Insulin Action on Activity and Cell Surface Disposition of Human HepG2 Glucose Transporters Expressed in Chinese Hamster Ovary Cells*

(Received for publication, November 6, 1988)

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Recent evidence suggests that a family of related proteins is responsible for the facilitative glucose transport activity in mammalian cells. Mueckler et al. (1985) first cloned the cDNA for a facilitative glucose transporter from a HepG2 cell line several years ago. Since that time, other glucose transporter cDNAs have been isolated from rat and rabbit brain (Birnbaum et al., 1986; Asano et al., 1988), human and rat liver (Fukumoto et al., 1988; Thorens et al., 1988), human fetal skeletal muscle (Kayano et al., 1988), rat adipocyte and muscle (James et al., 1989; Birnbaum, 1989; Charron et al., 1989), human muscle (Fukumoto et al., 1989), and mouse adipocyte (Kaestner et al., 1989). Amino acid sequences deduced from these cDNA sequences have revealed four distinct isotypes of glucose transporter. 1) A glucose transporter denoted as GLUT1 is prevalent in HepG2 cells, erythrocytes, and brain. The GLUT1 protein has been identified in human, rat, and mouse (Sogin and Hinkle, 1980; Oka et al., 1988; Joost et al., 1988; Blok et al., 1988; Calderhead and Lienhard, 1988; James et al., 1989; Kayano et al., 1989; Zorzano et al., 1989). 2) A glucose transporter denoted as GLUT2 is prevalent in liver, kidney, and intestine. The GLUT2 protein has been identified in rat (Thorens et al., 1988, 1989; Fuku- moto et al., 1989), rat adipocyte and muscle tissues (James et al., 1989; Birnbaum, 1989; Charron et al., 1989; Zorzano et al., 1989). 3) A glucose transporter denoted as GLUT3 is prevalent in human fetal skeletal muscle. The GLUT3 protein has been identified in rat, human, and mouse (James et al., 1988, 1989; Zorzano et al., 1989; Fukumoto et al., 1989). The sequence identity between members of one isotype in different tissues and species ranges from approximately 90 to 97%, whereas the identity between glucose transporter proteins from different isotypes, even within a given species, ranges from 50 to 65%. These preliminary classifications are not meant to imply that the identical

* This work was supported by National Institutes of Health Grants DK 30988 (to M. P. C.), DK 36081 (to A. C.), and National Institutes of Health Training Grant DK 07302 (to S. A. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: HepG2, a human hepatoma cell line; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RSV, Rous sarcoma virus.

§ GLUT1–4 are designations for the indicated glucose transporters or putative glucose transporters (GLUT9), adapted from the nomenclature of Fukumoto et al. (1989).
proteins will be the only ones expressed in those tissues. It seems quite probable that additional glucose transporter isoforms will be identified in the near future.

Several important questions about mammalian glucose transporters relate to the mechanisms that regulate the function of these proteins. Do hormones and other agents that modulate cellular sugar uptake regulate all of the different isotypes of glucose transporters present in a single cell? Are signaling pathways that regulate different transporter isoforms divergent? One of the major regulators of hexose uptake by insulin, which stimulates facilitative glucose transporter activity in skeletal muscle and in adipose tissues (Simpson and Cushman, 1986), is glucose. This effect contributes to the lowering of blood glucose in intact animals by this hormone. The signal transduction pathway or mechanism of activation of glucose transporters is not yet understood how insulin regulates the membrane distribution of glucose transporters, nor is it clear whether the intrinsic activity of the translocated glucose transporter proteins is also increased in response to insulin. Analysis of protein immunoblotting data obtained with antibodies directed against specific transporter isoforms suggests that insulin regulates both GLUT1 and GLUT4 in rat (Joost et al., 1988; James et al., 1989), and GLUT1 and GLUT4 in mouse (Blok et al., 1988).

It is not yet understood how insulin regulates the membrane distribution of glucose transporters, nor is it clear whether the intrinsic activity of the translocated glucose transporter proteins is also increased in response to insulin. Analysis of protein immunoblotting data obtained with antibodies directed against specific transporter isoforms suggests that insulin regulates both GLUT1 and GLUT4 in rat (Joost et al., 1988; James et al., 1989), and GLUT1 and GLUT4 in mouse (Blok et al., 1988). However, insulin regulation of GLUT1 has not been observed in a number of other cell types, including brain, human erythrocytes, and cultured HepG2 cells. Recent work by Oka and co-workers (Oka et al., 1988), Cushman and Wardzala (1980) and Suzuki and Kono (1980) originally proposed the hypothesis that insulin regulates glucose transport by stimulating the translocation of glucose transporter protein to an intracellular membrane pool to the plasma membrane in responsive adipoocytes. This hypothesis was based on measurements of cytochalasin binding to glucose transporters and reconstitution of transport activity from the different membrane fractions. Additional evidence for insulin-stimulated translocation was obtained by labeling glucose transporters covalently in intact cells (Oka and Czech, 1984; Holman et al., 1988; Calderhead and Lieuward, 1998) and by immunocytochemical analysis using electron microscopy (Blok et al., 1988).

