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

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Complementary DNA encoding a facilitative glucose transporter was isolated from a human hepatoma cell line (HepG2) cDNA library and subcloned into a metal-inducible mammalian expression vector, pLEN (California Biotechnology) containing human metallothionein gene II promoter sequences. Chinese hamster ovary (CHO) cells transfected with this transporter expression vector, pLENGT, exhibited a 2–17-fold increase in immunoreactive HepG2-type glucose transporter protein, as measured by protein immunoblotting with antipeptide antibodies directed against the HepG2-type glucose transporter C-terminal domain. Expression of the human glucose transporter was verified by protein immunoblotting with a mouse polyclonal antisera that recognizes the human but not the rodent HepG2-type transporter. 2-Deoxy-D-glucose uptake was increased 2–7-fold in transfected cell lines. Polyclonal antiserum directed against purified red blood cell glucose transporter were raised in several rabbits. Antiserum from one rabbit, 5, was found to bind to the surface of intact red cells but not to inside-out red cell ghosts. Using this 5-antiserum in intact cell-binding assays, 1.6–9-fold increases in cell surface expression of the human glucose transporter were measured in CHO-K1 cell lines transfected with the transporter expression vector. Measurements of total cellular glucose transporter immunoreactive protein using anti-HepG2 transporter C-terminal peptide serum, cell surface glucose transporter protein using 5-antiserum and 2-deoxyglucose uptake revealed proportional relationships among these parameters in transfected cell lines expressing different levels of transporter protein.

Insulin increased 2-deoxyglucose uptake 40% in control CHO-K1 cells and in CHO-K1 cells expressing modest levels of the human glucose transporter protein. However, stimulation of sugar uptake by insulin was only 10% in cells overexpressing human glucose transporter protein 9-fold, and no effect of insulin on sugar uptake was detected in several cell lines expressing very high levels (12–17-fold over controls) of human HepG2 glucose transporter protein. No insulin stimulation of anti-cell surface glucose transporter antibody binding was detected in any control or transfected CHO-K1 cell lines. These data indicate that a glucose transporter protein that is insensitive to insulin in HepG2 cells is regulated by insulin when expressed at low but not at high levels in insulin-response CHO-K1 cells. Additionally, the results suggest that insulin does not increase 2-deoxyglucose uptake by increasing the number of cell surface HepG2-type glucose transporters in CHO-K1 fibroblasts.

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 (Fukomoto et al., 1988; Thorens et al., 1988), human fetal skeletal muscle (Kayano et al., 1988), rat adipocyte and muscle (James et al., 1988, 1989; Charron et al., 1989), human muscle (Fukumoto et al., 1989), and mouse adipocyte (Kaeshter et al., 1989). Amino acid sequences deduced from these cDNA sequences have revealed four distinct isoatypes 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, 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) and mouse (Fukomoto et al., 1989; Zorzano et al., 1989). 3) A glucose transporter denoted as GLUT3 is prevalent in skeletal muscle, heart, and brain. The GLUT3 protein has been identified in rat, mouse, and human (James et al., 1988, 1989; Zorzano et al., 1989). 4) A glucose transporter denoted as GLUT4 is prevalent in adipose and muscle tissues. The GLUT4 protein has been identified in rat, mouse, and human (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 isoatypes, even within a given species, ranges from 50 to 65%. These preliminary classifications are not meant to imply that the identical...

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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 functions 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 isotypes divergent? One of the major regulators of hexose uptake in insulin, which stimulates facilitative glucose transporter activity in skeletal muscle and in adipose tissues (Simpson and Cushman, 1986). These effects contribute to the lowering of blood glucose in intact animals by this hormone. The signal transduction pathway or mechanism of activation of glucose transport by insulin has not yet been elucidated at the molecular level. 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 from an intracellular membrane pool to the plasma membrane in responsive adipocytes. This hypothesis was based on measurements of cytochalasin B binding to glucose transporters and by 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 Lieuhand, 1988) and by immunocytochemical analysis using electron microscopy (Blob 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 isotypes suggests that insulin regulates both GLUT1 and GLUT4 in rat (Joost et al., 1988; James et al., 1989) and mouse (Blob et al., 1988; James et al., 1989) adipocytes. However, insulin regulation of GLUT1 has not been observed in a number of other cells, including brain, human erythrocytes, and cultured HepG2 cells. Recent work by Oka and co-workers indicates that rabbit GLUT1 expressed in CHO fibroblasts is stimulated by insulin (Asano et al., 1989). Similarly, insulin causes translocation of human GLUT1 expressed in differentiated 3T3-L1 cells (Gould et al., 1989), but the regulatory mechanisms underlying hormonal sensitivity are unknown.

