

Isoform-specific Subcellular Targeting of Glucose Transporters in Mouse Fibroblasts

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Abstract. GLUT1, the erythrocyte glucose transporter, and GLUT4, the adipose/muscle transporter, were each expressed in NIH-3T3 cells by retrovirus-mediated gene transfer. In fibroblasts overexpressing GLUT1, basal as well as insulin-stimulated deoxyglucose uptake was increased. Expression of GLUT4 was without effect on either basal or hormone stimulated hexose uptake. Localization of each of the transporters by indirect immunofluorescence revealed that, whereas GLUT1 was found primarily on the cell surface, GLUT4 was directed to vesicles in a perinuclear distribution and throughout the cytoplasm. The GLUT4-containing compartment represented neither Golgi complex nor lysosomes, as evidenced by the failure of lgp10 or Golgi mannosidase to co-localize. However,

there was substantial overlap between the distribution of GLUT4 and the transferrin receptor, and some colocalization of the transporter isoform with the mannose-6-phosphate receptor. In addition, when FITC-wheat germ agglutinin bound to the cell surface was allowed to internalize at 37°C, it concentrated in vesicular structures coincident with GLUT4 immunoreactivity.

These data establish that GLUT1 and GLUT4 contain within their amino acid sequences information which dictates targeting to distinct cellular compartments. Moreover, GLUT4 can be recognized by those cellular factors which direct membrane proteins to the endosomal pathway.

ONE of the best-characterized actions of the anabolic hormone insulin is its ability to rapidly and reversibly increase the rate of glucose metabolism in peripheral target tissues (8). The flux of sugars across the plasma membrane of mammalian cells is catalyzed by a class of integral membrane glycoproteins, the facilitated hexose transporters. It is this process which is the primary site of action by which insulin modulates glucose uptake. A number of years ago, it became clear that in the basal state a substantial number of glucose transporters in adipose tissue and probably muscle reside within the cell, where they are inactive presumably solely because of their inaccessibility to extracellular sugars (36). Shortly after exposure to insulin, the adipose cell redistributes its glucose transporters such that a greater number are located on the cell surface and contribute to the influx of hexoses. Whereas it remains unclear as to whether the "translocation" model explains all of the increase in hexose uptake in response to hormone, redistribution certainly occurs and is important to the overall augmented glucose flux. Therefore, a major part of understanding the mechanism by which insulin regulates hexose metabolism involves characterizing the pathways of subcellular trafficking of glucose transport proteins.

Recently, the existence of a gene family encoding related glucose transport proteins has been firmly established (4). The two isoforms relevant to the experiments discussed be-

low are GLUT1 and GLUT4. cDNAs encoding the former were originally cloned from human hepatoma and rat brain, and probably also code for the well-characterized human erythrocyte hexose carrier (6, 32). Whereas GLUT1 is expressed fairly ubiquitously in adult mammalian tissues, GLUT4 is found exclusively in adipose tissue, muscle, and heart, those organs most responsive to insulin in terms of hexose uptake (5, 8, 24). These data have led to the idea that the expression of GLUT4 is an essential requirement for the full, hormonal stimulation. Consistent with this model, insulin effects a greater increase in GLUT4 than GLUT1 in the plasma membrane of adipocytes, though both are recruited to some extent (5, 23, 24). The precise contribution of each of the isoforms to the actual flux of hexose in the basal and insulin-stimulated states remains undetermined. In addition, the subcellular pathways through which each of the transporters is routed in the adipocyte are ill defined.

The major hypothesis which forms the basis for the studies reported herein is that each of the transporters contains intrinsic to its primary structure information dictating targeting to distinct cellular compartments. Our strategy to test this model has been to introduce the two isoforms into the same cellular context and examine the subcellular distributions and transport activity. In addition, we have addressed the question of whether expression of GLUT4 is sufficient to confer full insulin-responsive transport.

Materials and Methods

Materials

2-[1,2-³H]-deoxy-D-glucose (30.2 Ci/mmol) was purchased from New England Nuclear (Boston, MA), ³⁵S-Translabel and ¹²⁵I-protein A (>30 mCi/mg) were from ICN Radiochemicals (Irvine, CA). FITC and rhodamine-conjugated antibodies were purchased from Tago (Burlingame, CA), FITC-conjugated wheat germ agglutinin (WGA)¹ was from Molecular Probes (Eugene, OR), endoglycosidase H from Boehringer-Mannheim Biochemicals (Indianapolis, IN), SP6 RNA polymerase from New England Biolabs (Beverly, MA), Geneticin (G418) and Lipofectin reagent from Gibco-BRL (Gaithersburg, MD), and the bicinchoninic protein assay kit was purchased from Pierce Chemical Co. (Rockford, IL). Insulin was a gift from Dr. Ronald Chance of Lilly Laboratories (Indianapolis, IN). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Antisera directed against the transferrin receptor, Igpl10, Golgi mannosidase II, and the mannose-6-phosphate receptor were generously provided by Roger Davis (University of Massachusetts, Worcester, MA), Bruce Granger (Yale University School of Medicine, New Haven, CT), Brian Burke (Harvard Medical School, Boston, MA), and Thomas Bräulke (Georg-August-Universität Göttingen, Göttingen, Germany), respectively. Affinity purified anti-GLUT1 antipeptide antisera for immunofluorescence was a gift of Bernard Thorens (MIT, Cambridge, MA).