Isolation of HepG2 Glucose Transporter cDNA and Heterologous Expression of the Human Glucose Transporter Protein in CHO Fibroblasts—Human HepG2 transporter cDNA was isolated from a HepG2 cDNA library (Kwiatowski et al., 1986). The library (1 \times 10^5 recombinants) was screened at moderate stringency, 42°C, 5 \times 10^{-2} M sodium citrate (1 \times X) and 40% saline. The majority of our clones hybridized to the 3' but not the 5' probes (26 of 250 clones with 0.5% X), and the sizes of these 3' and 5' cDNA fragments suggested to us that the internal EcoRI cleavage site in the HepG2 glucose transporter protein was not preserved during construction of the cDNA library. Thus, the cloned cDNAs represented the two predicted EcoRI fragments of the full-length HepG2 glucose transporter mRNA. These two cDNA fragments were ligated into the expression vector pGEM-3Z, and subsequent double-stranded sequence analysis confirmed our hypothesis. The 5' and 3' EcoRI cDNA fragments were then excised from the plasmids by digestion with AoaI and EcoRI.
and HindIII and EcoRI, respectively, and ligated together into Avai-HindIII cut pGEM-3Z. Limited double-stranded sequence analysis and restriction analysis of our pGEM-3Z-GT construct confirmed that the cDNA (2.1 kilobase pairs) was identical to the published sequence (Muekler et al., 1985) and encompassed the entire coding region of the HepG2 glucose transporter mRNA.

The cDNA was then excised from pGEM-3Z-GT by digestion with SmaI and BamHI. BamHI linker was ligated to the 5' end of the cDNA, and the glucose transporter cDNA was ligated into the BamHI-cloning site of the mammalian expression vector pLEN. The resultant expression vector, pLENGT, was transfected into subconfluent CHO-K1 fibroblasts using the calcium phosphate precipitation method essentially as described previously (Davis et al., 1986). CHO-K1 cells were seeded in 10 cm dishes that were preincubated with pLEN expression vector containing the bacterial neomycin-phosphotransferase gene fused to the simian virus 40 early promoter at a ratio of 20:1. The transfected cells were grown in F-12 medium containing 250 μg/ml G418. G418-resistant clones were then picked and analyzed for expression of the human HepG2 glucose transporter protein and 2-deoxyglucose uptake activity.

Preparation of Polyclonal Antisera—Rabbit and mouse polyclonal sera were generated by hyperimmunizing the animals with injections of purified human erythrocyte glucose transporter protein (Carruthers and Helgerson, 1989). Primary injections and boosts in rabbits were subcutaneously and contained 200 μg of protein in Freund's complete adjuvant. As subcutaneous injections in mice were subcutaneous and contained 20 μg of protein in Freund's complete adjuvant, and boosts were intraperitoneal and contained 20 μg of protein in Freund's incomplete adjuvant. These antisera were analyzed as described under "Materials and Methods." The rabbit serum was collected 3 weeks after the last injection, and the mouse serum was collected 4 weeks after the last injection.

Competition ELISA—Competition ELISA measurements of rabbit α- and β-sera were performed using purified human erythrocyte glucose carrier and intact human and rat erythrocytes. 50 ng of purified glucose transporter was applied to individual ELISA plate wells (Carruthers and Helgerson, 1989). α-Serum and β-serum were diluted 1:1000, 1:2000, and 1:4000, respectively. Each of these sera and the control serum was preincubated with 25 strokes of a glass pestle in a glass Dounce homogenizer. The membrane proteins were solubilized in sample buffer at room temperature and analyzed by SDS-PAGE using 10% polyacrylamide gels as described by Laemmli (1970) and transferred electrochemically to nitrocellulose at 200 mA for 3 h, essentially as reported by Towbin et al. (1979). The nitrocellulose was blocked with gelatin/bovine serum albumin/Tween 20-containing buffer (0.5% gelatin, 0.5% bovine serum albumin, 0.05% Tween 20, 250 mM NaCl, and 10 mM Tris base, pH 7.5) for 1 h, and the membranes were incubated with 1 mg/ml anti-human GLUT1 serum (1/2000) overnight at 4 °C. The nitrocellulose was washed extensively with Tris/gelatin/Tween 20 (10 mM Tris base, pH 7.5, 0.05% gelatin, 250 mM NaCl, and 0.05% Tween 20) and then incubated with 125I-protein A (1/500 dilution, Du Pont-New England Nuclear, low specific activity) for 1 h at room temperature. The immunoreactive proteins were visualized by autoradiography using Kodak XAR film and intensifying screens at -70 °C.