The present work describes our recent progress toward the development of new CHO-K1 fibroblast phenotypes and novel antibody reagents that allow us to address some of these problems. Complementary DNA encoding a facilitative glucose transporter was cloned from an insulin-unresponsive HepG2 cell cDNA library and then subcloned into a mammalian expression vector, pLEN. CHO-K1 fibroblasts transfected with the HepG2 transporter expression vector pLENt exhibited high levels of expression of HepG2 glucose transporter protein and activity. A novel anti-glucose transporter antiserum that recognizes GLUT1 exposed on the exofacial surface of intact human erythrocytes and CHO fibroblasts was developed and used to assess cell surface expression of human GLUT1. We report here that insulin stimulates human GLUT1 expressed at low but not at high levels in hormonally responsive CHO fibroblasts and that this stimulation occurs without a concomitant increase in cell surface immunoreactive GLUT1 transporters.

**Experimental Procedures**

**Materials**—2-Deoxy-D-glucose, cytochalasin B, protein A-Sepharose, and phloretin were purchased from Sigma. Low specific activity insulin (Sigma) (5.31 μCi/μg) and NaCl and 100,000 Ci/mmol) and 40% deoxynucleotides (800 Ci/mmol) were purchased from Du Pont-New England Nuclear. 2-Deoxy-D-[2-3H]glucose (30-60 Ci/mmol) was purchased from Amersham Corp. Restriction enzymes, 14 DNA ligase, polynucleotide kinase, klenow fragment of DNA polymerase, and BamHI linkers were purchased from Boehringer Mannheim and New England Biolabs. CHO-K1 fibroblasts and the expression vector pRSVneo were purchased from American Type Culture Collection. Porcine insulin was a gift from Dr. Ronald Chance, Lilly Research Laboratories, Inc. Rabbit antisera R495 and R820 were purchased from East Acres Biologicals. Double-stranded DNA sequencing vectors, pGEM-3Z, deoxynucleotides, and dideoxynucleotides were purchased from Promega Biotec. The mammalian expression vector pLEN was a gift from Dr. Ty White, California Biotechnology. DNA synthesis was performed by the Recombinant DNA Core Facility at the University of Massachusetts Medical Center. Tissue culture media, antibiotics, and fetal bovine serum were purchased from Gibco.

The HepG2 cDNA library cloned into A/10 was a gift from Dr. D. Kwiatkowski.

**Assay of 2-Deoxyglucose Uptake in CHO Cells**—CHO-K1 cells were maintained in culture in Ham's F-12 medium containing 10% fetal bovine serum, and sugar transport assays in CHO fibroblasts were performed essentially as described previously by Oka and co-workers (James et al., 1988). Cells (1.5 × 10^6) were plated with 0.5% agar in 24-well cluster plates and grown in culture for 2 or 3 days, respectively. Prior to initiating uptake assays, cells were washed three times with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM NaH2PO4, 0.88 mM CaCl2, and 0.49 mM MgCl2, pH 7.4) and incubated in 0.5 ml of serum-free F-12 medium for 1.5-2 h at 37 °C. The cells were washed once with 0.4 ml of Krebs-Ringer phosphate buffer containing 130 mM NaCl, 5 mM KCl, 1.3 mM CaCl2, 1.3 mM MgSO4, and 10 mM NaH2PO4, pH 7.4, and incubated in 0.5 ml of this buffer ± 100 ng insulin for 20 min at 37 °C. Sugar uptake was initiated by the addition of 2-deoxy-D-[2-3H]glucose, pH 7.4, and incubated in 0.5 ml of this buffer ± 100 ng insulin for 20 min at 37 °C. Assays were terminated by two rapid washes (0.5-1 ml/well each) with ice-cold Krebs-Ringer phosphate buffer. Cells were solubilized with 0.4 ml of 0.1% SDS, and 3H was detected in 4 ml of scintillant. Nonspecific deoxyglucose uptake was measured in the presence of 20 μM cytochalasin B and 300 μM phloretin and was subtracted from each determination to obtain specific uptake.