DNA Constructs

The rat GLUT4 glucose transporter cDNA was isolated by excision from the plasmid pSM1-1-1 (5) by first digesting with XbaI, filling in the ends using the Klenow fragment of DNA polymerase I, adding Sall linkers, and re-ligating the plasmid. The resultant plasmid was digested with AhaIII, BamHI linkers were added, and the plasmid was religated. The resulting 1.8-kb BamHI-Sall fragment, containing the GLUT4 cDNA coding region as well as 12 and 272 bp of 5-prime and 3-prime flanking DNA, respectively, was ligated into the multiple cloning site of pDO-R, and called pDOJ-SM. The retroviral expression vector, pDO-R, which was kindly provided by Dr. Connie Cepko (Harvard Medical School, Boston, MA) contains sites for insertion of a cloned cDNA immediately downstream of the retroviral LTR, as well as a neomycin resistance gene driven by the SV-40 early promoter, which allows selection of transfected cells by growth in media containing the antibiotic G418 (31). A retroviral expression plasmid containing the rat GLUT1 cDNA, pDOJ-GT, was constructed from a fragment of the GLUT1 cDNA (6) extending from the unique XhoI site (changed to a BglII site) to the natural BglII site; this insert contains the complete coding region as well as 17 and 97 bp of 5-prime and 3-prime non-translated sequences, respectively.

To construct a vector suitable for the expression of a cloned cDNA by a strong promoter using histidinol for positive selection, pCMV1 (provided by Dr. M. F. Stinski, University of Iowa, Iowa City, IA), which contains a cytomegalovirus promoter followed by a human growth hormone termination sequence, was used (1). The EcoRI site in the polylinker was destroyed, and the remaining EcoRI-SfiI fragment isolated and ligated to the EcoRI-SfiI fragment of pSV2-His, which contains the *hisD* gene of *Salmonella typhimurium*, encoding histidinol dehydrogenase (21). The fragment of the rat GLUT4 cDNA described above was cloned into the polylinker following the CMV promoter using the unique BglII restriction site.

Cell Culture and Gene Transfer

NIH-3T3 fibroblasts and ψ 2 cells were grown in DME supplemented with 10% calf serum in an atmosphere of 8% CO₂ at 37°C. Cells transfected or infected with vectors encoding *neo* resistance were grown in the same medium supplemented with 400 μ g/ml G418 (active concentration); for cells expressing histidinol resistance, 400 μ g/ml histidinol was added to the media.

The retroviral packaging cell line ψ 2 (29), obtained from C. Cepko (Harvard Medical School), was transfected with either pDOJ-SM, pDOJ-GT, or pDOJ using the Lipofectin reagent as suggested by the manufacturer. After selection in medium containing 400 μ g/ml G418, the clones were expanded and screened for retrovirus. Clones were grown to over-confluence, the virus-containing medium was harvested and polybrene (8 μ g/ml) was added, and this medium used to infect NIH-3T3 fibroblasts. After 3 h, fresh medium was added to dilute the polybrene to 2 μ g/ml and the cells were

allowed to divide twice before they were split 1:20 into selection medium containing 400 μ g/ml G418 (active concentration). Colonies were observed 10–14 d after selection. These were pooled or cloned, and continued to be grown in medium containing 400 μ g/ml G418. NRK cells expressing GLUT4 were made by lipofectin-mediated gene transfer of pDOJ-SM DNA.

NIH-3T3 HIR3.5 and CHO HIRC cell lines were graciously provided by Drs. Jonathan Whittaker (State University of New York, Stony Brook, NY) and Morris White (Joslin Diabetes Center, Boston, MA), respectively.

Analysis of Proteins

Crude total cellular membranes were prepared and assayed for glucose transporter content by SDS-PAGE and Western blot analysis as described (22). Rabbit polyclonal antisera raised against bacterial fusion proteins including the cytoplasmic carboxy terminus of GLUT4 have been described (5), and rabbit anti-GLUT1 polyclonal antibodies purchased from East Acres (Southbridge, MA) were used for Western blotting at a dilution of 1:2,000.

For the determination of sensitivity to endoglycosidase H, cells plated on 10-cm dishes (GLUT4) or 6-well dishes (GLUT1) and grown to confluence were washed with PBS and incubated in methionine-free DME (Gibco-BRL) for 45 min. They were exposed to ³⁵S-Translabel (0.5 mCi/ml) in DME with 2.5% BSA for 15 min and chased with DME containing 0.2 mg/ml methionine and 0.2 mg/ml cysteine. The chase was terminated by placing the cells on ice and washing with cold PBS. For GLUT1, the cells were solubilized directly and used for immunoprecipitation. For GLUT4, crude membranes were prepared from each plate (22). Membrane pellets were resuspended in solubilization buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM DTT, 0.4% SDS, 1 mM PMSF) and immunoprecipitation with GLUT4 was carried out (5). The protein A-Sepharose pellets were resuspended in 0.05% SDS, and boiled for 4 min. The enzymatic reaction was carried out in the presence of 50 mM sodium citrate, 10 μ g/ml protease inhibitors (chymostatin, leupeptin, antipain, pepstatin, and PMSF), and 2 mU endoglycosidase H for 17 h at 37°C. After digestion, the samples were submitted to 10% SDS-PAGE, treated with Enhance (New England Nuclear), and exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) at -70°C.

