient Human Fibroblasts. In Figure 1, we show, using Western analysis, the GLUT 1 glucose transporter content of the plasma membrane fractions of the WG750 versus MCH55 cell strains (Fig. 1A). It can be seen clearly that the P1 fraction of the WG750 cell strain (Fig. 1B; Lane 2) contained more than a three-fold increase in GLUT 1 transporter when compared with the control cells (Fig.1B; Lane 1.). Additionally, it is evident that the P1 fraction of both cell types is enriched in 5′-nucleotidase activity (Fig.1C) (15). The lysate values of both cell types was <1 unit of the 5′-nucleotidase activity (data not shown). This indicates that the increased whole cell GLUT 1 transporter content observed earlier (7) resides in the plasma membrane (i.e., the 5′-nucleotidase–enriched fraction) and that the elevation in 2-DG transport is due to the membrane-located GLUT 1 transporter content.

Transport Characteristics. With respect to the elevated 2-DG transport observed in the respiration-deficient cells, the insulin responsiveness of both cell types was investigated. The data in Table II clearly indicate that the normal cells are insulin-responsive, exhibiting a significantly increased rate of 2-DG transport (>2-fold) after short-term insulin exposure whereas the respiration-deficient cells exhibited no significant increase (one-way ANOVA; $P < 0.05$; $n = 5$) in 2-DG transport post-insulin exposure. Previously, we have shown that the increase in glucose transport in response to insulin is the result of translocation of GLUT 1 (6). Thus, it is apparent that no transporter translocation is taking place in these respiration-deficient cells in response to insulin. Interestingly, dexamethasone (100 n*M*) can result in a reverse translocation of the GT into intracellular vesicles (14, 22). To test for the ability of the transporters to be translocated at all, we examined the effects of a 6-hr exposure to 100 n*M* dexamethasone on 2-DG transport in both the WG750 and MCH55 cell strains. Irrespective of the basal rate of sugar transport, exposure to dexamethasone decreased basal sugar transport ≈ 50% in both cell types. For example, in MCH55 cells, 2-DG transport in the presence of 100n*M* dexamethasone exhibited a decreased 2-DG transport from 2.8 ± 0.2 to 1.5 ± 0.1 nmoles 2-DG/mg protein/2min whereas in the WG750 cells, a dexamethasone-induced decrease from 9 ± 4 to 5 ± 3 nmoles 2-DG/mg protein/2 min was observed. Previously, we had observed no change in the K_m for glucose transport, the K_I for glucose, nor the specific binding of ^{125}I-insulin (7).

Glucose Transporter Content of Membrane Fractions Derived from Normal and Respiration-Deficient Chinese Hamster Fibroblasts. The data in Figure 2 do not indicate a pattern of transporter content and membrane association that would be expected from the high rate of basal 2-DG transport observed in respiration-deficient cells (e.g., parental, 8.0 ± 0.5; respiration-deficient, 23.2 ± 1.0). The respective plasma membrane–enriched fraction showed an enhanced ouabain-sensitive Na^+ ATPase (>8-fold over the lysate activity; data not shown). This membrane marker–enriched band represents the P1 fraction in hamster cells. Clearly, the glucose transporter in the respiration-deficient cells migrates differently on a polyacrylamide gel when compared with the glucose transporter in parental cells (Fig. 2A; Lane 2 vs 1). Additionally, it can be seen that the amount of GLUT 1 transporter protein in both the P1 fractions was equivalent in the