Protein immunoblot analysis of human erythrocyte glucose transporters, using rabbit α- and β-antisera, was performed as follows. Human red cell ghosts were prepared as described above. Ghosts (3 × 108) and CHO-K1 cells (3 × 106 cells) were washed and serum starved as described above. Total membrane immunoreactivity was prepared from control and transfected cells by scraping the cells off the culture plates into 5 ml of ice temperature Tris/EDTA/sucrose buffer (10 mM Tris base, pH 7.4, 1 mM EDTA, and 0.25 M sucrose). All subsequent membrane preparation procedures were performed at ice temperature. The protease inhibitor phenylmethylsulfonyl fluoride was added to the cell suspension (0.1 mM final concentration) immediately after harvesting to completely inhibit any protease activity with 25 strokes of a glass pestle in a glass Dounce homogenizer. The homogenate was then separated into supernatant and pellet fractions by centrifugation at 200,000 × g for 1.5 h. The high speed pellet was resuspended in Tris/EDTA buffer plus phenylmethylsulfonyl fluoride (0.1 mM final concentration) and then resuspended with 200 μl of ice-cold saline and resuspended in 200 μl of saline, and the radioactivity in 60 μl aliquots determined in triplicate. The experiment was repeated using two separate membrane and red cell preparations.

Anti-glucose Transporter Antibody Binding to Intact CHO-K1 Cells—CHO-K1 control and HepG2 glucose transporter-overexpressing CHO-K1 cell lines were plated at 100,000 or 50,000 cells/well in 24-well culture plates, grown for 48 or 72 h, and assayed for 2-deoxyglucose uptake and β-antibody binding. Cells were washed three times with phosphate-buffered saline (buffer A) and incubated in serum-free F-12 medium for 2 h at 37 °C. In studies involving insulin treatment, cells were serum starved for 2 h, and β-antibody binding was measured following incubation of the cells with 10−7 M insulin for 20 min at 37 °C. Antibody and 125I-protein A buffers also contained 10 mM sodium azide and 0.25 mM phenylmethylsulfonyl fluoride. Cells were then washed three times with Krebs-Ringer phosphate buffer and then poisoned for 20 min in Krebs-Ringer phosphate buffer containing 10 mM sodium azide and 20 mM 2-deoxyglucose at 37 °C. Following this incubation, the cells were washed with buffer A and incubated with either rabbit preimmune serum or β-antiserum (diluted from 1/250 to 1/2000) for 2 h at room temperature. The cells were then washed three times with buffer A and incubated for 1 h with 125I-protein A (1/50 dilution of Du Pont-New England Nuclear low specific activity preparation) at room temperature. The wells were then washed again three times with buffer A, and the bound 125I-protein A was solubilized in 0.1% SDS and the radioactivity determined in a γ-counter. Nonspecific binding (preimmune serum) was subtracted from total binding for each cell line.

Each assay point was determined in triplicate.

Glucose Transporter Protein Immunoblot Analysis Human erythrocyte ghosts were prepared as described by Carruthers and Melchior (1983). Control and transfected CHO-K1 cell membrane proteins were solubilized in sample buffer at room temperature for 40 min, resolved by SDS-PAGE using 10% polyacrylamide gels as described by Laemmli (1970), and transferred electrochemically to nitrocellulose at 200 mA for 3 h, essentially as reported by Towbin et al. (1979). The nitrocellulose was blocked with gelatin/bovine serum albumin/Tween 20-containing buffer (0.5% gelatin, 0.5% bovine serum albumin, 0.05% Tween 20, 250 mM NaCl, and 10 mM Tris base, pH 7.5) for 1 h, and the membranes were incubated with 1 mg/ml anti-human GLUT1 serum (1/2000) overnight at 4 °C. The nitrocellulose was washed extensively with Tris/gelatin/Tween 20 (10 mM Tris base, pH 7.5, 0.05% gelatin, 250 mM NaCl, and 0.05% Tween 20) and then incubated with 125I-protein A (1/500 dilution, Du Pont-New England Nuclear, low specific activity) for 1 h at room temperature. The immunoreactive proteins were visualized by autoradiography using Kodak XAR film and intensifying screens at -70 °C.

Quantitative immunoblot analysis of human erythrocyte glucose transporters, using rabbit α- and β-antisera, was performed as follows. Human red cell ghosts were prepared as described above. Ghosts (3 × 106 cells) and CHO-K1 cells (3 × 106 cells) were dissolved in sample buffers at ice temperature for 5 min, resolved by SDS-PAGE using 10% polyacrylamide gels as described by Laemmli (1970), and transferred electrochemically to nitrocellulose for 1 h. The nitrocellulose was divided in two, and each half was immunoblotted using either α- or β-serum (1000-fold dilution). The immunoreactive proteins were visualized for autoradiography using Kodak XAR film and intensifying screens at -70 °C.

