Intracellular Targeting of the Insulin-regulatable Glucose Transporter (GLUT4) Is Isoform Specific and Independent of Cell Type

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Abstract. Insulin stimulates glucose transport in adipocytes via the rapid redistribution of the GLUT1 and GLUT4 glucose transporters from intracellular membrane compartments to the cell surface. Insulin sensitivity is dependent on the proper intracellular trafficking of the glucose transporters in the basal state. The bulk of insulin-sensitive transport in adipocytes appears to be due to the translocation of GLUT4, which is more efficiently sequestered inside the cell and is present in much greater abundance than GLUT1.

The cell type and isoform specificity of GLUT4 intracellular targeting were investigated by examining the subcellular distribution of GLUT1 and GLUT4 in cell types that are refractory to the effect of insulin on glucose transport. Rat GLUT4 was expressed in 3T3-L1 fibroblasts and HepG2 hepatoma cells by DNA-mediated transfection. Transfected 3T3-L1 fibroblasts over-expressing human GLUT1 exhibited increased glucose transport, and laser confocal immunofluorescent imaging of GLUT1 in these cells indicated that the protein was concentrated in the plasma membrane. In contrast, 3T3-L1 fibroblasts expressing GLUT4 exhibited no increase in transport activity, and confocal imaging demonstrated that this protein was targeted almost exclusively to cytoplasmic compartments. 3T3-L1 fibroblasts expressing GLUT4 were unresponsive to insulin with respect to transport activity, and no change was observed in the subcellular distribution of the protein after insulin administration. Immunogold labeling of frozen ultrathin sections revealed that GLUT4 was concentrated in tubulo-vesicular elements of the *trans*-Golgi reticulum in these cells. Sucrose density gradient analysis of 3T3-L1 homogenates was consistent with the presence of GLUT1 and GLUT4 in discrete cytoplasmic compartments. Immunogold labeling of frozen thin sections of HepG2 cells indicated that endogenous GLUT1 was heavily concentrated in the plasma membrane. Sucrose density gradient analysis of homogenates of HepG2 cells expressing rat GLUT4 suggested that GLUT4 is targeted to an intracellular location in these cells. The density of the putative GLUT4-containing cytoplasmic membrane vesicles was very similar in HepG2 cells, 3T3-L1 fibroblasts, 3T3-L1 adipocytes, and rat adipocytes. These data indicate that the intracellular trafficking of GLUT4 is isoform specific. Additionally, these observations support the notion that GLUT4 is targeted to its proper intracellular locale even in cell types that do not exhibit insulin-responsive glucose transport, and suggest that the machinery that regulates the intracellular targeting of GLUT4 is distinct from the factors that regulate insulin-dependent recruitment to the cell surface.

GLUCOSE, an essential nutrient for many mammalian cells, is transported across the plasma membrane via facilitative carrier proteins. Five glucose transporter isoforms have been described thus far (for reviews, see Gould and Bell, 1990; Mueckler, 1990). These are named GLUT1 through GLUT5 in order of their identification by means of cDNA cloning. The five transporter isoforms are distinguished by their discrete tissue distributions, kinetic properties, and regulation. In most cell types, such as endothelial cells, hepatocytes, and parenchymal cells of the brain, glucose transport is probably not rate limiting for glucose metabolism and is not subject to acute regulation. The major glucose transporter isoforms present in these cells (GLUT1, GLUT2, and GLUT3, respectively) are constitutively localized to the plasma membrane, and the flux of glucose across the membrane is largely dependent upon the circulating glucose concentration. Muscle and fat cells, on the other hand, exhibit an acutely regulated glucose transport system that responds within minutes to factors such as insulin (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Wardzala and Jeanrenaud, 1981).

The regulation of glucose transport in muscle cells and

adipocytes appears to reflect the unique subcellular targeting of the glucose transporter in these cells (Blok et al., 1988; Slot et al., 1991*a*,*b*). In the basal state the glucose transporters are enriched in tubulo-vesicular structures of the *trans*-Golgi reticulum. After stimulation of the cells with insulin, the number of glucose transporters at the cell surface is markedly increased, presumably as a consequence of accelerated exocytosis from the intracellular pool. This mechanism for rapidly increasing cellular glucose uptake does not occur in most other cell types. It is currently unknown whether the capacity for this acute regulation is the consequence of a cell-specific targeting/translocation apparatus, or alternatively, reflects the presence of a ubiquitous cellular machinery that acts in an isoform-specific manner.

The transport of glucose in insulin-sensitive tissues is mediated by two transporter isoforms, GLUT1 and GLUT4. GLUT4 is the major isoform present in fat and muscle and its expression is restricted to insulin-sensitive tissues (James et al., 1989; Birnbaum, 1989; Charron et al., 1989; Fukumoto et al., 1989). GLUT1 is expressed in many cell types, including those that do not normally exhibit insulin-sensitive glucose transport (Mueckler et al., 1985; Birnbaum et al., 1986; Flier et al., 1987; Fukumoto et al., 1988). Nevertheless, both isoforms undergo insulin-dependent movement to the cell surface in adipocytes (Zorzano et al., 1989; Holman et al., 1990; Calderhead et al., 1990; Piper et al., 1991). These observations suggest that the potential for insulin-regulated translocation of the glucose transporter is conferred by cell-specific factors. However, recent evidence indicates that there are targeting differences between the two glucose transporter isoforms in insulin-sensitive cells. GLUT1 and GLUT4 are located in distinct intracellular vesicles in rat adipocytes (Zorzano et al., 1989) and 3T3-L1 adipocytes (Piper et al., 1991). Furthermore, in both cell types the intracellular sequestration of GLUT4 is more efficient than for GLUT1 in the basal state, and because of this the relative increase at the cell surface is greater for GLUT4 than GLUT1 after exposure to insulin. On the basis of these data, it has been proposed that GLUT4 has evolved as a glucose transporter that is specifically adapted to acute regulation via alterations in its subcellular distribution. It is unclear, however, whether the mechanisms of transporter sequestration and insulin-sensitive movement operate in cells that do not normally express GLUT4 or exhibit insulin-sensitive glucose transport.

We have addressed this question by studying the distribution of GLUT1 and GLUT4 in transfected cell lines that do not exhibit insulin-stimulated glucose transport. The two cell types that we have selected for study are 3T3-L1 preadipocytes and HepG2 cells. 3T3-L1 cells replicate in culture as fibroblast-like preadipocytes and can be induced to differentiate into insulin-sensitive adipocyte-like cells after achieving confluence (Kehinde and Green, 1975). The preadipocytes are insensitive to insulin with respect to glucose transport and express only the GLUT1 glucose transporter. During differentiation, GLUT4 expression increases in parallel with insulin-stimulated glucose transport, and GLUT1 expression declines in parallel with basal transport (Tordjman et al., 1989). HepG2 cells express high levels of GLUT1 and, as is the case for 3T3-L1 preadipocytes, do not exhibit insulin-stimulated glucose transport although they do respond to insulin in a variety of other ways (Conover and Lee, 1990; Adeli and Sinkevitch, 1990).