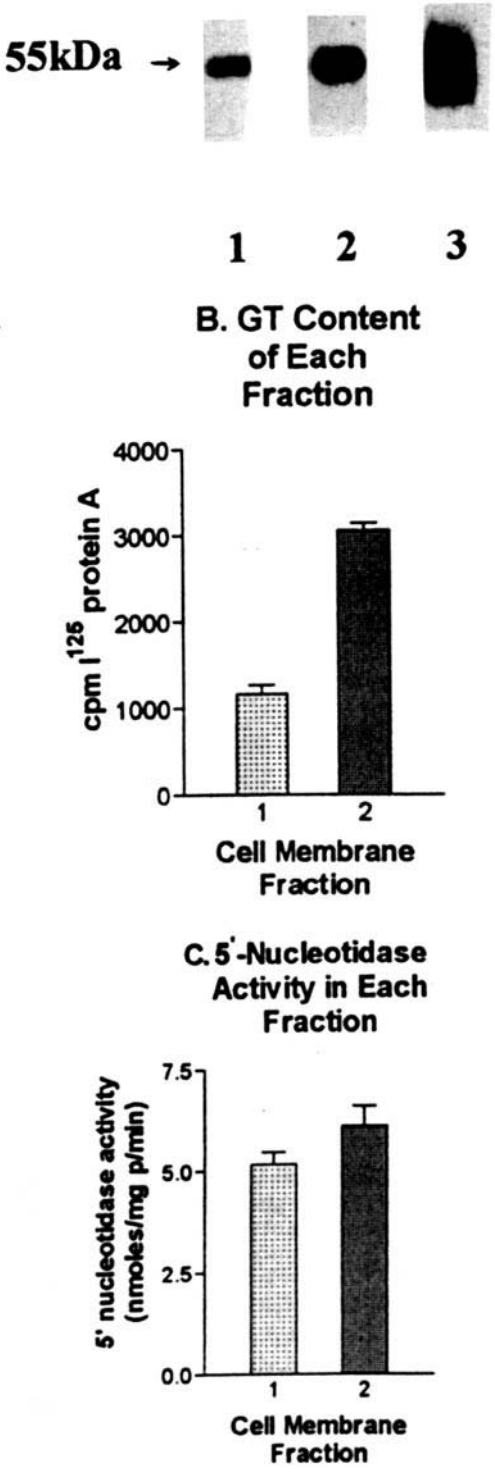


Figure 1. The distribution of the GLUT 1 glucose transporter in plasma membrane–enriched fractions derived from MCH55 and WG750 cell strains. (Panel A) Representative experiment: Western analysis of plasma membrane–enriched fractions of MCH55 and WG750 cells. Lanes 1 and 2 represent plasma membrane–enriched fractions of MCH55 cells and WG750 cells, respectively. Lane 3 represents 100ng of the purified human erythrocyte glucose transporter (i.e., GLUT 1). (Panel B) Representative GLUT 1 transporter content of the plasma membrane–enriched fractions of MCH55 and WG750 cells. Lanes 1 and 2 represent plasma membrane–enriched fractions of MCH55 cells and WG750 cells, respectively. Data were quantitated by cutting out the corresponding spots on the nitrocellulose membranes. The samples were counted in a gamma counter, and the data represent the average ± SEM ($n = 4$). *$P < 0.05$; two-tailed t test for Lanes 1 and 2. (Panel C) 5′ nucleotidase activity of the plasma membrane–enriched fractions (Lanes 1 and 2; MCH55 and WG750 cells, respectively). Data ± SEM ($n = 4$). Bars as in panel B.

respective cell types (Fig. 2B). We have demonstrated that this difference in migration is related to differential glucose transporter glycosylation (e.g., treatment of the membranes with endoglycosidase F results in a band exhibiting the same core protein on Western analysis for both cell types; data not shown) (5).

Transport Characteristics. In the data shown in Table III, the insulin responsiveness of the V79 and G14 cell lines was compared. As seen in the mutant human cells, the G14 respiration-deficient cell line exhibited no increase in insulin-mediated sugar transport. On the other hand a >2-fold increase in insulin-stimulated sugar transport was

Table II. The Effect of Insulin On Sugar Transport in Normal (MCH55) and Respiration-Deficient (WG750) Human Fibroblasts

Cell type	2-DG transport (nmoles/mg protein/2 min)	
	– Insulin	+ Insulin[a,b]
MCH 55	3.2 ± 0.5[a]	6.6 ± 0.8[a,c]
MG750	17.1 ± 4.3[a]	18.1 ± 6.1[a]

Note. Human fibroblasts were serum deprived for 24 hr in 0% MEM. At that time the cell monolayers were exposed to the various treatments for 1 hr, after which 2-DG transport was determined. Additional details can be found under Materials and Methods. All data represent the average ± SEM.