**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 (Kwiatkowski et al., 1986). The library (1 × 10^7 recombinants) was screened at moderate stringency, 42 °C, 5 × saline sodium citrate (1 × = 0.14 M NaCl and 0.01 M Na citrate), 2× sodium phosphate, and 0.5% sodium dodecyl sulfate, using 5′- and 3′-coding region oligonucleotide probes, essentially as described previously (Maniatis et al., 1982). The 5′′ oligonucleotide sequence was 5′-GCGTAGATGACTCCAGTGTT-3′, and the 3′ sequence was 5′-GAGGACGAACACGACCACACAA-3′, and 5′-CTCGAG-GTTTCTGCTGCTGGTCTGGCAGTTAGCGCTACGTGCTGAGCCAGC-3′. These probe sequences were derived from the published HepG2 glucose transporter cDNA sequence of Muekler et al. (1985). Approximately 100 putative positive plaques were identified from 1.5 × 10^9 plaques screened by hybridization to a mixture of the three 32P-labeled probes. Individual phage colonies were cloned from these plaques by limiting dilution, and the DNA was then digested with the restriction enzyme EcoRI. The cDNA fragments were then resolved by electrophoresis on 1% agarose gels, transferred to nitrocellulose, and hybridized to the individual 5′ and 3′ probes. None of the isolated clones contained full-length coding cDNAs for the HepG2 glucose transporter.

The majority of our clones hybridized to the 3′ but not the 5′ probe. A few clones did hybridize to the 5′ probe; however, these were not retained due to the sizes of the 5′ and 3′ cDNA fragments suggested to us that the internal EcoRI cleavage site in the HepG2 glucose transporter mRNA (Muekler et al., 1985) was not protected 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 fragments were inserted into the mammalian 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 AvoI 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 (Mueckler 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 SstI and BamHI, BamHI linkers were 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 pLLEN. The resulting 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 infected with 200 ng of the 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 cloned and analyzed for expression of the human HepG2 glucose transporter protein and 2-deoxyglucose uptake activity.

Preparation of Polyclonal Antiserum—Rabbit and mouse polyclonal sera were generated by hyperimmunizing the animals with injections of purified human erythrocyte glucose transporter protein (Carruthers and Helgerson, 1989). The antisera were then diluted 5,000- and 4,000-fold, respectively, in 500 μl of phosphate buffer (Carruthers and Helgerson, 1989) and incubated for 2 h at room temperature. The sera were then washed three times with buffer A and incubated for 1 h with 125I-protein A (1/500 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 y-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 10 min, resolved by SDS-PAGE using 10% polyacrylamide gels as described by Laemmli (1970), and transferred electrophoretically 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, 0.5% gelatin, 250 mM NaCl, and 0.05% Tween 20) and then incubated with anti-human GLUT1 serum (1/2000 dilution) or mouse anti-human GLUT1 serum (1/2000 dilution) overnight at 4 °C. The nitrocellulose was washed extensively with Tris/gelatin/Tween 20 (20 mM Tris base, pH 7.5, 0.5% 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 using 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 harboring 5 × 10^10 human (3 mg of membrane protein/lane) were dissolved in sample buffers at ice temperature for 5 min, resolved by SDS-PAGE using 10% polyacrylamide gels as described above, transferred to nitrocellulose with 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 α-antisera (diluted from 1/250 to 1/1000) 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 (1/500 dilution of Du Pont-New England Nuclear low specific activity preparation) at room temperature. Each assay point was determined in triplicate.

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100 μg of each protein sample were loaded onto the gels along with three different amounts of the human erythrocyte ghost protein used as immunoreactive protein standard.