In the present study we show that human GLUT1 is targeted to the cell surface in both HepG2 cells and 3T3-L1 preadipocytes. Conversely, GLUT4 is excluded from the plasma membrane and its steady-state distribution in these cells is indistinguishable from that observed in insulin-sensitive adipocytes. These data strongly suggest that the intracellular sequestration of GLUT4, which is fundamental to insulin regulation, is mediated by factors common to many cell types. Furthermore, because insulin-dependent movement of GLUT4 to the plasma membrane does not occur in fibroblasts, it is likely that insulin-sensitive cells express a unique machinery that acts to recruit transporters from the intracellular compartment to the plasma membrane.

Materials and Methods

Cell Culture and 2-Deoxyglucose Uptake Measurements

3T3-L1 preadipocytes were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine as described previously (Tordjman et al., 1989). HepG2 cells were cultured in the same medium supplemented with 10% fetal calf serum rather than calf serum. [^{3}H]-2-Deoxyglucose uptake was measured as previously described (Tordjman et al., 1989). Non-carrier-mediated uptake was measured in the presence of 20 μM cytochalasin B. Cells were incubated in serum-free medium for 1 h before measurement and insulin-stimulated uptake was determined after an additional 20-min incubation with 10^{-7} M insulin in serum-free medium. To induce expression of GLUT4 from the metallothionein promoter of the recombinant bovine papilloma virus vector, confluent 3T3-IRGT25 cells were exposed to medium containing 7 mM sodium butyrate and 100 μM $ZnCl_2$ for 16 h.

Construction of Glucose Transporter Expression Vectors

Human GLUT1 cDNA was obtained by gel purification of a 2.4-kb BamHI fragment from pSPGT (Mueckler and Lodish, 1986). GLUT4 cDNA was obtained by gel purification of a 2-kb EcoRI fragment from pIRGT (James et al., 1989). The vector used for expression in 3T3-L1 cells was pBPV-MTHXho (kindly provided by Dr. D. H. Hamer, National Institutes of Health, Bethesda, MD). This vector contains the origin of replication and the ampicillin-resistance gene of pML, the 5.5-kb subgenomic transforming fragment of BPV, and the mouse metallothionein gene (Glanville et al., 1981) modified by replacement of the BglII site upstream of the metallothionein gene initiator ATG with a unique XhoI site. pBPV-MTHXho replicates within cells in a stable manner as a multicopy episome and allows continuous expression of foreign cDNAs independent of chromosomal controls (Campo, 1985). GLUT1 and GLUT4 cDNAs were blunt-end subcloned into the XhoI site of pBPV-MTHXho in the proper orientation for expression from the metallothionein promoter.

The plasmid vector pSSFV-neo (kindly provided by Dr. Dennis Loh, Washington University School of Medicine, St. Louis, MO) was used for expression of GLUT4 in HepG2 cells. The vector codes for neomycin resistance and expression of foreign cDNAs subcloned into a unique EcoRI site is driven by the Splenic Focus-Forming Virus LTR (Yamamoto et al., 1981). The vector contains the polyadenylation and splice sites of the SV-40 large T antigen downstream of the EcoRI cloning site. All DNA constructions were verified by restriction endonuclease mapping.

Transfection of Mammalian Cell Lines

Confluent NIH 3T3-L1 fibroblasts were split 1:5 into 10-cm dishes 24 h before cotransfection (Chen and Okayama, 1987) with 22 μg of a calcium phosphate precipitate of recombinant BPV vector and pWLneo (Stratagene, San Diego, CA) in a 10:1 molar ratio. Transfectants were selected by culture

in 1.0 mg/ml G418 (active fraction 0.52 mg/ml) (Gibco Laboratories, Grand Island, NY) (Gorman, 1985). G418-resistant colonies were isolated with cloning rings after 2 wk of selection, grown to confluence in 24-well tissue culture plates, and then grown in mass. Confluent HepG2 cells were split 1:3 into 15-cm dishes on the day prior to transfection. Approximately 10^7 cells were transfected with 20 μg of SalI-linearized pSSFV-GLUT4 using Lipofectin (Bio-Rad Laboratories, Richmond, CA) as described by the manufacturer. A population of stable transfectants was selected in media containing G418 at 1.0 mg/ml for 1 mo.

Western Blot Analysis

Transfected cell lines were characterized by SDS-PAGE and Western blot analysis (James et al., 1989), using one or more of the following primary antibodies: a rabbit polyclonal antiserum (F349) raised against a synthetic peptide corresponding to the COOH-terminal 16 residues of rat GLUT4; a rabbit polyclonal antiserum (F350) raised against a synthetic peptide corresponding to the COOH-terminal 16 residues of human GLUT1; affinity-purified IgG from a rabbit polyclonal antiserum raised against the purified human erythrocyte glucose transporter (a kind gift of Dr. G. Lienhard, Dartmouth Medical School); a monoclonal antibody species specific for the human GLUT1 transporter (Allard and Lienhard, 1985).

The quantities of the glucose transporter proteins expressed in transfected cell lines were determined relative to standards consisting of either purified human erythrocyte glucose transporter (to quantitate GLUT1) or low density microsomes from rat adipocytes (to quantitate GLUT4). The total glucose transporter content of rat adipocyte low density microsomes consists of at least 90% GLUT4 (Zorzano et al., 1990). The microsomal fraction was titrated with cytochalasin B as described (Cushman and Wardzala, 1980) and was found to contain 60 pmol of D-glucose-displaceable binding sites (i.e., total glucose transporter) per mg protein. Blots were quantitated using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Confocal Immunofluorescence Microscopy

Sample preparation and confocal immunofluorescence microscopy of transfected cells were conducted essentially as described previously (Piper et al., 1991; Tordjman et al., 1990). Briefly, fibroblasts were grown on glass coverslips in 35-mm tissue culture dishes. The slides were rinsed twice with serum-free medium and then incubated in serum-free medium for 1 h before fixation. Cells were fixed in 4% paraformaldehyde at room temperature for 20 min, treated with 100 mM glycine for 10 min, and then permeabilized with 1% Triton X-100 at room temperature for 15 min before incubation with primary antibody at 4°C overnight. Slides were then incubated with fluorescein-conjugated goat anti-rabbit IgG at room temperature for 1 h. The primary antibody for GLUT1 was obtained by affinity purification of R493 antiserum using the peptide antigen coupled to acrylamide beads. The primary antibody for detection of GLUT4 was an IgG fraction of F349 GLUT4 antiserum prepared using protein A-Sepharose.

Sucrose Density Gradient Analysis

Two confluent 15-cm dishes of 3T3-IRGT25 fibroblasts were homogenized (20 strokes) in 3 ml of HES buffer (20 mM Hepes, 250 mM sucrose, 1 mM sodium EDTA) using a ground glass homogenizer with a teflon dounce. The resulting homogenates were layered onto 10-ml linear gradients (15–37% [wt/vol]) containing 20 mM Hepes, pH 7.4, and 1 mM sodium EDTA. Gradients were centrifuged for 10 h at 100,000 *g* at 4°C in a rotor (model SW41; Beckman Instruments, Inc., Palo Alto, CA). Fractions were collected from the top of each gradient using a gradient collector (Buchler Instruments, Fort Lee, NJ) and subjected to SDS-PAGE and immunoblotting using either R493 or R820 as the primary antibody. Gradients were aligned by both protein concentration profiles and fraction density.