[a] The data of five experiments are shown (triplicate plates in each experiment).

[b] The insulin concentration used in all experiments was 667 n*M*.

[c] $P < 0.05$: A one-way analysis of variance (ANOVA) was performed with the MCH55 + I being significantly greater than the noninsulin-exposed control. No significant difference was seen in the WG750 ± I.

seen in the parental V79 cells when exposed to insulin for 1 hr (one-way ANOVA; $P < 0.05$; $n = 5$).

GLUT 1 Transporter mRNA Regulation. Next, we investigated the effect of insulin on GLUT 1 transporter gene expression. Both the MCH55 and WG750 cell strains were exposed to 100 n*M* insulin for 8 hr in 0% DMEM (Figs. 3A & 3B). At each time interval, total RNA was isolated (20) and probed with a single-stranded GLUT 1 RNA probe. The size of the message for the GLUT 1 mRNA was 2.4 kb (17). In Figure 3B, an increase in GLUT 1 mRNA was seen in the WG750 (i.e., respiration-deficient) cells versus the MCH55 (i.e., normal) cells at 0 time insulin exposure ($P < 0.05$, $n = 4$). After 8 hr exposure to insulin, we observed an insulin-induced increase in the expression of the GLUT 1 mRNA (Fig. 3B) in the MCH55 normal cell strain and a dramatic increase (Fig. 3B) in the WG750 cell strain versus the MCH55 mRNA. The lower group of bands in the same blot were stripped and reprobed with human β-actin cDNA (2.2kb), which is constitutively expressed in these cells. In the hamster parental and respiration-deficient cells, the GLUT 1 mRNA levels were not different in the presence or absence of insulin (unpublished data).

Discussion

From the elegant studies of Cushman and Kono (23, 24), insulin has been shown to activate the sugar transport system through the translocation of sugar carriers from intracellular sites to the plasma membrane of cells. However, the movement of sugar transporters may not explain all of the effects of insulin on sugar transport activation. Other evidence has been reported indicating that alternative methods of transporter activity regulation exist. Whitesell and Abumrad (25) suggested that insulin stimulates glucose transport by inducing changes in the K_m for transport rather than transporter numbers. Others have proposed that catecholamines inhibit transport activity by decreasing the intrinsic activity of the transporter rather than by altering its subcellular distribution (26). Certain agents such as phenylarsine oxide, diacylglycerol, or cycloheximide have been shown to stimulate sugar transport without an effect on transporter translocation (27–29). Thus, it still remains to be ascertained how insulin effects such changes in different cell types.