The immunoreactive proteins were visualized by autoradiography, and the films were used to locate glucose transporter proteins on the nitrocellulose filters. The glucose transporter proteins were then excised from the filters and bound 125I in a γ-counter. Nitrocellulose with surface areas equal to those of the excised bands were cut from the same filters immediately above and below each glucose transporter protein band. These control blanks were analyzed in the γ-counter, the cpm were divided by 2, and the specific antibody binding was determined by subtracting these nonspecific control values. Results from the assays were normalized to cpm/mg of total membrane protein for each plate of cells.

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 utility 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 antibodies in the δ-serum were binding to extracellular epitopes or domains of the erythrocyte glucose transporter protein. Furthermore, assuming an equal stoichiometry of δ-IgG binding to isolated and erythrocyte-associated glucose transporter protein, these titration curves indicate that human and rat erythrocytes contain 1.4 × 10^6 and 680 glucose transporter proteins/cell, respectively. These results are very close to those obtained in measurements of D-glucose-inhibited cytochalasin B binding to human (Helgerson and Carruthers, 1987) and rat (Helgerson and Carruthers, 1989) erythrocytes (1.5 × 10^6 and 600 glucose transporter proteins/cell, respectively).

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-serum 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 40 μg of purified glucose transporter for 30 min at 37 °C. That had been first treated with rabbit β-antiserum was additionally incubated with 40 μg of purified glucose transporter for 30 min at 37 °C and then washed as before. The Sepharose beads were finally incubated with human red cells (1 × 10⁶ 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, β-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.

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 × 10⁶ 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, β-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.

FIG. 4. Rabbit α- and δ-antiserum binding to intracellular and extracellular domains of the glucose transporter protein in human erythrocyte membranes. Intact human 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 X 10⁶ 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.

Intact red cells, sealed red cell ghosts, leaking 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 X 10⁶ 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.

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Porter domains are accessible to the antibodies and that the leaky ghosts bound roughly equal amounts of α- and δ-immunoglobulins. Similar results were obtained when fluorescein-conjugated goat anti-rabbit IgG was substituted for ¹²⁵I-protein A as the reporter molecule (not shown).

Generation of CHO-K1 Phenotypes That Express Human GLUT1 Protein—CHO-K1 control cells and CHO-K1 cells transfected with a mammalian expression vector containing the HepG2 glucose transporter cDNA pLENGT were grown in culture, and the total membrane proteins from these cells were harvested as described under "Experimental Procedures." Expression of total immunoreactive GLUT1 protein was assayed by protein immunoblot analysis using an antiserum raised against recombinant human GLUT1 C-terminal peptide (Fig. 5A and B) and a mouse polyclonal antiserum that is specific for human GLUT1 protein (Fig. 5C). Fig. 5A demonstrates that the apparent M₅ values of the CHO-K1 host glucose transporter (lanes 2 and 3) and of the expressed human glucose transporter (lanes 4–8) are similar, and both have the same apparent M₅ as the human erythrocyte glucose transporter (lanes 1, 9, and 10). These data suggested that the transfected CHO cells express very different amounts of GLUT1 protein. Subsequent immunoblotting of identical preparations of mem-

brane proteins with either a GLUT1 C-terminal peptide antiserum (Fig. 5B) or an antisera specific for human GLUT1 (Fig. 5C) confirmed that it was the human glucose transporter protein that was overexpressed (Fig. 5C, lanes 4-9) and not host glucose transporter protein. Note that no CHO host glucose transporter 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 antisera (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 1F8 or with anti-rat GLUT4 C-terminal peptide serum (not shown).4

Quantitative immunoblot analysis indicates that overexpression of the human GLUT1 protein in these cell lines ranges from 2-fold (CHO-GT26) to 17-fold (CHO-GT3). Assuming equivalent binding of the C-terminal peptide antibodies to the denatured erythrocyte ghost glucose transporter and to the expressed HepG2 glucose transporter protein (Gould et al., 1989) and a $M_r = 55,000$ 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 $7.3 \times 10^4$ and $1.2 \times 10^6$, respectively.