HepG2 cells were homogenized by passing 12 times through an LSC homogenizer (Yamato Scientific, Northbrook, IL) equipped with an LSC loose-fit pestle at a speed of 1,200 rpm. The homogenate was then subjected to sucrose density gradient fractionation as described above. GLUT4 was immunoprecipitated from each fraction using monoclonal antibody 1F8 (James et al., 1988) as detailed previously (Piper et al., 1991). Immunoprecipitates were subjected to SDS-PAGE and were then immunoblotted using R820 antiserum as the primary antibody. The GLUT 1 distribution in the gradients was determined by direct immunoblotting using R493 antiserum as the primary antibody.

Electron Microscopy

Confluent 10-cm dishes of 3T3-IRGT25 fibroblasts were fixed with 2% paraformaldehyde/0.2% glutaraldehyde in phosphate-buffered saline, pH 7.2, at room temperature for several hours. Cells were scraped off the dish and stored in 0.2% paraformaldehyde in phosphate-buffered saline, pH 7.2. Cells were embedded in 10% gelatin as described previously (Slot et al., 1988), but without fixation of the gelatin (Peters, 1991). Briefly, the cells were suspended in gelatin at 37°C. After pelleting the cells, the gelatin was solidified on ice. Blocks for ultracryotomy were prepared and infused with 2.3 M sucrose overnight at 4°C. Ultrathin sections were prepared, immunolabeled, washed, stained with uranyl acetate, embedded in methyl cellulose, examined, and photographed as previously described (Slot et al., 1991*a*). The antibody used to localize GLUT 4 (anti-$GLUT4_{19}$) was kindly provided by Dr. G. E. Lienhard. It was prepared by affinity purification of a rabbit antiserum against a peptide consisting of the 19 COOH-terminal amino acids of rat GLUT 4. The secondary reagent was protein A conjugated to 10 nm gold.

Results

Expression of Human GLUT1 and Rat GLUT4 in 3T3-L1 Preadipocytes

3T3-L1 fibroblasts were cotransfected with a plasmid encoding neomycin resistance and a plasmid containing either human GLUT1 or rat GLUT4 cDNA downstream of the murine metallothionein-I promoter. Several G418-resistant cell lines were cloned that expressed either the human GLUT1 or rat GLUT4 proteins. Control cell lines transfected only with the neomycin-resistance plasmid were also isolated.

The levels of human and total (human + endogenous murine) GLUT1 protein expressed in the parental 3T3-L1 preadipocytes, a control transfected line (Neo-1), and two human GLUT1 transfectants (3T3-Hep4 and 3T3-Hep18) are shown in Fig. 1. Total cellular homogenates (30 μg protein per lane) corresponding to each transfectant were subjected to SDS-PAGE and immunoblotted either with a monoclonal antibody specific for human GLUT1 or a polyclonal antibody that recognizes both the human and endogenous murine GLUT1 proteins. Aliquots of purified erythrocyte glucose transporter were analyzed on the same gel in order to quantitate the amount of transporter expressed in transfected lines. Parental 3T3-L1 fibroblasts and the control Neo-1 line expressed ~170 ng of murine GLUT1 per mg of total protein. The level of total GLUT1 was increased 1.6- and 3.2-fold in 3T3-Hep4 and 3T3-Hep18 cells, respectively, relative to control cells. Consistent with the differential expression of GLUT1 in the two lines, glucose uptake was increased by 2.7- and 4.1-fold, respectively, in 3T3-Hep4 and 3T3-Hep18 relative to Neo-1 (Fig. 2). The quantitative difference between the increase in total GLUT1 protein and the increase in transport activity in the transfectants may reflect more efficient targeting of the human GLUT1 to the cell surface in 3T3-L1 fibroblasts relative to the endogenous murine GLUT1, or may simply be due to the accuracy of the methods used.

Targeting of GLUT1 to the plasma membrane was confirmed by laser confocal immunofluorescence imaging (Fig. 3) using an affinity-purified antibody raised against a 12–amino acid synthetic peptide corresponding to the COOH terminus of GLUT1. The plasma membrane of 3T3-Hep4 preadipocytes was most prominently labeled, but intracellular staining was also evident. This is qualitatively similar to the GLUT1 labeling that is observed in 3T3-L1 adipocytes, except that in adipocytes the ratio of plasma membrane to intra-

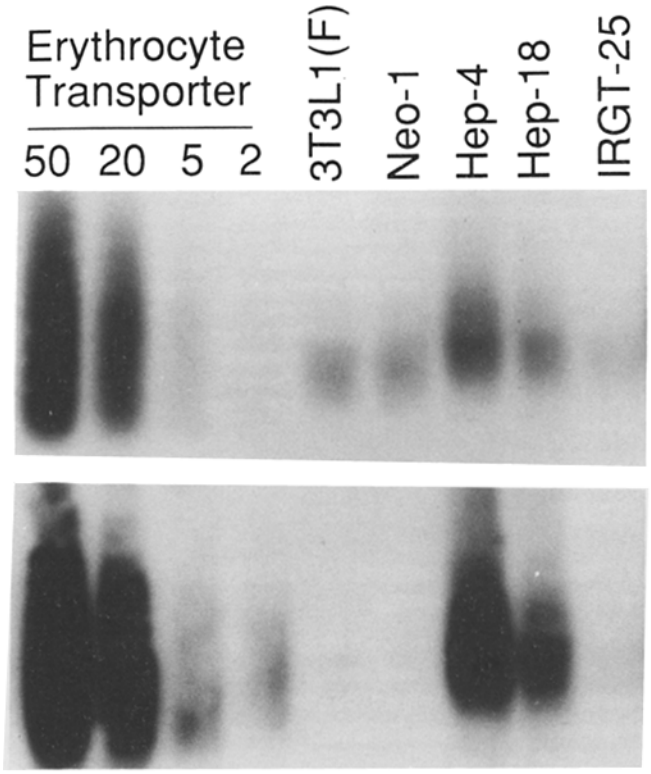


Figure 1. GLUT1 expression in 3T3-L1 cell lines. Duplicate 30-μg aliquots of total detergent-soluble protein fractions were electrophoresed on each of two 10% polyacrylamide gels and the gels subjected to Western blotting as described in Materials and Methods. The upper blot was incubated with a polyclonal antibody (*PAB*) that recognized both the human and the endogenous murine GLUT1 proteins. The lower blot was probed with a monoclonal antibody (*MAB*) that recognized only human GLUT1. The numbers above the blots represent nanograms of purified erythrocyte glucose transporter loaded on the gels as quantitative standards. *3T3L1(F)*, parental 3T3-L1 fibroblasts.

cellular labeling is considerably lower. The labeling observed was specific for GLUT1 as it was completely quenched in the presence of the peptide antigen (data not shown).