For quantitation of transporter isoforms by Western immunoblot, standards were prepared. These consisted of β -galactosidase fused to the cytoplasmic carboxy terminus of each of the transporters. The construction of the β -galactosidase-GLUT4 fusion protein has been described (5). For construction of the GLUT1 fusion protein, the *lacZ*-containing plasmid, pUR291 (35), was digested with BamHI, the staggered ends filled in with the Klenow fragment of DNA polymerase I, and the plasmid digested with PstI. The 625-bp fragment of a *RsaI/PstI* digest of the rat GLUT1 cDNA was isolated and ligated into pUR291. This resulted in a construct encoding the terminal 51 amino acids of the rat GLUT1 protein fused in frame to β -galactosidase. Both fusion proteins were partially purified from *Escherichia coli* inclusion bodies by extraction with 6 M urea and β -mercaptoethanol (5), and quantitated by SDS-polyacrylamide electrophoresis followed by staining with Coomassie blue using β -galactosidase as a standard.

Hexose Transport

The rate of hexose transport was determined by measuring the uptake of 2-deoxy-D-[³H]glucose (15). Varying concentrations of insulin were added for the last 20 min of incubation. Uptake was initiated by the addition of 0.1 ml KRP containing 1 or 2 mCi/ml 2-deoxy-D-[³H]glucose and 0.5 mM or 1.0 mM deoxyglucose. After 4 min, during which time the uptake was linear, the reaction was stopped by three successive immersions of the plate into ice-cold TBS (154 mM NaCl, 20 mM Tris, pH 7.4). The cells were solubilized in 0.75 ml 1% Triton X-100 for 20 min at 37°C, and radioactivity determined in a 0.5-ml aliquot by liquid scintillation spectrometry. Uptake was measured in duplicate. The protein concentration of the solubilized cells was determined using the bicinchoninic assay (Pierce Chemical Co.), with BSA as a standard.

Immunofluorescence

Cells plated on glass coverslips 2 d before an experiment were washed with PBS and fixed in 3% paraformaldehyde for immunofluorescence (15). For the immunofluorescent localization of the transferrin receptor, cells were permeabilized with 0.1% NP-40 instead of Triton X-100. For detection of Igpl10, fixation was accomplished by immersion in ice cold methanol for 5 min, followed by acetone for 30 s. In all cases, coverslips were mounted

1. Abbreviation used in this paper: WGA, wheat germ agglutinin.

in Mowiol and visualized with an Axiophot fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

For endocytosis studies, JSM8 cells were plated onto glass coverslips 2 d before an experiment. The coverslips were transferred to 6-well dishes containing L15 (Gibco-BRL) media with 2% calf serum and 5 μ g/ml FITC-conjugated WGA (FITC-WGA) at 4°C for 1 h. The cells were washed three times with cold L15 media, and then transferred to a 6-well plate containing L15 media plus 2% calf serum at 37°C for the times indicated. The uptake was terminated by return of the cells to L15 with 2% calf serum at 4°C, followed by washing of the cells twice with cold PBS containing 50 mM *N*-acetyl glucosamine. These washes removed most, but not all of the extracellular bound FITC-WGA. The cells were then fixed and processed for immunofluorescence as described above.

Expression in *Xenopus* Oocytes

GLUT1 and GLUT4 RNA were synthesized in vitro and injected into oocytes prepared from gravid *Xenopus laevis* frogs (Xenopus I, WI) as has been described (5, 30). Hexose transport assays were performed 3 d after injection.

Results

Two glucose transporter retroviral expression vectors, pDOJ-GT (GLUT1) and pDOJ-SM (GLUT4) were constructed using the plasmid pDO-R, in which the viral long terminal repeat serves as promoter for inserted cDNAs (31). Stable cell lines were established by transfection of the retroviral packaging cell line ψ 2 followed by infection of NIH-3T3 cells with the viral supernatant. A control cell line, DOJ, was generated by infection of NIH-3T3 cells with parental virus. Colonies were selected in G418, and both pooled and clonal cell lines established. Overexpression of the transporters was analyzed by Western blot of GLUT1 and GLUT4 using polyclonal antisera directed against the carboxy terminus of each protein. GLUT4 was not detected in total membranes prepared from NIH-3T3 infected with parental virus (DOJ), but was present as a single immunoreactive species in membranes from a pool of G418-resistant cells infected with DOJ-SM (Fig. 1). On the other hand, GLUT1 was readily detected in DOJ membranes; the amount of GLUT1 was significantly greater in membranes from DOJ-GT infectant cells. For the interpretation of experiments described below, it was essential to ascertain the relative levels of overexpression and expression of GLUT1 and GLUT4, respectively. Since different antisera were used for immuno-detection of the transporter isoforms, a simple comparison of the Western blots was not adequate. Instead, standards were prepared; these consisted of the cytoplasmic "tails" of each of the proteins fused to β -galactosidase, expressed in *E. coli*, and partially purified. This method provided a means of comparing the relative affinities of the non-crossreactive antisera for GLUT1 and GLUT4. Total membranes from two clonal lines of NIH-3T3 cells expressing GLUT4 (JSM8) or overexpressing GLUT1 (JGT2) were submitted to Western immunoblot analysis along with the several known amounts of β -galactosidase-glucose transporter fusion proteins. Quantitation of the radioactivity in each band indicated that the JGT2 clone of DOJ-GT infected NIH-3T3 fibroblasts expressed about 16-fold more GLUT1 than the parental cells. A cell line (JSM8) derived from DOJ-SM infected fibroblasts expressed approximately threefold higher levels of total cellular GLUT4 than GLUT1 (data not shown).