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Quantitative immunoblot analysis of CHO-K1 control and four pLENGT-transfected CHO-K1 cell lines was performed as follows. Cells were plated at 50,000 cells/150-mm plate, grown to near confluence, and assayed for total GLUT1 immunoreactive protein. Cells were washed and serum starved as described above. Total membrane immunoreactivity was prepared from control and transfected cells by scraping the cells off the culture plates into 5 ml of ice temperature Tris/EDTA/sucrose buffer (10 mM Tris base, pH 7.4, 1 mM EDTA, and 0.25 M sucrose). All subsequent membrane preparation procedures were performed at ice temperature. The protease inhibitor phenylmethylsulfonyl fluoride was added to the cell suspension (0.1 mM final concentration) immediately after harvesting to completely inhibit any protease activity with 25 strokes of a glass pestle in a glass Dounce homogenizer. The homogenate was then separated into supernatant and pellet fractions by centrifugation at 200,000 × g for 1.5 h. The high speed pellet was resuspended in Tris/EDTA buffer plus phenylmethylsulfonyl fluoride (0.1 mM final concentration) and then resuspended with 200 μl of ice-cold saline and resuspended in 200 μl of saline, and the radioactivity in 60 μl aliquots determined in triplicate. The experiment was repeated using two separate membrane and red cell preparations.
RESULTS

Development of a Novel Antitransporter Antiserum That Recognizes Exofacial Epitopes—Erythrocyte glucose transporter protein was purified from human red cells and injected into rabbits as described under "Experimental Procedures." Preimmune and immune sera harvested from these animals were analyzed for their ability in competition ELISA and protein immunoblots and for their ability to bind to intact human red blood cells. Two of the rabbit antisera, α- and δ-, were found to be useful in these immunoassays of the erythrocyte glucose transporter GLUT1. Human erythrocyte proteins were solubilized in SDS sample buffer, resolved on 10% acrylamide gels, transferred to nitrocellulose filters, and immunoblotted with either α- or δ-antisera. As can be seen in Fig. 1, both of these polyclonal sera recognize a broad band of erythrocyte protein with an apparent molecular weight of 45,000-60,000. Identical results were observed using purified erythrocyte glucose transporter protein (data not shown).

The α- and δ-antisera were then tested by competition ELISA for their ability to recognize purified human erythrocyte glucose transporter protein. Fig. 2A demonstrates that both α- and δ-antisera bind to nondenatured glucose transporter protein to a similar extent and that purified erythrocyte glucose transporter competes equally for α- and δ-antibody binding to the solid phase glucose transporter protein. In a parallel experiment, intact erythrocytes were substituted for purified glucose transporter protein and incubated with α- or δ-antisera prior to the addition of the antisera to microtiter wells. Results from this experiment are shown in Fig. 2B. Intact human (circles) and rat (triangles) red blood cells depleted the δ- but not the α-serum, of anti-human erythrocyte glucose transporter immunoglobulins. This unexpected finding suggested that all of the anti-glucose transporter antibodies in the δ-serum and few or no anti-glucose transporter antibodies in the α-serum were binding to extracellular epitopes or domains of the erythrocyte glucose transporter protein.

An alternative method for testing the ability of the δ-antisera to bind to an extracellular domain(s) on the glucose transporter protein was to immunoprecipitate intact red blood cells bound to protein A-Sepharose beads. In this experiment, protein A-Sepharose beads were incubated with either α- or δ-serum preincubated with purified red cell glucose carrier, washed extensively, and then incubated with intact human red blood cells. These beads were then washed, placed on microscope slides, and examined by phase-contrast microscopy. Results are shown in Fig. 3 for protein A-Sepharose beads treated with α-serum, δ-serum, or δ-serum plus purified human glucose carrier protein. The results shown here are typical of two experiments made using separate batches of red cells and protein A-Sepharose. No red cell binding to Sepharose beads was observed for the α-serum control (left panel). Preimmune serum resulted in red cell adsorption that was indistinguishable from that observed with α-serum (not shown). δ-Serum-treated beads were extensively decorated with human red cells (center panel). Preadsorption of the δ-serum-treated beads with purified glucose transporter protein effectively competed for all red cell binding to the protein A-Sepharose (right panel).

Finally, in order to compare quantitatively the binding of the α-serum and δ-serum with intracellular and extracellular glucose transporter domains, these sera were incubated with
C and then washed as above. The Sepharose beads were finally incubated with human red cells (1 x 10^7 cells in saline) for 30 min at 37 °C. Rabbit α-antiserum or 8 ~1 of rabbit α-antiserum for 30 min at 37 °C. That had been first treated with rabbit α-antiserum was additionally incubated with 40 μg of purified glucose transporter for 30 min at 37 °C and then washed as above. The Sepharose beads were finally incubated with human red cells (1 × 10^7 cells in saline) for 30 min at 37 °C. Samples were placed on microscope slides, examined by phase-contrast microscopy at a magnification of ×400, and photographed. Results are shown for protein A-Sepharose beads treated with α-antiserum, δ-antiserum, and δ-antiserum plus purified glucose carrier. The results shown here are typical of two experiments performed using separate batches of red cells and protein A-Sepharose.