The levels of rat GLUT4 in two transfected cell lines (3T3-IRGT14 and 3T3-IRGT25) and several control lines are shown in Fig. 4. Aliquots of rat adipocyte low density microsomes, previously subjected to a cytochalasin B binding assay to estimate the GLUT4 protein content, were included on the gel as quantitative standards (see Materials and Methods). As shown previously (James et al., 1989; Tordjman et al., 1989), GLUT4 was not detected in 3T3-L1 preadipocytes and was present at 65 ng per mg total cellular protein in differentiated adipocytes. The level of expression of GLUT4 in 3T3-IRGT25 (60 ng per mg total protein) was similar to that in 3T3-L1 adipocytes and was induced 3.3-fold

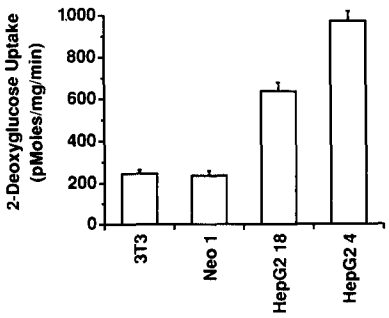


Figure 2. Glucose transport activity in 3T3-L1 cell lines expressing human GLUT1. [^{3}H]-2-Deoxyglucose uptake assays were conducted as described in Materials and Methods on cell lines grown to confluence in 35-mm plates. Values represent means ± SE of six determinations.

in response to a combination of butyrate and $ZnCl_2$ treatment of the cells. Induced 3T3-IRGT25 cells exhibited about a twofold increase in the expression of total glucose transporter (endogenous murine GLUT1 + rat GLUT4) relative to nontransfected preadipocytes and the control Neo-1 line. In contrast to the GLUT1 transfectants, no increase in basal glucose transport was observed in GLUT4-transfected lines (Fig. 5). The rate of 2-deoxyglucose uptake measured in the transfected lines was unaffected by acute treatment of the cells with 100 nM insulin. Induction of GLUT4 protein by incubation of cells in the presence of butyrate and zinc had no effect on transport activity in the presence or absence of insulin (data not shown).

Consistent with these observations, GLUT4 appeared by laser confocal immunofluorescence imaging to be localized almost entirely within cytoplasmic structures in 3T3-IRGT25 cells (Fig. 3). The staining was concentrated in a discrete perinuclear region and was also observed in a punctate pattern throughout the cytoplasm. This staining pattern is very similar to that observed for the endogenous murine GLUT4 in the differentiated adipocytes (Piper et al., 1991). However, in contrast to our observations in 3T3-L1 adipocytes, no change was seen in the distribution of GLUT4 in 3T3-IRGT25 preadipocytes following stimulation of the cells with insulin or IGF-I (data not shown).

Because of the similarity in GLUT4 labeling by indirect immunofluorescence between 3T3-L1 adipocytes and the transfected preadipocytes, the distribution of rat GLUT4 in 3T3-IRGT25 cells was investigated at the ultrastructural level by immunogold labeling of frozen thin sections (Fig. 6, *bottom*). Staining was heavily concentrated in tubulo-vesicular elements of the *trans*-Golgi reticulum. Labeling of the cell surface was very low. The staining pattern was very

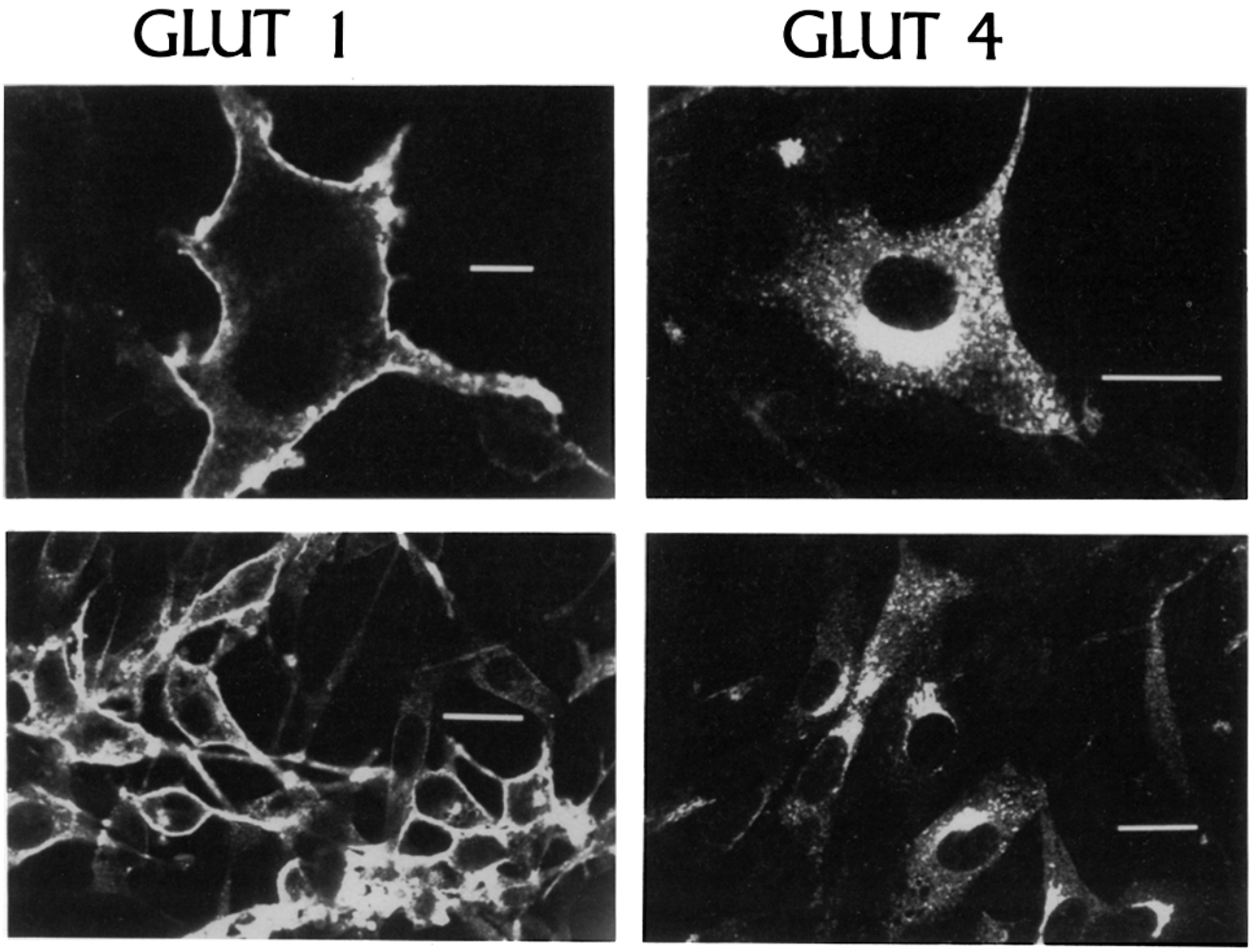


Figure 3. Visualization of GLUT1 and GLUT4 transfected preadipocyte cell lines. 3T3-Hep4 (*left*) and 3T3-IRGT25 (*right*) cells were grown on glass coverslips and processed for indirect immunofluorescence-laser confocal microscopy as described in Materials and Methods. Representative lower (*bottom*) and higher (*top*) magnification fields are shown. Note that staining is not apparent in cells that are out of the focal plane of the laser. Control slides incubated with antibody mixed with an excess of a rat COOH-terminal GLUT1 peptide did not exhibit any immunofluorescence, and the control 3T3-Neo-1 line did not exhibit any background immunofluorescence with COOH-terminal GLUT4 antibody (data not shown). Bars: (*top right* and *bottom*) 25 μm; (*top left*) 10 μm.

similar to that observed previously for endogenous GLUT4 in various insulin-sensitive cells, i.e., basal (non-insulin treated) brown adipocytes (Slot et al., 1991*a*), 3T3-L1 adipocytes (Piper et al., 1991), and cardiac muscle (Slot et al., 1991*b*).