The biochemical effects of overexpression of each transporter isoform in these cell lines were examined using 2-de-

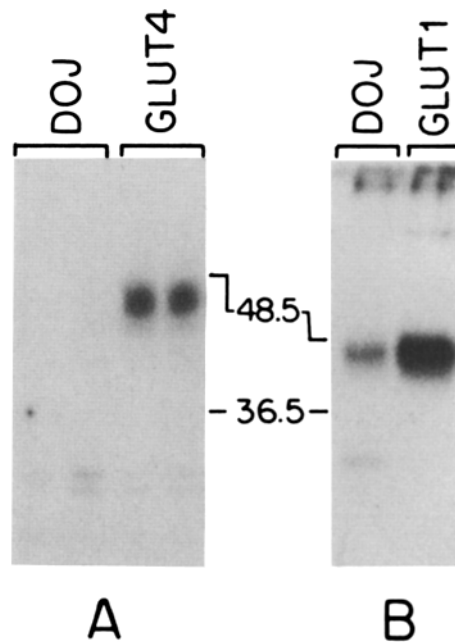


Figure 1. Western immunoblot of glucose transporter isoforms in NIH-3T3 fibroblasts. 40 μ g total membranes prepared from pools of G418-resistant fibroblasts infected with DOJ, DOJ-SM (GLUT4), or DOJ-GT (GLUT1) recombinant retrovirus were submitted to SDS-polyacrylamide electrophoresis, transferred to nitrocellulose, and probed by polyclonal antisera specific for GLUT4 (A) or GLUT1 (B). The samples in A represent duplicate preparations of membranes. Sizes of two molecular weight standards are indicated in kD.

oxyglucose uptake in the presence of increasing insulin concentrations. Basal levels of glucose uptake in JGT2 cells were approximately seven times greater than in control cell line, DOJ (Fig. 2). Furthermore, insulin-stimulated hexose uptake significantly; the magnitude of the increase was greater, but the fold stimulation less than in parental fibroblasts. There was no significant difference in the rates of hexose uptake between the GLUT4 expressing JSM8 cells and control fibroblasts when the assay was performed in the absence or in the presence of several concentrations of insulin (Fig. 2). Apparent minor differences in transport between DOJ and JSM8 in the series of experiments represented in Fig. 2 were not supported by study of other independently isolated cell lines (for example see Fig. 4). Hexose uptake in clonal or pooled fibroblast cell lines infected with DOJ-GT in which the level of GLUT1 was increased only two- to fourfold demonstrated a commensurate increase in hexose uptake (data not shown). This is similar to results reported by other investigators following introduction of GLUT1 into other fibroblast cell lines (2, 19, 20).

One trivial explanation for the lack of increased hexose uptake in JSM8 cells in spite of the overexpression of GLUT4 was that the GLUT4 cDNA had sustained an inactivating mutation in the course of molecular cloning. To exclude this possibility, mRNA was synthesized in vitro from the cloned GLUT1 and GLUT4 cDNAs and injected into *Xenopus laevis* oocytes. Oocytes injected with either transporter mRNA catalyzed significantly more 2-deoxyglucose uptake than water injected oocytes, and uptake in GLUT1-expressing oocytes was about 1.5-fold greater than that in

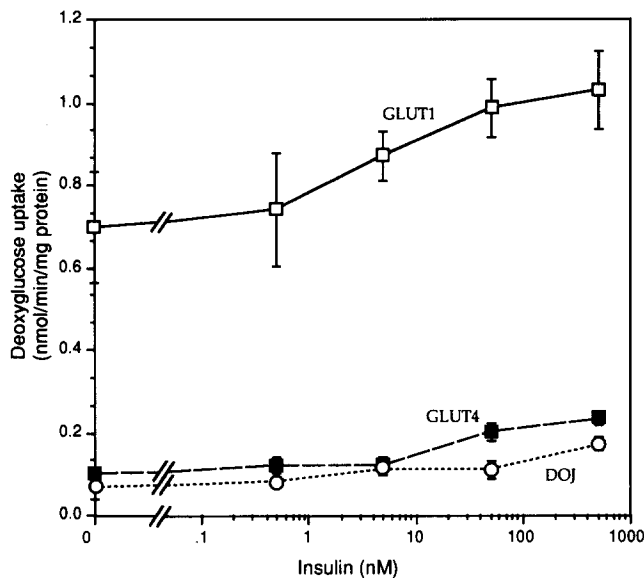


Figure 2. Hexose uptake into glucose transporter expressing NIH-3T3 fibroblasts. 2-Deoxyglucose uptake into clonal lines of fibroblasts expressing vector alone (\circ), GLUT1 (JGT2, \square) and GLUT4 (JSM8, \blacksquare) was measured in the presence of varying concentrations of insulin. Values are the means \pm SEM of four experiments, performed in duplicate.