The results are shown as mean ± standard deviation. Preimmune serum bound only to a glucose transporter domain or domains located inside red cells, as evidenced by 125I-protein A for 1 h at 20 °C. Parallel experiments demonstrated that protein A binding reached equilibrium by this time and that protein A binding was not limited by the concentration of protein A in solution (not shown). The membranes were washed five times in 10 ml of ice-cold saline, resuspended in 200 μl of saline, and 60-μl aliquots counted in triplicate. The experiment was repeated using two separate membrane and red cell preparations. The results are shown as mean ± standard deviation. Preimmune serum control determinations resulted in background activities of 343–388 cpm.

Intact red cells, sealed red cell ghosts, leaky red cell ghosts, and sealed inside-out red cell membrane vesicles were incubated in the presence of α- or δ-serum for 1 h at 20 °C (10 μl/1 × 10^6 packed red cells or 10 μl/60 μg of membrane protein). These conditions were determined in preliminary experiments to reflect both equilibrium and saturated IgG binding (not shown). The cells and membranes were washed in ice-cold saline and then incubated with 125I-protein A for 1 h at 20 °C. Parallel experiments demonstrated that protein A binding reached equilibrium by this time and that protein A binding was not limited by the concentration of protein A in solution (not shown). The membranes were washed five times in 10 ml of ice-cold saline, resuspended in 200 μl of saline, and 60-μl aliquots counted in triplicate. The experiment was repeated using two separate membrane and red cell preparations. The results are shown as mean ± standard deviation. Preimmune serum control determinations resulted in background activities of 343–388 cpm.

FIG. 3. Immunoprecipitation of intact human red cells by rabbit δ-antibody-protein A-Sepharose bead complexes and its inhibition by purified glucose transport protein. Protein A-Sepharose beads were incubated in 1 ml of saline containing 10 μl of rabbit α-antiserum or 8 μl of rabbit δ-antiserum for 30 min at 37 °C. The beads were then washed in ice-cold saline. One aliquot of beads that had been first treated with rabbit δ-antiserum was additionally incubated with 40 μg of purified glucose transporter for 30 min at 37 °C and then washed as above. The Sepharose beads were finally incubated with human red cells (1 × 10^7 cells in saline) for 30 min at 37 °C. Samples were placed on microscope slides, examined by phase-contrast microscopy at a magnification of ×400, and photographed. Results are shown for protein A-Sepharose beads treated with α-antiserum, δ-antiserum, and δ-antiserum plus purified glucose carrier. The results shown here are typical of two experiments performed using separate batches of red cells and protein A-Sepharose.
brane proteins with either a GLUT1 C-terminal peptide anti-
serum (Fig. 5B) or an antisem specific for human GLUT1
(Fig. 5C) confirmed that it was the human glucose transport
protein that was overexpressed (Fig. 5C, lanes 4–9) and not
host glucose transporter protein. Note that no CHO host
glucose transport protein was detected by the mouse serum
(Fig. 5C, lanes 2 and 3), but the CHO GLUT1 protein was
clearly detected by the C-terminal peptide antisem (Fig. 5B,
lanes 2 and 3). This experiment also illustrates the low level
of expression of human GLUT1 in the CHO GT26 cells (Fig.
5, B and C, lane 9). Additionally, no rat GLUT4 protein was
detected in the CHO-K1 cells by immunoblot analysis with
the monoclonal antibody IF8 or with anti-rat GLUT4 C-
terminal peptide serum (not shown).4

Quantitative immunoblot analysis indicates that overex-
pression of the human GLUT1 protein in these cell lines
ranges from 2-fold (CHO-GT26) to 17-fold (CHO-GT3). As-
suming equivalent binding of the C-terminal peptide antibo-
dies to the denatured erythrocyte ghost glucose transporter
and to the expressed HepG2 glucose transporter protein (Gould et
al., 1980) and a M, = 55,000 for each of these proteins (Fig.
5), and assuming that 2% of the erythrocyte ghost protein
preparation is GLUT1 protein (Helgerson and Carruthers,
1987), the calculated numbers of GLUT1 proteins/cell in the
control CHO-K1 and in CHO-GT3 cells are approximately
7.3 × 10⁴ and 1.2 × 10⁵, respectively.

Quantitative Comparisons among Total Cellular Expressed
GLUT1 Protein, Cell Surface GLUT1 Protein, and 2-Deoxy-
glucose Uptake—Experiments were conducted to determine
whether the polyclonal antibody-binding assay could detect the
expressed human glucose transporter protein on the surface
of CHO-GT cells (Fig. 6). Cell surface expression of GLUT1 was
measured in CHO-K1 control, CHO-GT1, and CHO-GT3 cells,
using a range of dilutions of 125I-serum from 250- to 2000-
fold. Specific, saturable polyclonal antibody binding was ob-
served for each of the three cell lines tested. At these antibody dilutions,
overexpression of cell surface GLUT1 was observed in cell
lines CHO-GT1 and CHO-GT3 relative to the CHO-K1 con-
trol. The magnitude of ¹²⁵I-protein A binding in the cell
culture wells was dependent upon polyclonal antibody dilution (Fig. 6).