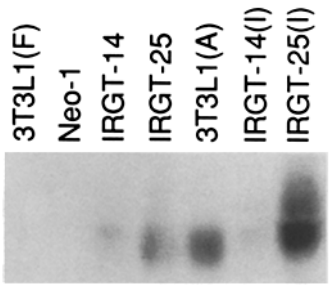


Figure 4. GLUT4 expression in 3T3-L1 cell lines. 30-μg aliquots of total cellular protein from each cell line were electrophoresed on 10% polyacrylamide gels and the gels subjected to Western blotting as described in Materials and Methods. The primary antibody used (F349) was specific for the GLUT4 transporter. *3T3L1(F)*, parental 3T3-L1 preadipocytes; *3T3L1(A)*, parental 3T3-L1 adipocytes; *IRGT-14(I)*, 3T3-IRGT14 cells induced with butyrate and $ZnCl_2$; *IRGT-25(I)*, 3T3-IRGT25 cells induced with butyrate and $ZnCl_2$.

Unfortunately, we were unable to immunolocalize GLUT1 in 3T3-L1 fibroblasts at the EM level because of the low efficiency of labeling of the protein in frozen thin sections. Therefore, to confirm the differential targeting of GLUT1 and GLUT4 in the fibroblasts we performed sucrose density gradient centrifugation (Fig. 7). Total cellular homogenates

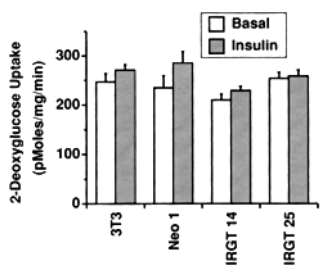
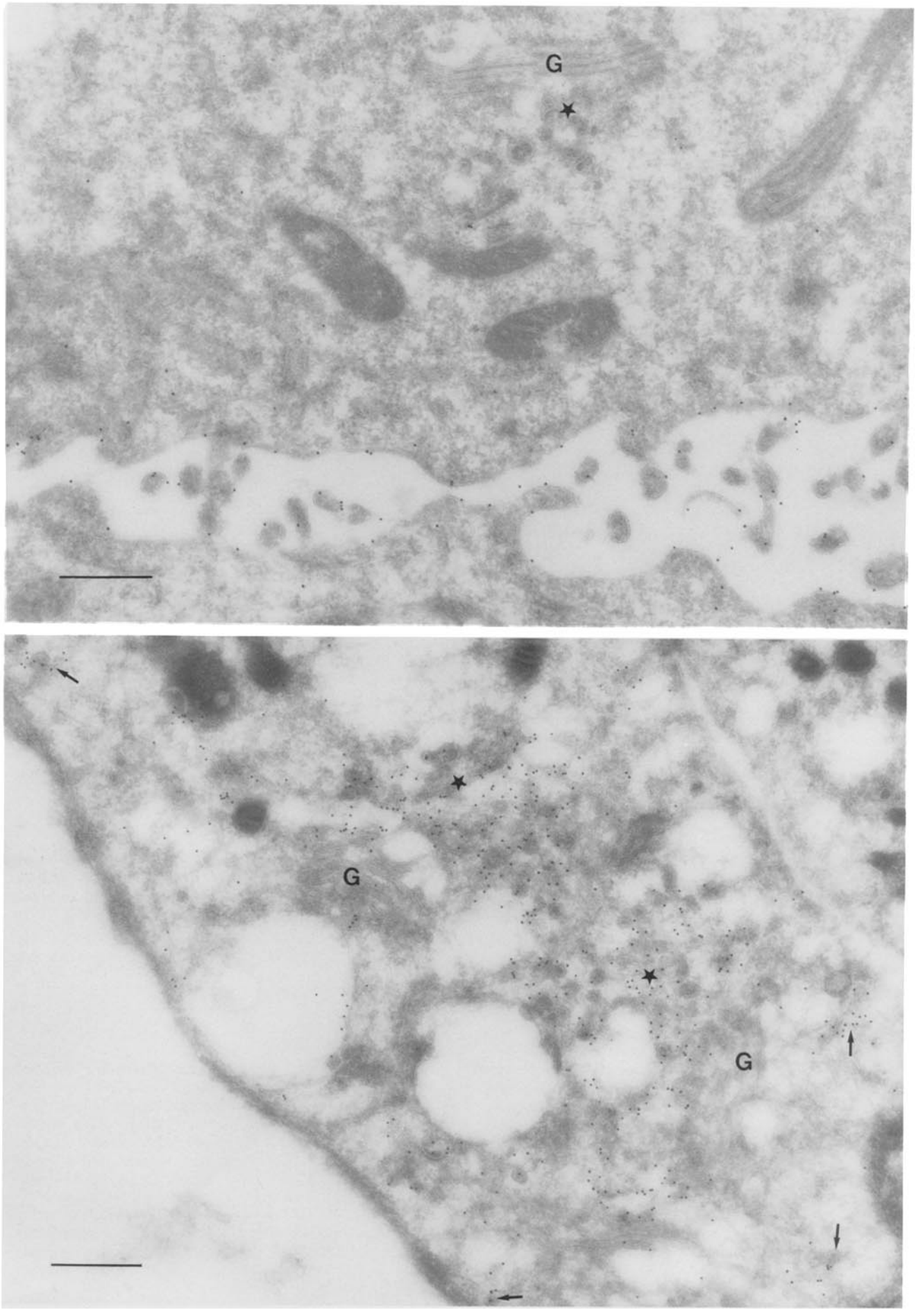