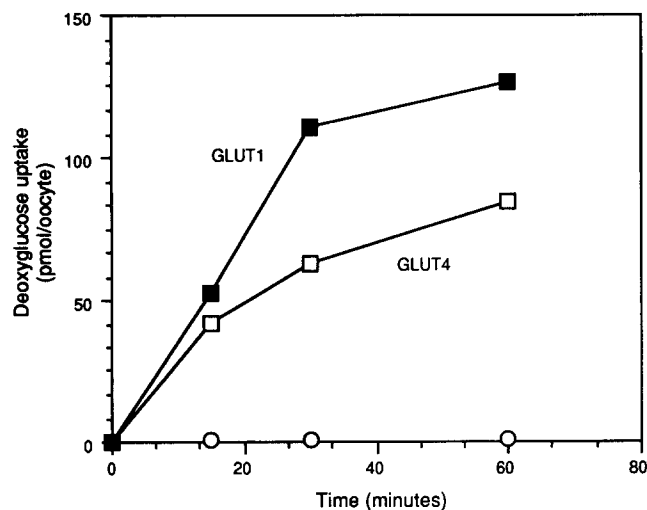


Figure 3. Uptake of hexose into *Xenopus laevis* oocytes. Oocytes injected with H_2O (\circ) or 50 ng mRNA encoding GLUT1 (\blacksquare) or GLUT4 (\square) were incubated for 3 d at 19°C before assay for 2-deoxyglucose uptake as a function of time. Each point represents the mean of 5 oocytes, and is typical of at least four independent experiments.

GLUT4 (Fig. 3). These data indicate that both cDNAs are capable of encoding catalytically active hexose transport proteins and are consistent with previous reports of expression in oocytes (5, 16, 27, 41).

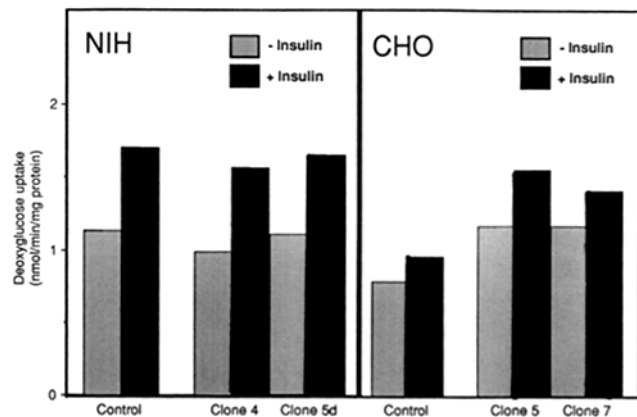


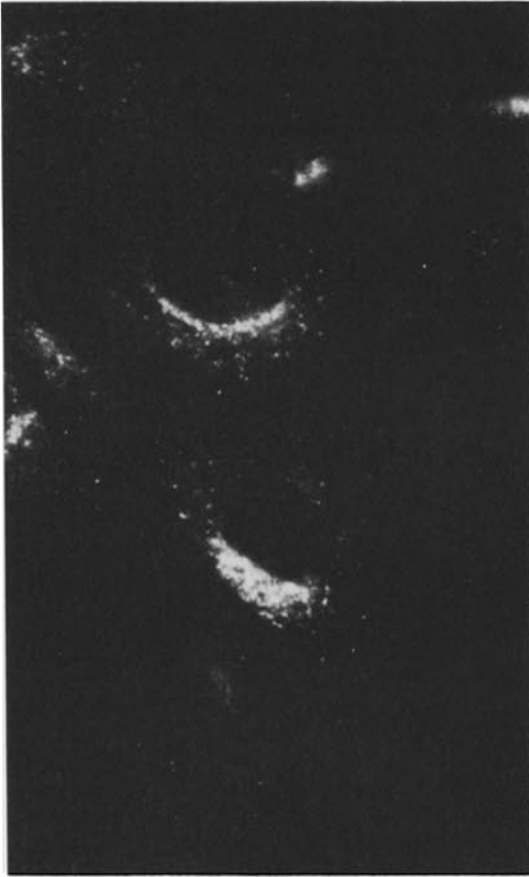
Figure 4. Hexose uptake into fibroblasts overexpressing insulin receptors and GLUT4. NIH-3T3 HIR3.5 cells or CHO HIRC cells overexpressing human insulin receptors were transfected with a plasmid encoding rat GLUT4. Clonal cell lines expressing GLUT4 protein were identified by immunofluorescence, grown to mass culture, and assayed for 2-deoxyglucose uptake in the absence (\square) and presence (\blacksquare) of 1 μ M insulin. For each parental fibroblast line, uptake data from a control cell line as well as two independently isolated GLUT4 expressing clones are shown.