The relationship among levels of expression of total immu-
noreactive GLUT1 protein, cell surface δ-antiserum binding,
and 2-deoxyglucose uptake activity were measured in
various transfected cell lines (Fig. 7). CHO-K1 control and
CHO-GT cell lines were plated on the same day in paired 24-
well culture dishes, grown in culture for 48 h, and assayed for
δ-antiserum binding and 2-deoxyglucose uptake, as described un-
der "Experimental Procedures." Total immunoreactive
GLUT1 was determined by quantitative immunoblot analysis
using anti-GLUT1 C-terminal peptide serum. The results of
these three assays are plotted as fold increases over values
measured in control cells (CHO-K1 = 1). Levels of expression
of total immunoreactive GLUT1 protein ranged from approxi-
mately 2-fold over those of control in cell line CHO-GT26 to
17-fold in cell line CHO-GT3, with two other cell lines
expressing intermediate levels of GLUT1. CHO-GT26 cells
expressed 1.6-fold greater 2-deoxyglucose uptake activity and
1.6-fold greater cell surface antibody binding than CHO-K1
controls, and similar good correlations between levels of
expression of sugar uptake and intact cell δ-antibody binding
were observed in the CHO-GT cell lines expressing much
higher levels of human GLUT1 protein (Fig. 7). It was also
observed that increasing levels of expression of total immu-
noreactive glucose transporter protein in different cell lines
resulted in increasing levels of expression of 2-deoxyglucose
uptake and cell surface antibody binding to intact cells. Note
that at low levels of human GLUT1 expression, 1.9-fold
increases in sugar transporter protein result in 1.6-fold
increases in sugar uptake, whereas 9–12-fold increases in
GLUT1 protein expression result in 4–5-fold increases in
sugar uptake. Similar relationships between 2-deoxyglucose
uptake by CHO-K1, CHO-GT1, and CHO-GT3 were observed
when uptake was measured at 2 mM substrate (not shown),4
suggesting that hexokinase activity was not saturated in these
cells under our experimental conditions.

Fig. 6. Rabbit δ-antiserum binding to intact CHO-K1 cells.
CHO K1 control and transfected CHO K1 cell lines were grown in
culture for 48 h as described in detail under "Experimental Proce-
dures." Cells were washed three times with phosphate-buffered saline
and serum starved for 2 h at 37°C. Cell monolayers were washed
again once and then poisoned for 20 min in buffer containing 10 mM
sodium azide and 20 mM 2-deoxyglucose at 37°C. Following this
incubation, the cells were washed with phosphate-buffered saline,
incubated with either rabbit preimmune serum or δ-antiserum (di-
luted from 1/250 to 1/2000) for 2 h at room temperature. The cells
were then washed with phosphate-buffered saline and incubated for
1 h with ¹²⁵I-protein A at room temperature. The wells were washed
three times with phosphate-buffered saline, and the bound ¹²⁵I-protein
A was solubilized in 0.1% SDS and counted in a γ-counter. Nonspe-
ific binding was subtracted from total binding for each cell line.
Each assay point is the average of three determinations.

Fig. 7. Levels of 2-deoxyglucose uptake, cell surface
GLUT1, and total immunoreactive GLUT1 protein in trans-
fected CHO-K1 cells. CHO-K1 control and pLENGT-transferred
CHO-K1 cell lines were assayed for 2-deoxyglucose uptake, rabbit δ-
antibody binding, and total GLUT1 immunoreactive protein, as de-
scribed in detail under "Experimental Procedures." Specific 2-deoxy-
glucose uptake and antibody-binding values were determined by
subtracting appropriate nonspecific control values, as described under
"Experimental Procedures." The results of these three assays are
plotted as fold increases over control (CHO-K1 = 1). These results
are representative of two experiments, with eight determinations of
2-deoxyglucose uptake and six determinations of ¹²⁵I bound to intact
cells for each experiment.

4 S. A. Harrison, J. M. Buxton, and M. P. Czech, unpublished data.
is not accompanied by increases in immunoreactive GLUT1 ~3.5%. These data demonstrate that insulin-stimulated glucose transport activity in control and transfected CHO cells h-antibody-binding determinations (six determinations in cell lines tested in these experiments. In the absence of insulin, increases of 1.6- and 5-fold in s-antibody binding to