Figure 5. Glucose transport activity in 3T3-L1 cell lines expressing rat GLUT4. Confluent 35-mm plates of cells were either untreated or treated with 100 nM insulin for 20 min. 2-Deoxyglucose uptake assays were performed as described in Materials and Methods. Values represent means ± SE of six measurements.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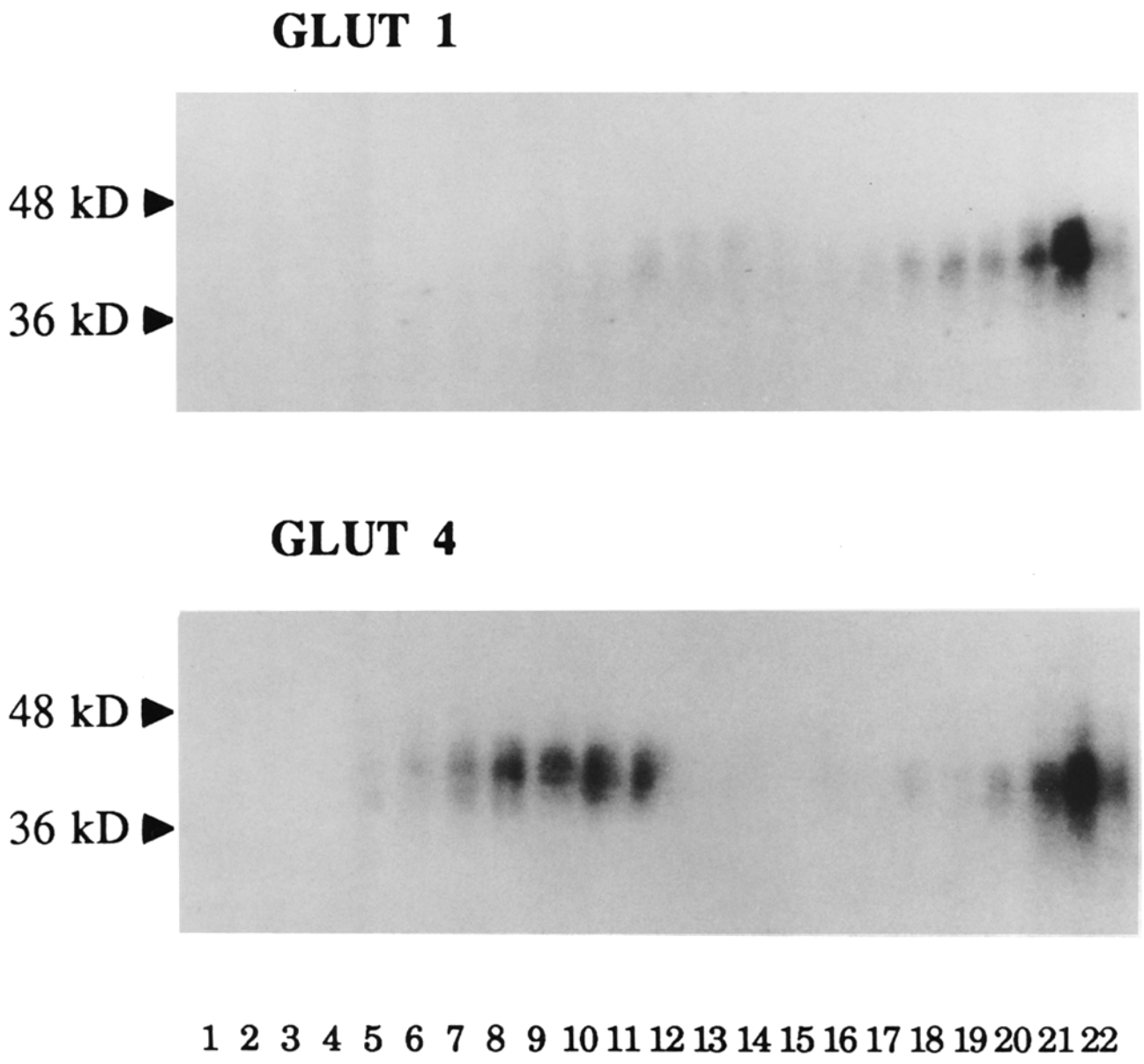
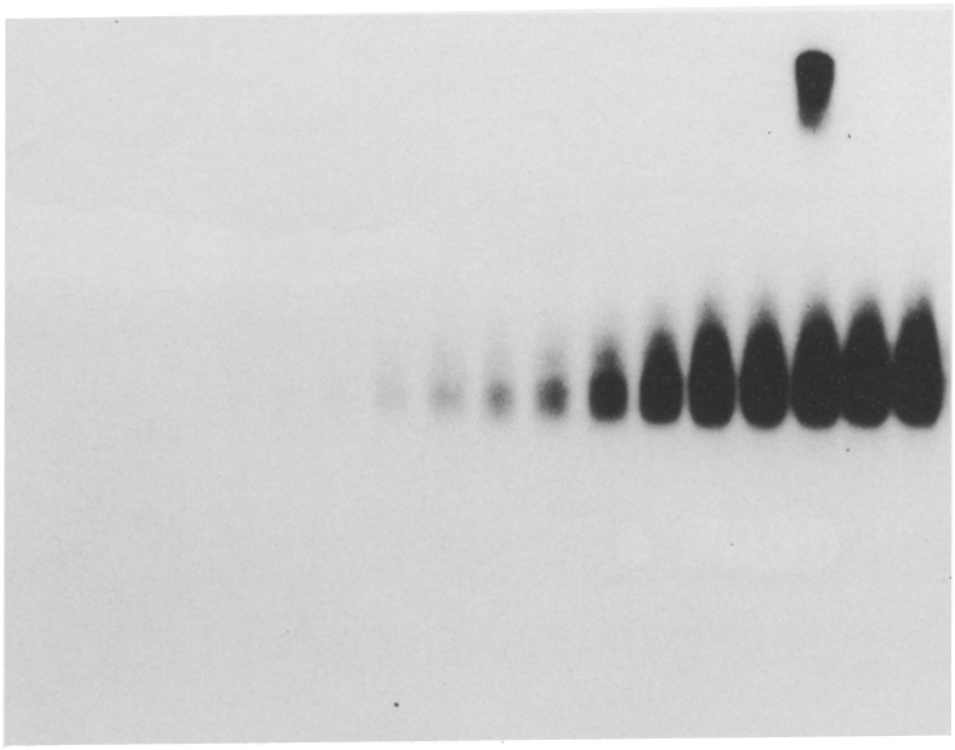


Figure 7. Sucrose density gradient analysis of GLUT1- and GLUT4-containing membranes in 3T3-IRGT25 preadipocytes. A total cellular homogenate was prepared from 3T3-IRGT25 fibroblasts and layered onto a 10–37% sucrose gradient. After centrifugation to equilibrium, gradient fractions were collected and subjected to SDS-PAGE and immunoblotting using antibodies specific for either GLUT1 (*top*) or GLUT4 (*bottom*). The mobility of molecular mass standards is indicated on the left. The numbers below the autoradiograms represent the fractions collected beginning from the top of the gradient.