Quantitative Comparisons among Total Cellular Expressed GLUT1 Protein, Cell Surface GLUT1 Protein, and 2-Deoxyglucose Uptake—Experiments were conducted to determine whether the $\delta$-antisera-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 $\delta$-serum from 250- to 2000-fold. Specific, saturable $\delta$-antibody binding was observed 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 control. The magnitude of $^{125}$I-protein A binding in the cell culture wells was dependent upon $\delta$-antibody dilution (Fig. 6).and upon cell density (not illustrated). The results appeared to correlate well with levels of expression of total immunoreactive GLUT1 protein measured by immunoblot analysis in these cells.

The relationships among levels of expression of total immunoreactive GLUT1 protein, cell surface $\delta$-antisera 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 $\delta$-serum binding and 2-deoxyglucose uptake, as described under “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 approximately 2-fold over those of control in cell line CHO-GT26 to 17-fold in cell line CHO-GT3, with two other cells 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 $\delta$-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 immunoreactive 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 glucose 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), suggesting that hexokinase activity was not saturated in these cells under our experimental conditions.

**Fig. 6. Rabbit $\delta$-antisera 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 Procedures.” 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 $\delta$-antisera (diluted 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 $^{125}$I-protein A at room temperature. The wells were washed three times with phosphate-buffered saline, and the bound $^{125}$I-protein A was solubilized in 0.1% SDS and counted in a $\gamma$-counter. Nonspecific 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 transfected CHO-K1 cells.** CHO-K1 control and pLENGT-transfected CHO-K1 cell lines were assayed for 2-deoxyglucose uptake, rabbit $\delta$-antibody binding, and total GLUT1 immunoreactive protein, as described in detail under “Experimental Procedures.” Specific 2-deoxyglucose 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 $^{125}$I-bound to intact cells for each experiment.

*S. A. Harrison, J. M. Buxton, and M. P. Czech, unpublished data.*
at the cell surface. Is not accompanied by increases in immunoreactive GLUT1 cose transport activity in control and transfected CHO cells increases in basal sugar uptake measured in those cells. In all each experiment) the standard errors of the mean were always served compared with control cells, consistent with the in-
cell lines tested in these experiments. In the absence of
The absolute increase in the rate of deoxyglucose uptake due protein (9- and 17-fold over controls, respectively, Fig. 7).
cells with lo-'
2-deoxyglucose uptake and antibody binding in CHO-Kl cells, and CHO-GT3 cells were insensitive to insulin in these experiments. These latter two cell lines exhibited much higher levels of human GLUT1 expressed human GLUT1 was also detected by a mouse pol-
epitopes has been described previously by Burdett and Klp (1988). However, the binding of that antibody to intact red cells was very low, only 0.25% of the total GLUT1 protein/ cell. Rabbit δ-antiserum appears to bind quantitatively, and it was used to calculate the number of cell surface glucose transporter proteins on human and rat red cells, resulting in an estimate of 1.4 × 10⁶ and 680 glucose transporter proteins/ cell, respectively. These data confirm independent measurements of red cell glucose transporter proteins determined by δ-glucose-inhibited cytochalasin B binding to human (Hel-
ger and Carruthers, 1987) and rat (Helgeron and Car-
ruthers, 1989) erythrocytes.