The absolute increase in the rate of deoxyglucose uptake due to protein (9- and 17-fold over controls, respectively, Fig. 7). Cells were serum starved for 2 h, and sugar uptake was measured following incubation of the cells with 10^{-7} M insulin for 20 min at 37 °C. As shown in Fig. 8, insulin stimulated 2-deoxyglucose uptake 40% in both CHO-K1 control cells and in CHO-GT26 cells that express human GLUT1 at levels about 2-fold over controls. Insulin stimulated 2-deoxyglucose uptake 10% in CHO-GT1 cells, and CHO-GT3 cells were insensitive to insulin in these experiments. These latter two cell lines exhibited much higher levels of human GLUT1 protein (9- and 17-fold over controls, respectively, Fig. 7). The absolute increase in the rate of deoxyglucose uptake due to insulin in CHO-GT1 cells were approximately equal to that measured in the CHO-GT26 cells (Fig. 8A, legend). Insulin failed to detectably stimulate β-antibody binding to any of the cell lines tested in these experiments. In the absence of insulin, increases of 1.6- and 5-fold in β-antibody binding to CHO-GT26 cells and CHO-GT1 cells, respectively, were observed compared with control cells, consistent with the increase in basal sugar uptake measured in those cells. In all β-antibody-binding determinations (six determinations in each experiment) the standard errors of the mean were always <3.5%. These data demonstrate that insulin-stimulated glucose transport activity in control and transfected CHO cells is not accompanied by increases in immunoreactive GLUT1 at the cell surface.

**DISCUSSION**

The results presented in this report demonstrate the development of a novel anti-GLUT1 antisera that recognizes one or more extracellular epitopes on the GLUT1 protein. Intracellular epitopes on GLUT1 are not recognized by the antisera. This rabbit β-antisera preparation is unique in that most antisera raise against the erythrocyte glucose transporter previously appear to recognize intracellular domains on the red cell glucose transporter. The rabbit α-antisera described in the present work is an example of this type of antisera. The GLUT1 protein was examined in control CHO fibroblasts and in CHO fibroblasts transfected with the human GLUT1 expression vector and pLENGT. Using specific antibody reagents, we were able to monitor total cellular and cell surface host (hamster) GLUT1 in control cells, total cellular and cell surface hamster plus human GLUT1 in transfected cells, and total human GLUT1 in transfected cells. Hoet and heterologously expressed human GLUT1 proteins were detected by antipeptide antisera directed against GLUT1 C-terminal 12 amino acids and by the rabbit polyclonal antisera (β) described above (Fig. 5, A and B). The expressed human GLUT1 was also detected by a mouse polyclonal antisera raised against purified human erythrocyte glucose transporter. This latter antisera is highly selective for human GLUT1, and it did not react with the rodent GLUT1 (Fig. 6C). James and co-workers (1988) developed a monoclonal antibody, IF8, by immunizing mice with partially purified low density microsomal proteins from insulin-treated rat adipocytes. Their antibody is specific for GLUT4 protein, prevalent in fat and muscle, and it does not recognize GLUT1 from rat, mouse, or human tissues (James et al., 1988, 1989; Zorzano et al., 1989). In our studies, no GLUT4 protein was detected in CHO-K1 cells using the antiadipocyte glucose transporter monoclonal antibody or an anti-rat GLUT4 C-terminal peptide serum (James et al., 1989).

The specific antibody reagents described above were used to determine whether 1) increased expression of GLUT1 in transfected CHO cells leads to increased numbers of cell surface GLUT1 proteins; and 2) the expressed GLUT1 transporters are functional. We observed excellent correlations in numerous cell lines among overexpression of human GLUT1.
total immunoreactive protein, cell surface GLUT1 protein, and 2-deoxyglucose uptake (Fig. 7). These data demonstrate that the expressed human GLUT1 protein is processed and exported to the surface of the transfected fibroblasts and that these cell surface carrier proteins are capable of transporting deoxyglucose across the plasma membranes of these cells.

The pLENGT mammalian expression vector contains metal-inducible human metallothionein gene II-promoting sequences, and zinc and calcium were expected to induce expression of GLUT1 in cells transfected with this vector. As expected, transporter expression from this vector in 3T3-L1 cells exhibited very low constitutive expression, and high levels of zinc (75–125 μM) were required to achieve 4–8-fold overexpression of glucose transporter protein.5 However, in the transfected CHO cells used in the present study, expression of the transporter protein was constitutively very high in the absence of the added metals. Addition of zinc or cadmium had little or no further effect on GLUT1 expression. Similarly, Oka and co-workers observed constitutively high levels of rabbit GLUT1 in CHO cells transfected with an expression vector containing mouse metallothionein gene I-promoting sequences (Asano et al., 1989). We have no documented explanation for this phenomenon at present.