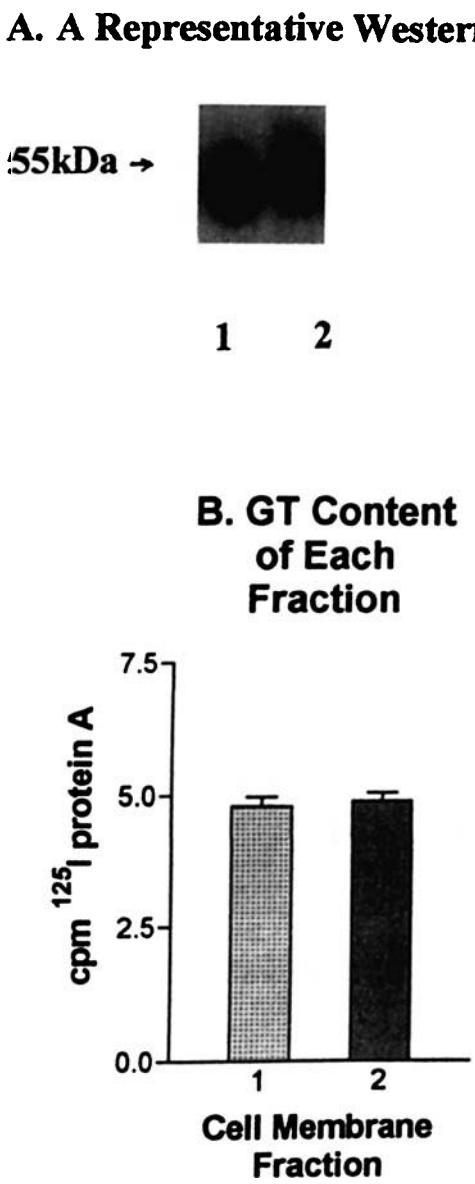


Figure 2. The distribution of the GLUT 1 glucose transporter in cell fractions derived from the V79 and G14 cell lines. (Panel A) Representative experiment: Western analysis of different membrane fractions of V79 and G14 cells. Lane 1 represents fraction P1 of V79 cells whereas Lane 2 represents the same fraction of the G14 cells, respectively. (Panel B) Representative glucose GLUT 1 transporter content of different membrane fractions of the V79 (i.e., Lane 1) and G14 cells (i.e., Lane 2). The GLUT 1 transporter content of the membrane fraction, P1 of the V79 and G14 cells (Lanes 1 and 2) were not significantly different ($P > 0.05$, $n = 7$, one-way ANOVA, data ± SEM).

In the present study, we showed that the permanently upregulated increase in 2-DG transport in human fibroblasts was membrane associated (Figs. 1A–C). The WG750 mutant did not respond to insulin as measured by an increased 2-DG transport but did express an increased GLUT 1

Table III. The Effect of Insulin On Sugar Transport in Normal (V79) and Respiration Deficient (G14) Chinese Hamster Fibroblasts

Cell type	2-DG transport (nmoles/mg protein/2 min)	
	– Insulin	+ Insulin[a,b]
V79	11.0 ± 0.3	24.0 ± 1.3[c]
G14	22.2 ± 1.8	24.1 ± 1.4

Note. Chinese Hamster fibroblasts were serum deprived for 24 hr in 0% MEM. At that time, the cell monolayers were exposed to the various treatments for 1 hr, after which 2-DG transport was determined. Additional details can be found under Materials and Methods. All data represent the average ± SEM.

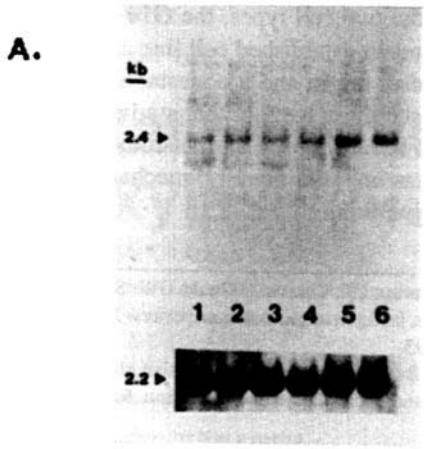
[a] The data of five experiments are shown (triplicate plates in each experiment).

[b] The insulin concentration used in all experiments was 667 n*M*.

[c] $P < 0.05$: A one-way analysis of variance (ANOVA) was performed with the V79 + I being significantly greater than the noninsulin-exposed control. No significant difference was seen in the G14 ± I.

mRNA both in the absence and presence of insulin (i.e., for up to 8 hr) when compared with the MCH55 cell strain. Of interest here is the observation that a Chinese hamster fibroblast cell line (i.e., the G14 cell line) possessing the same enzyme defect in the respiratory chain (8, 9) as WG750, and expressing an increased 2-DG transport, showed no increased content of whole-cell or membrane-located GLUT 1 glucose transporter. As observed with the human cell strains, the mutant G14 cell line showed no increase in insulin-stimulated sugar transport. The GLUT 1 transporter was found to be differentially glycosylated in the hamster cell line, and additional studies (5) indicated that the mechanism of the sugar transport increase was quite different (i.e., the intrinsic activity of the transporter appeared to be changed) from that seen in the WG750 cell strain. In the hamster G14 cell line, no increased GLUT 1 mRNA was seen under basal conditions (data not shown) whereas in the WG750 cell strain the GLUT 1 mRNA was greater than the normal in the absence of insulin. The GLUT 1 mRNA in the human mutant cell line, though increased in the absence of insulin, still responded to insulin exhibiting significant increases in mRNA after 8 hr in the presence of insulin. This in conjunction with previous observations (7) that insulin-stimulated amino acid uptake is not affected leads us to conclude that although sugar transport is modulated in different ways, the insulin-resistant states exhibited by the two respiration-deficient mutants relate directly to the perturbations in sugar uptake induced by the gene defect in each cell type.