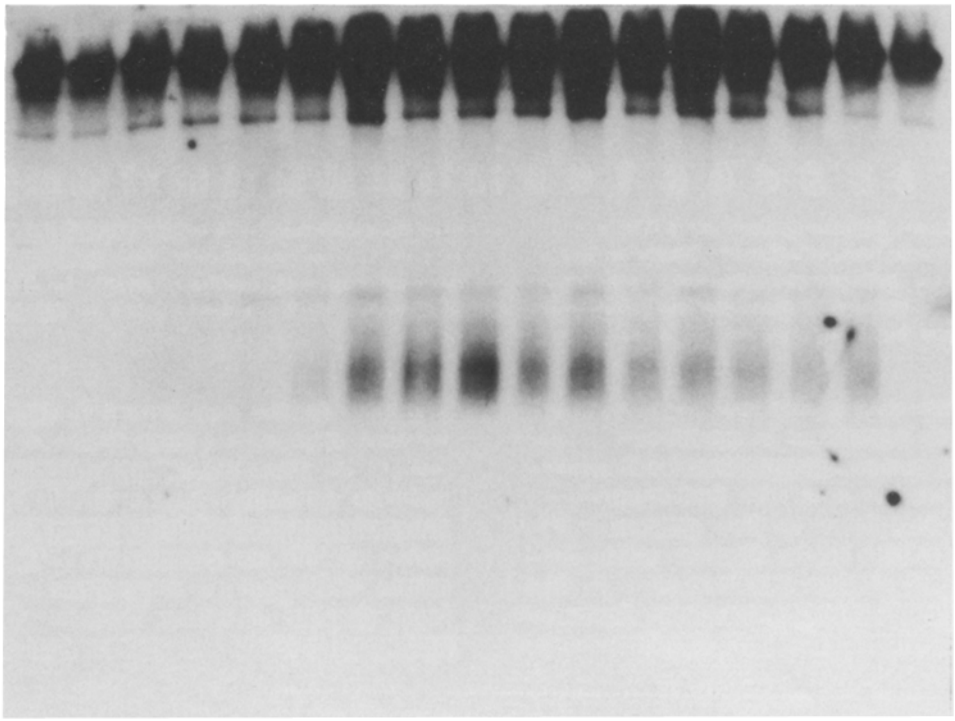
of 3T3-IRGT25 cells were layered onto 15–37% linear sucrose gradients and centrifuged to equilibrium. Gradient fractions were collected and immunoblotted with antibodies specific for either GLUT1 or GLUT4. Both transporters exhibited a bimodal distribution on these gradients. GLUT1 was concentrated in high density fractions (mean density ~1.15 g/cm^3) in each gradient examined (n = 4). Based upon the immunofluorescence data (see above), this presumably reflects the distribution of plasma membrane in these gradients. Another peak of GLUT1, representing ~10% of the total, migrated at a mean density of ~1.10 g/cm^3 (fractions 11–13). These lower density fractions presumably represent the intracellular pool of GLUT1. The smaller size of this pool is consistent with the immunofluorescence data (see above). GLUT4 was concentrated in fractions 5–11 (mean density ~1.09 g/cm^3) of all gradients (n = 4) with ~30% being detected in denser fractions. The fibroblast-like preadipocytes are difficult to break open so that a portion of

Figure 6. Ultrastructural localization of GLUT4 and GLUT1. Frozen thin sections of HepG2 cells (*top*) or 3T3-IRGT25 preadipocytes (*bottom*) were stained with an antibody specific for the COOH terminus of GLUT1 or GLUT4, respectively, followed by protein A complexed to 10-nm gold particles. *G*, Golgi cisternae; *asterisks*, *trans*-Golgi reticulum; *arrows*, tubulo-vesicular elements. Bar, 500 nm.

GLUT 1



GLUT 4



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

the localization of GLUT4 in the denser fractions may be due to the presence of unbroken and partially broken cells near the bottom of the gradient. However, we cannot rule out the possibility that some GLUT4 in the denser fractions may represent plasma membrane localization that was too low to detect by immunofluorescence or a denser pool of intracellular vesicles. It is also conceivable that the COOH-terminal epitope of GLUT4 is selectively masked in the plasma membrane of 3T3-L1 preadipocytes. We consider this unlikely because the epitope is not masked in the plasma membrane of insulin-treated 3T3-L1 adipocytes (Piper et al., 1991) or in the intracellular pool of 3T3-IRGT25 preadipocytes.

The immunofluorescence, EM, and sucrose gradient data together suggest that the lower density fractions containing the bulk of GLUT4 represent the distribution of the *trans*-Golgi reticulum and tubulo-vesicular elements of 3T3-L1 fibroblasts. It is noteworthy that the profiles of GLUT1 and GLUT4 in the putative intracellular fractions do not coincide. This is consistent with the distinct distribution of these two isoforms in 3T3-L1 adipocytes (Piper et al., 1991) and rat adipocytes (Zorzano et al., 1990). We also subjected purified intracellular membranes from both rat and 3T3-L1 adipocytes to sucrose density gradient analysis in parallel with total cellular homogenates from the 3T3-IRGT25 clone. The density of the putative intracellular GLUT4-containing vesicles from each cell type was identical (data not shown). In contrast to what we previously observed in adipocytes (Piper et al., 1991), there was no significant change in the sucrose gradient distribution of GLUT1 or GLUT4 in 3T3-IRGT25 following incubation with insulin/IGF-I (data not shown).

Distribution of GLUT1 and GLUT4 in HepG2 Hepatoma Cells

To extend our findings concerning the localization of the glucose transporters to an additional cell type that is refractory to insulin-stimulated glucose transport, we investigated the distribution of GLUT1 and GLUT4 in a human hepatoma cell line, HepG2. GLUT4-expressing HepG2 cells were obtained after transfection of cells with an expression vector containing GLUT4 cDNA downstream of the Splenic Focus Forming Virus long terminal repeat and also containing the neomycin-resistance gene driven by the SV-40 large T antigen promoter. Because of the very slow growth rate of HepG2 cells, we were unsuccessful in obtaining single GLUT4 positive clones. Therefore, we performed our analyses using a pooled stock of G418-resistant clones. Only a subset of these cells actually expressed the GLUT4 protein, so that the overall expression was quite low. Total homogenates of this HepG2 GLUT4 pool were subjected to sucrose density gradient analysis as described above for 3T3-L1 cells. The level of GLUT4 expression was too low in these cells to visualize by direct immunoblotting or microscopy. Therefore, in order to detect the distribution of GLUT4 by sucrose density gradient centrifugation, an equal volume of each fraction was solubilized and subjected to quantitative immunoprecipitation using a monoclonal antibody specific for GLUT4 (James et al., 1988). The immunoprecipitates were then analyzed by SDS-PAGE and immunoblotting using a polyclonal antibody specific for GLUT4. GLUT1 was detected by directly immunoblotting a smaller proportion of the identical gradient fractions used for detection of GLUT4.

The two transporter isoforms exhibited distinct distribution patterns on the sucrose gradients (Fig. 8). GLUT1, which appears to be a marker for the plasma membrane in these cells (see below), was present almost exclusively in dense fractions at the very bottom of the gradient. There was no detectable GLUT4 in these fractions. A high molecular mass band (M_r ~170 kD) corresponding to the IgG from the beads used for immunoprecipitation was detected in each lane of the GLUT4 immunoblot. A protein of the appropriate molecular mass for GLUT4 (~43 kD) was concentrated in fractions 7–11 after immunoprecipitation. The density of these fractions (mean ~1.09 g/cm^3) was similar to that of the GLUT4 peak in 3T3-IRGT25 preadipocytes. These data demonstrate that GLUT1 and GLUT4 are targeted to distinct subcellular compartments in HepG2 cells.