Another explanation for the inability of overexpression of the GLUT4 isoform to alter hexose uptake could have been that fibroblasts lack sufficient number of insulin receptors to stimulate recruitment of transporters to the cell surface. To test this possibility, GLUT4 was introduced into fibroblast lines already expressing high numbers of functional human insulin receptors by DNA-mediated gene transfer. The rat GLUT4 cDNA was cloned into an eucaryotic expression vector with the following characteristics: transcription of the transporter cDNA was driven by the strong CMV promoter, since preliminary experiments had shown that this achieved higher levels of GLUT4 protein than either the viral LTR or the SV-40 early promoter; and the plasmid contains the *hisD* gene of *Salmonella typhimurium*, encoding resistance to histidinol and thus permitting introduction of a second cloned gene into cells already G418 resistant. Two such cell lines were used for this purpose, NIH-3T3 HIR3.5 cells and CHO HIRC cells (42, 43). Fig. 4 shows deoxyglucose uptake into control cells (vector alone) and two independent clonal cell populations of either CHO HIRC or NIH-3T3 HIR3.5 cells which express rat GLUT4 in addition to human insulin receptors. Even in the presence of insulin receptors, the expression of GLUT4 did not augment the increase in hexose uptake in response to hormone.

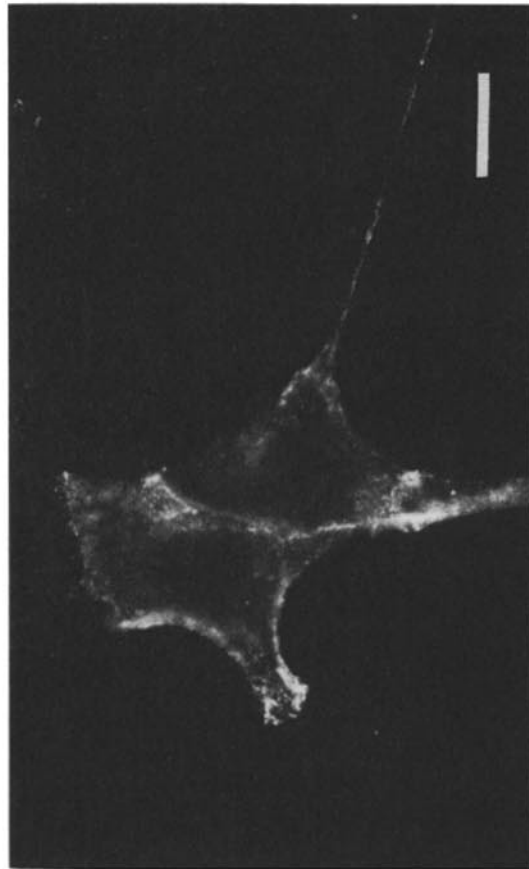
To clarify the mechanism underlying the differential effect on hexose uptake in GLUT1 vs. GLUT4 expressing cells, the intracellular localization of each transporter isoform was examined by indirect immunofluorescence. Pools of NIH-3T3 cells infected with DOJ-GT displayed increased expression of GLUT1 in >90% of the cells. In essentially all overex-

Figure 5. Immunofluorescent localization of glucose transporter isoforms in 3T3-L1 fibroblasts. (a). Fibroblasts overexpressing GLUT1 (NIH-JGT) or GLUT4 (NIH-JSM) were assayed for the distribution of transporter using antisera directed against GLUT1 (left) or GLUT4 (right). Phase micrographs of the corresponding fields are shown below. For GLUT1, a non-expressing cell can be visualized in the upper part of the micrograph, and several non-expressing cells are seen in the lower part of the panel containing the DOJ-SM-infected cells. Bar, 20 μ m.