Others employing inhibitors of respiration (30, 31) and hypoxia (32) induced increased sugar transport (30) and expression of GLUT 1 mRNA (32) in clone 9 cells. In these studies, conditions were used to inhibit oxidative phosphorylation such as rotenone and azide (30–32). The mutant cell lines employed herein express the same enzymatic mutation (8, 9, 11) and express increased sugar transport employing different mechanisms. These are stable cell lines that do not require chemical or physical modifications to achieve the



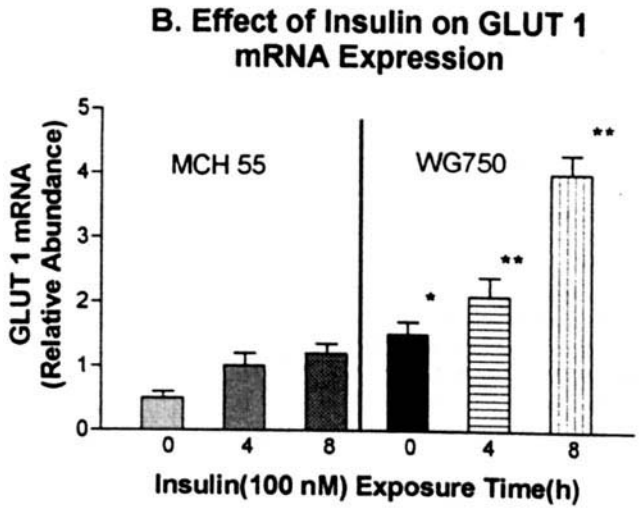


Figure 3. The effect of insulin on *GLUT 1* gene expression in MCH55 and WG750 cell strains. (Panel A) A representative Northern blot depicting the GLUT 1 mRNA (2.4kb) and human β-actin mRNA (2.2kb). Lanes 13 represent 0-, 4-, and 8-hr exposure to 100n*M* insulin in the MCH55 cells, whereas Lanes 4–6 represent 0-, 4-, and 8-hr exposure to the 100 n*M* insulin in the WG750 cells. (Panel B) Densitometric scans of the blots for the normal (MCH55) and mutant (WG750) cell strains. Data ± SEM ($n = 4$). One-way ANOVA. *$P <$ 0.05, 0 hr WG750 vs 0 hr MCH55; **$P < 0.05$, 0 hr WG750 vs 4- or 8-hr WG750 + insulin.

modulation of glucose transport response. The human and hamster enzymatic mutants of NADH CoQ reductase both need excess amounts of glucose to compensate for the energy requirements for survival and growth. It is of interest to note that while sugar transport is increased in the G14 and WG750 mutant cells, their compensation routes are quite different. The human mutant apparently has increased its GLUT 1 transporter mRNA and the membrane content of the transporter protein. This is similar to what has been shown in clone 9 cells (30–33). On the other hand, the hamster cell mutant has modulated its transporter by changing its glycosylation patterns that we have seen for other hamster cell respiration mutants (data not shown). This association between altered glycosylation and increased sugar transport has been reported recently with human cell hybrids that have been induced to tumorigenesis with radiation (34). Of course, there may be a relationship to species dif-

ferences in the two cell types, the G14 cells being of hamster origin and an established cell line and the WG750 cells being of human origin and a nonestablished cell strain. All that notwithstanding, we feel that studies of the respiratory mutants will be very useful in the study of various insulin-resistant states and additionally, mechanism(s) involved in transport regulation.

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