The GLUT1 protein was examined in control CHO fibro-
blasts and in CHO fibroblasts transfected with the human GLUT1 expression vector and pLENGT. Using specific an-
tibody 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. Host and heterologously expressed human GLUT1 proteins were detected by antipeptide antiserum directed against GLUT1 C-terminal 12 amino acids and by the rabbit polyclonal antiserum (δ) described above (Fig. 5, A and B). The expressed human GLUT1 was also detected by a mouse pol-
cyclonal antiserum raised against purified human erythrocyte glucose transporter. This latter antiserum 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, lF8, by immunizing mice with partially purified low density microsomal proteins from insulin-treated rat adipocytes. Their antibody is specific for GLUT4 protein, but not albumin immobilized onto protein A-Sepharose beads was able to immunoprecipitate intact red cells (Fig. 9). An antibody reagent that may recognize exofacial (GLUT1) epitopes has been described previously by Burdett and Klp (1988). The results presented in this report demonstrate the develop-
ment of a novel anti-GLUT1 antiserum that recognizes one or more extracellular epitopes on the GLUT1 protein. Intracellular epitopes on GLUT1 are not recognized by the anti-
serum. This rabbit δ-antiserum preparation is unique in that most antisera raised against the erythrocyte glucose transporter previously appear to recognize intracellular domains on the red cell glucose transporter. The rabbit α antiserum described in the present work is an example of this type of antiserum. Both α- and δ-antisera recognize denatured GLUT1 protein in immunoblots (Fig. 1), and both antisera recognize similar amounts of nondenatured red cell glucose transporter measured by competitive ELISA and by antibody binding to leaky red cell ghosts (Figs. 2 and 4). However, not α-antiserum binds to intact red cells and sealed right side-out red cell ghosts, and the δ-antiserum binding to the glucose transporter extracellular domain(s) was blocked by prior incubation of the antibody preparation with purified human erythrocyte glucose transporter protein. Additionally, α- but not δ-antiserum immobilized onto protein A-Sepharose beads was able to immunoprecipitate intact red cells (Fig. 9). An antibody reagent that may recognize exofacial (GLUT1) epitopes has been described previously by Burdett and Klp (1988). However, the binding of that antibody to intact red cells was very low, only 0.25% of the total GLUT1 protein/cell. Rabbit δ-antiserum appears to bind quantitatively, and it was used to calculate the number of cell surface glucose transporter proteins on human and rat red cells, resulting in an estimate of 1.4 × 10⁶ and 680 glucose transporter proteins/cell, respectively. These data confirm independent measurements of red cell glucose transporter proteins determined by δ-glucose-inhibited cytochalasin B binding to human (Helgeron and Carruthers, 1987) and rat (Helgeron and Carruthers, 1989) erythrocytes.

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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

**FIG. 8.** Insulin regulation of 2-deoxyglucose uptake and rabbit δ-antiserum binding in CHO-K1 cells. CHO-K1 control and three pLENGT-transfected CHO-K1 cell lines were assayed for 2-deoxyglucose uptake and rabbit δ-antiserum binding, as described in detail under “Experimental Procedures.” 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. A, 2-deoxyglucose uptake results are averages of 12 experiments (CHO-K1 control and CHO-K1 GT26 cells) or three experiments (CHO-K1 GT26 and CHO-K1 GT1 cells), with eight determinations for each experiment. The data are plotted as percentages of basal uptake measured in each cell line. The actual uptake values in pmol/min/10⁶ cells are: CHO-K1, 131 ± 10 and 179 ± 13; CHO-GT26, 222 ± 31 and 305 ± 58; CHO-GT1, 641 ± 46 and 728 ± 21; and CHO GT3, 908 ± 66 and 901 ± 60, for basal and insulin-treated cells, respectively. B, rabbit δ-antiserum-binding results are averages of two experiments with six determinations for each experiment. The data are plotted as percentages of basal antibody binding measured in each cell line. The actual binding values in cpm/10⁶ cells are: CHO-K1, 478 and 440; CHO-GT26, 614 and 559; CHO-GT1, 2545 and 2253; and CHO-GT3, 4493 and 4166 for basal and insulin-treated cells, respectively. In δ-antiserum-binding exper-
iments, the standard errors of the mean were always <3.5%. Specific uptake and antibody binding were determined by subtracting appropriate nonspecific control values, as described under “Experimental Procedures.” Nonspecific binding (preimmune) was <25% to CHO-K1 cells; <10% to CHO-GT1 GT26 cells; <5% to CHO-K1 GT1 cells; and <2% to CHO-K1 GT3 cells.
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 I-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. 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 1-promoting sequences (Asano et al., 1989). We have no documented explanation for this phenomenon present 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 fibroblasts (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 HepG2 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 isoform 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 (Jooost 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 δ 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 δ-antibody binding was detected in any of the several control or transfected CHO-K1 cell lines employed in these studies (Fig. 8D). No difference in δ-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 δ-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 (Hiraki 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 δ-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.

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