NIH-JSM α GLUT4



NIH-JGT α GLUT1



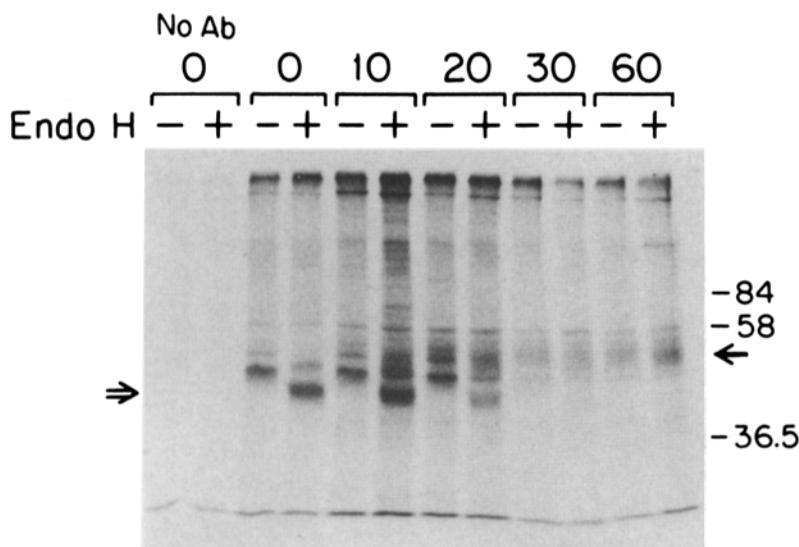
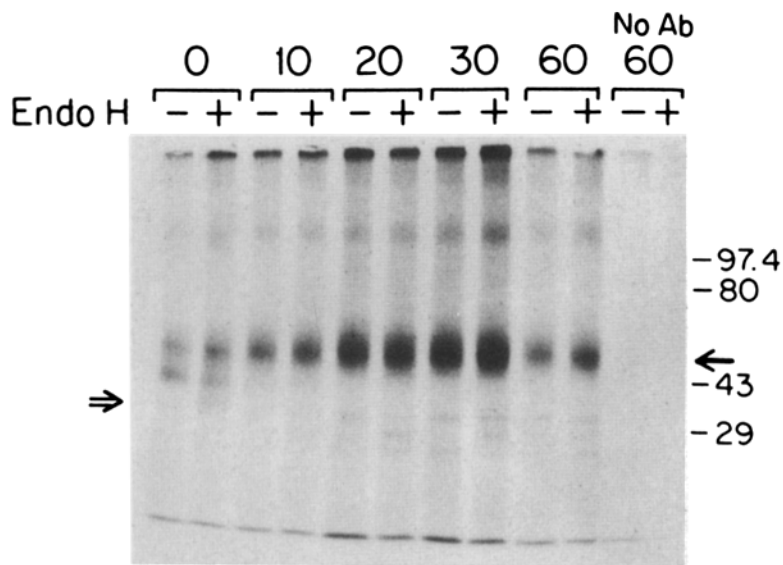


Figure 6. Acquisition of resistance to endoglycosidase H. Fibroblasts infected with recombinant retroviruses were labeled with ^{35}S -Translabel for 15 min, incubated with non-radioactive amino acids for the times indicated, immunoprecipitated and treated with endoglycosidase H. Molecular weight standards are shown in kD. The approximate mobility of the mature transporter is indicated by an arrow, and the earliest synthesized precursor by an open arrow. (a) Fibroblasts expressing GLUT1, immunoprecipitated with an antisera against this isoform. Immunoabsorption of extract from the 60 min chased cells to protein A-Sepharose in the absence of antisera is shown (*No Ab*). (b) Fibroblasts expressing GLUT4, immunoprecipitated with an antisera against this isoform. Immunoabsorption of extract from the "non-chase" cells to protein A-Sepharose in the absence of antisera is shown (*No Ab*).

pressing cells as well as parental fibroblasts, GLUT1 appeared to be substantially on the cell surface (Fig. 5). There was some perinuclear staining in most of the GLUT1-overexpressing cells (data not shown). The general subcellular distribution of GLUT1 appeared to be independent of the level of expression in each of the individual infected cells. The cellular location of GLUT4 was strikingly different. This transporter isoform was detected predominantly in a perinuclear region in a distinctly punctate distribution (Fig. 5). GLUT4 was also distributed throughout the cell, again in a punctate pattern. Whereas the intensity of the more peripheral GLUT4 immunofluorescence was somewhat variable among different experiments (for example compare Figs. 5 and 10), the general pattern of staining was relatively independent of the level of expression. However, the highest expressing cells in the mixed polyclonal population often displayed some cell surface staining.

We sought evidence as to whether the distribution of GLUT4 was a result of the foreign protein being arrested in a biosynthetic compartment. Acquisition of resistance to

digestion by endoglycosidase H is a characteristic feature of a glycoprotein which has undergone processing in the *cis*- and middle Golgi complex. Fibroblasts expressing either rat GLUT1 or GLUT4 were metabolically labeled for 15 min, and then "chased" with non-radioactive amino acids as indicated. At each time, transporter was immunoprecipitated, and half of the carrier enzymatically deglycosylated by digestion with endoglycosidase H. Fig. 6 A shows the acquisition of resistance to endoglycosidase H in GLUT1 overexpressing cells. By the end of the labeling period, much of the GLUT1 was already resistant to endoglycosidase H, and the remainder of the transporter acquired resistance in the subsequent 10 min. As shown in Fig. 6 B, GLUT4 was processed considerably more slowly, requiring 30 min to acquire complete resistance to endoglycosidase H. In addition, it appeared that the amount of GLUT4 protein decreased during the course of the experiment, suggesting that some of the GLUT4 protein may have been degraded before complete maturation. Nonetheless, these data demonstrate that foreign GLUT4 does ultimately acquire resistance to en-