It is unclear whether the insulin sensitivity of sugar uptake in various types of cells, including muscle, adipocyte, and fibroblasts, is dependent upon glucose transporter protein primary structure, cell-specific regulatory machinery, or both. Insulin stimulation of glucose uptake in adipocytes occurs in association with recruitment of GLUT4 from intracellular membranes to membranes at the cell surface (James et al., 1988; Zorzano et al., 1989). Only 5–10% of the glucose transporters present in these cells are GLUT1 proteins (Oka et al., 1988; Zorzano et al., 1989). Insulin stimulation of glucose uptake has also been observed in CHO (Fig. 5) and 3T3-L1 fibroblasts7 (James et al., 1989) that contain GLUT1 but not GLUT4. However, insulin stimulation of glucose uptake has not been observed in a number of other GLUT1-containing cells including brain, human erythrocytes, and cultured HepG2 cells. Recent data by Oka and co-workers indicate that when GLUT1 isolated from rabbit brain (97.5% identity with the IhepG2 glucose transporter (Asano et al., 1989)) is expressed in CHO fibroblasts, both the CHO control and the expressed rabbit GLUT1 deoxyglucose uptake activities are stimulated approximately 40% by insulin (Asano et al., 1989). Similarly, insulin caused translocation of human GLUT1 expressed in differentiated 3T3-L1 cells (Gould et al., 1989), but the GLUT1 contribution to insulin-stimulated sugar uptake was not examined. We found that 100 nM insulin increased 2-deoxyglucose uptake approximately 40% in control CHO-K1 cells and in CHO-K1 cells expressing the human GLUT1 protein 2-fold over endogenous host GLUT1 protein (Fig. 8A). These results strongly suggest that a human glucose transporter protein, GLUT1, which is not responsive to insulin in HepG2 cells, is regulated by insulin when expressed at low levels in Chinese hamster ovary cells. Thus, specific insulin-responsive cellular processes rather than transporter isoyme structure appear to be involved in transporter regulation by this hormone.

Although the evidence cited above supports the conclusion that membrane distributions of both GLUT4 and GLUT1 are acutely regulated by insulin fat and muscle (Joost et al., 1988; James et al., 1988, 1989; Blok et al., 1988; Gould et al., 1989; Birnbaum, 1989; Zorzano et al., 1989), it is not yet clear whether insulin regulates GLUT4 and GLUT1 by similar or different mechanisms. Additionally, the mechanism(s) of insulin stimulation of glucose uptake in fibroblasts is unknown.

We have used control and pLENGT-transfected CHO fibroblasts and the rabbit 5 antibody that binds to an extracellular GLUT1 domain to ask whether insulin stimulates membrane redistribution of GLUT1 protein in these fibroblasts. No insulin stimulation of cell surface rabbit 5-antibody binding was detected in any of the several control or transfected CHO-K1 cell lines employed in these studies (Fig. 8B). No difference in 5-antibody binding was observed between control and insulin-treated cells poisoned with sodium azide and 2-deoxyglucose or cooled rapidly to 4 °C with ice-cold buffer prior to assay with 5-antibody. These data suggest that unlike rat and mouse adipocytes, insulin does not increase 2-deoxyglucose uptake by stimulating the membrane redistribution of GLUT1 proteins in CHO-K1 fibroblasts.

The data presented in this report also indicate that the HepG2 glucose transporter protein is regulated by insulin when expressed at low but not at high levels in insulin-responsive CHO-K1 cells. In cells expressing human GLUT1 protein 9-fold over controls, insulin stimulation of sugar uptake was only 10%, and no insulin stimulation of sugar uptake was detected in several cell lines expressing very high levels (12–17-fold) of human GLUT1 protein (Fig. 8A). This loss of insulin-regulated sugar transport in cells expressing very high levels of glucose transporter protein suggests that the cell-specific machinery responsible for insulin stimulation of glucose transport may be down-regulated or inhibited by chronically high levels of intracellular glucose, glucose metabolites, or glucose transporter proteins. Oka and co-workers observed insulin regulation of the heterologously expressed rabbit GLUT1 activity at high (4-fold) and low (2-fold) levels of overexpression (Asano et al., 1989). It is not yet clear why our results differ from theirs, but we have examined a number of variables including cell density, insulin concentration dependence, assay time, 2-deoxyglucose concentration, and time of serum starvation for possible effects on insulin sensitivity of our cells. None of these variables appears to account for the differences between our findings.

The presence of multiple forms of mammalian facilitative glucose transporters raises interesting questions regarding their structural, functional, and regulatory characteristics. Little is known about the developmental regulation of the different glucose transporter isoforms or about their distinctive functional characteristics. A number of recent reports suggest that specific glucose transporter isoforms predominate in individual cells and that transformation (Birnbaum et al., 1987; Flier et al., 1987), hormonal stimulation (Hirakii et al., 1988; Garcia de Herreros and Birnbaum, 1989; Walker et al., 1989), and a variety of metabolic stresses (Walker et al., 1989; Rhoads et al., 1988) may differentially regulate their expression. The rabbit 5-antiserum developed here should be a useful tool for monitoring the cell surface disposition of GLUT1 under a variety of such conditions. It should also be useful in assessing the contribution of cell surface GLUT1 to the regulation of glucose transport by insulin in highly sensitive systems such as differentiated mouse 3T3-L1 cells.

Acknowledgments—We wish to thank Dr. T. White for his gift of the mammalian expression vector pLENG, Dr. F. Felch for his gift of the monoclonal antibody IF8; Dr. D. Kwiatkowski for his gift of the HepG2 cDNA library; and Drs. R. Heller-Harrison, B. Clancy, C. W. Woon, and K. Davis for helpful discussions.

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