To clarify the nature of the different glucose transporter-containing subcellular compartments in HepG2 cells, GLUT1 was localized in these cells by immunogold labeling of frozen thin sections. The transporter was found predominantly in the plasma membrane (Fig. 6, *top*). Gold particles were concentrated on the cytoplasmic face of the membrane, consistent with the cytoplasmic localization of the COOH-terminal domain of the erythrocyte transporter (Davies et al., 1990). Taken as a whole the HepG2 data suggest that GLUT4 is present in intracellular vesicles, perhaps derived from the *trans*-Golgi reticulum. Unfortunately, GLUT4 could not be localized directly in the HepG2 transfectants by electron microscopy because of its low level of expression.

Discussion

The major mechanism by which insulin stimulates glucose transport in adipocytes is by eliciting the redistribution of GLUT4 from an intracellular compartment to the cell surface (Holman et al., 1990; Calderhead et al., 1990; Piper et al., 1991). What makes this process so dramatic is the virtual exclusion of GLUT4 from the cell surface in the absence of insulin. Our data demonstrate that the appropriate intracellular targeting of GLUT4 occurs in cells that do not exhibit insulin-sensitive glucose transport and do not normally

Figure 8. Sucrose density gradient analysis of GLUT1- and GLUT4-containing membranes in HepG2 cells. A total cellular homogenate from a population of HepG2 cells expressing rat GLUT4 was layered onto a 10–37% sucrose gradient and centrifuged to equilibrium. Gradient fractions were subjected to quantitative immunoprecipitation with monoclonal antibody 1F8 and the immunoprecipitates were then analyzed by SDS-PAGE and immunoblotting using polyclonal antibody R820 specific for GLUT4. The distribution of GLUT1 in the gradients was determined by direct SDS-PAGE and immunoblotting of gradient fractions using a primary antibody (R493) specific for GLUT1. The numbers below the autoradiograms represent the collected fractions beginning from the top of the gradient. The mobility of molecular mass standards of 36 and 48 kD is indicated on the left. The high molecular mass bands in the GLUT4 autoradiogram correspond to the IgG present in the beads used for immunoprecipitation of GLUT4 from the gradient fractions.

express this transporter isoform. In contrast, GLUT1 is sorted principally to the plasma membrane under the same conditions.

We have previously characterized the intracellular compartment containing GLUT4 (GTV, glucose transporter vesicle) in a variety of insulin-sensitive cells, including 3T3-L1 adipocytes (Piper et al., 1991; Gould et al., 1989), rat adipocytes (Slot et al., 1991*a*; James et al., 1987), and rat cardiac muscle (Slot et al., 1991*b*). Similar experimental protocols were applied to study the intracellular location of GLUT4 in 3T3-L1 fibroblasts and HepG2 cells. Both the morphological and biochemical characteristics of the GTV appeared identical between insulin-sensitive and non-insulin-sensitive cells. Using immunocytochemistry we found that GLUT4 is enriched in tubulo-vesicular elements in 3T3-L1 fibroblasts and adipocytes as well as in rat brown adipocytes and cardiac myocytes. Furthermore, the density in sucrose gradients of the GTV is very similar among different cell types. These data strongly suggest that the targeting of GLUT4 to a common or similar GTV occurs in both insulin-sensitive and insensitive cells.

At present little is known about the nature of the intracellular GTV. Although it may represent a unique organelle, we cannot exclude the possibility that it corresponds to a subcompartment of an organelle such as the *trans*-Golgi reticulum. The major problem in characterizing the GTV is that apart from GLUT4 there are no known markers that distinguish it from other elements of the endocytic pathway. Whereas most of the cellular GLUT1 is targeted to the plasma membrane in HepG2 cells and 3T3-L1 fibroblasts, a smaller intracellular pool of GLUT1 is also evident. A characteristic of insulin-sensitive cells is that GLUT1 is primarily located within intracellular vesicles that are distinct from those vesicles containing GLUT4. Similarly, in non-insulin-sensitive cells the intracellular pool of GLUT1 is distinct from the intracellular pool of GLUT4. This is an important criterion which lends support to the notion that GLUT4 is targeted correctly in cells that do not normally express this molecule. It remains to be determined whether the primary function of the GTV is for the intracellular sequestration of GLUT4 under basal conditions or to shuttle GLUT4 molecules to the cell surface after exposure to insulin. It has been proposed that GLUT4 constitutes a substantial proportion of GTV protein (up to 15%) in adipocytes (Zorzano et al., 1989). By analogy with secretory granules, this could be an indication that a major function of the GTV is related to GLUT4. This raises the question as to whether the GTV is a compartment or organelle present in all cells that has functions unrelated to GLUT4. Alternatively, as has been proposed for secretory granules (Wagner et al., 1991), the expression of GLUT4 in a particular cell might trigger the biogenesis of the GTV.

The subcellular targeting of GLUT4 appears to be isoform specific. Thus, GLUT4 presumably contains a subcellular targeting motif, absent in other transporter isoforms, that is recognized by a ubiquitously expressed cellular targeting machinery. This motif could be a linear stretch of amino acids or a posttranslational modification. The N- and C-terminal cytoplasmic domains of GLUT4 are unique compared to the other transporter isoforms, and these regions are likely candidates for containing a sorting motif. The observations described here provide an obvious method for the identification of the targeting signal.

A major property of insulin-sensitive cells that was not apparent in 3T3-L1 fibroblasts or HepG2 cells is the regulated movement of GLUT4 to the cell surface following exposure to insulin or IGF-I. We were unable to detect movement of GLUT4 to the cell surface by measurement of glucose transport, sucrose density gradient analysis, or immunofluorescence microscopy. These are all techniques that have previously been used to document insulin-dependent translocation of GLUT4 in 3T3-L1 adipocytes (Piper et al., 1991). It is likely that a similar magnitude of GLUT4 translocation in the preadipocytes could have been detected, since the level of expression of the transporter in the fibroblasts was comparable to that observed in either 3T3-L1 or rat adipocytes. 3T3-L1 adipocytes possess a markedly insulin-sensitive glucose transport system, and in these cells a smaller percentage of the intracellular GLUT4 is translocated to the cell surface after maximal stimulation with insulin (Piper et al., 1991) compared with that seen in rat adipocytes (James et al., 1988, 1989). Thus, we cannot exclude the possibility that in 3T3-L1 preadipocytes a very small percentage of the intracellular GLUT4 may have been translocated to the membrane in a regulated manner. Furthermore, insulin-stimulated movement of GLUT4 into other intracellular compartments, such as endosomes and coated vesicles, has also been observed in adipocytes (Slot et al., 1991*a*). The resolution of the techniques used in the present studies may have been insufficient to resolve changes of this nature.

Our data suggest that non-insulin-sensitive cells express the appropriate factors required for the intracellular targeting of GLUT4, but the additional machinery required to recruit GLUT4 from this compartment to the cell surface in response to insulin is missing. This is an important observation because it suggests that the targeting and recruitment of GLUT4 are independent phenomena. The absence of the translocation response is not due to the lack of insulin receptors in the preadipocytes (Reed et al., 1976); however, it is possible that the signaling pathway used by insulin to promote GLUT4 translocation is not present.

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