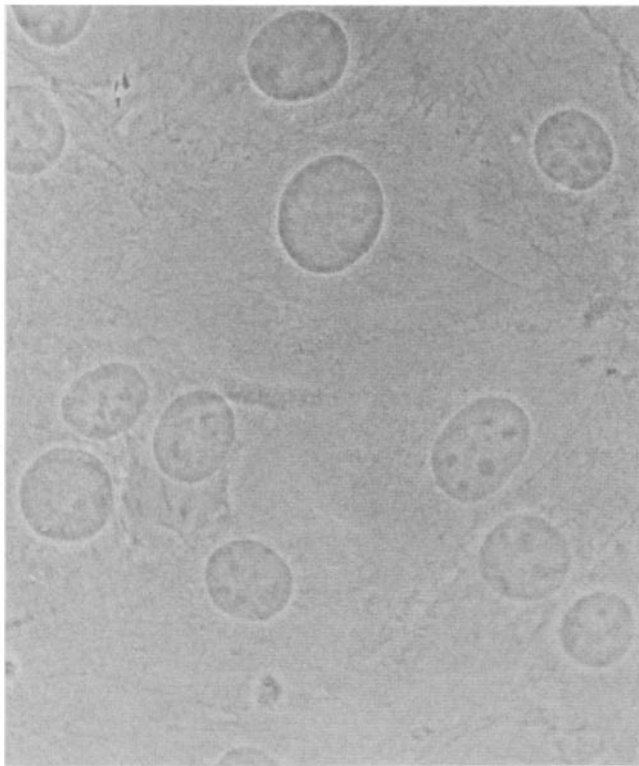
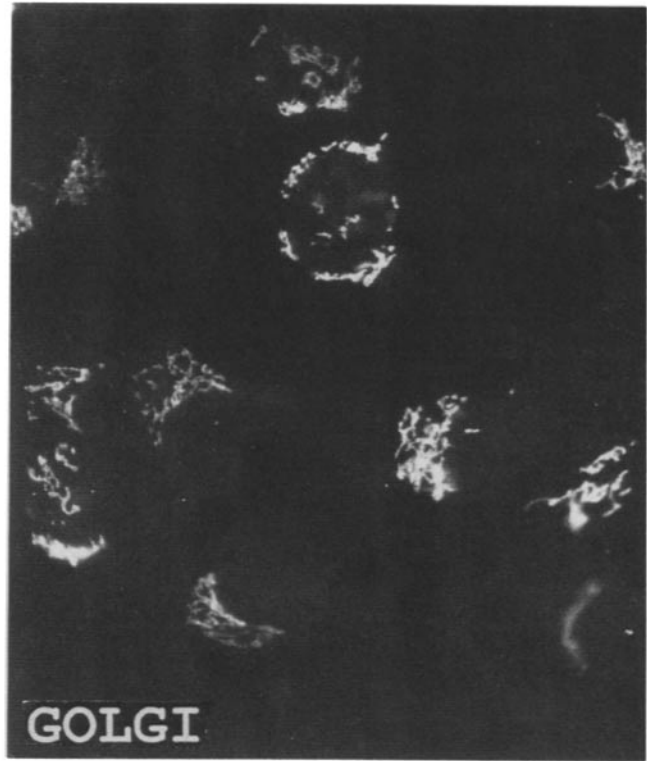
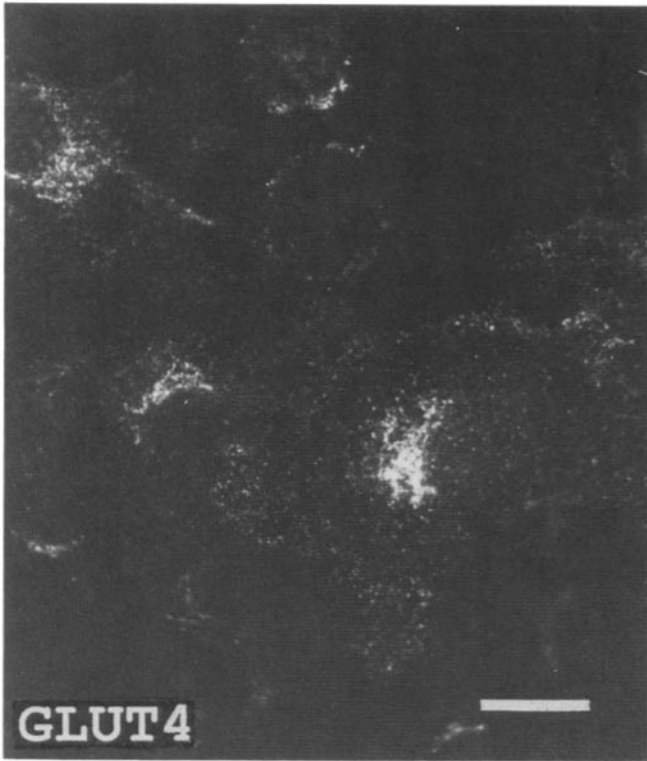


Figure 7 Immuno-localization of the Golgi complex and GLUT4. Pools of NRK fibroblasts stably expressing rat GLUT4 were stained with antisera against GLUT4 (*left*) and Golgi mannosidase II (*right*). The corresponding phase micrograph is shown on the bottom. Bar, 20 μ m.

doglycosidase H in murine fibroblasts, and thus traverses the medial Golgi complex.

In view of the relatively slow posttranslational processing of GLUT4 protein, further studies were performed to determine whether the steady state GLUT4 staining pattern

reflects transient accumulation in the Golgi complex. A mAb directed against rat Golgi mannosidase II was used to identify the Golgi complex in double labeling experiments (3, 7). Since the α -Golgi antibody is specific for rat cells, GLUT4 was stably expressed in NRK fibroblasts. The same cells

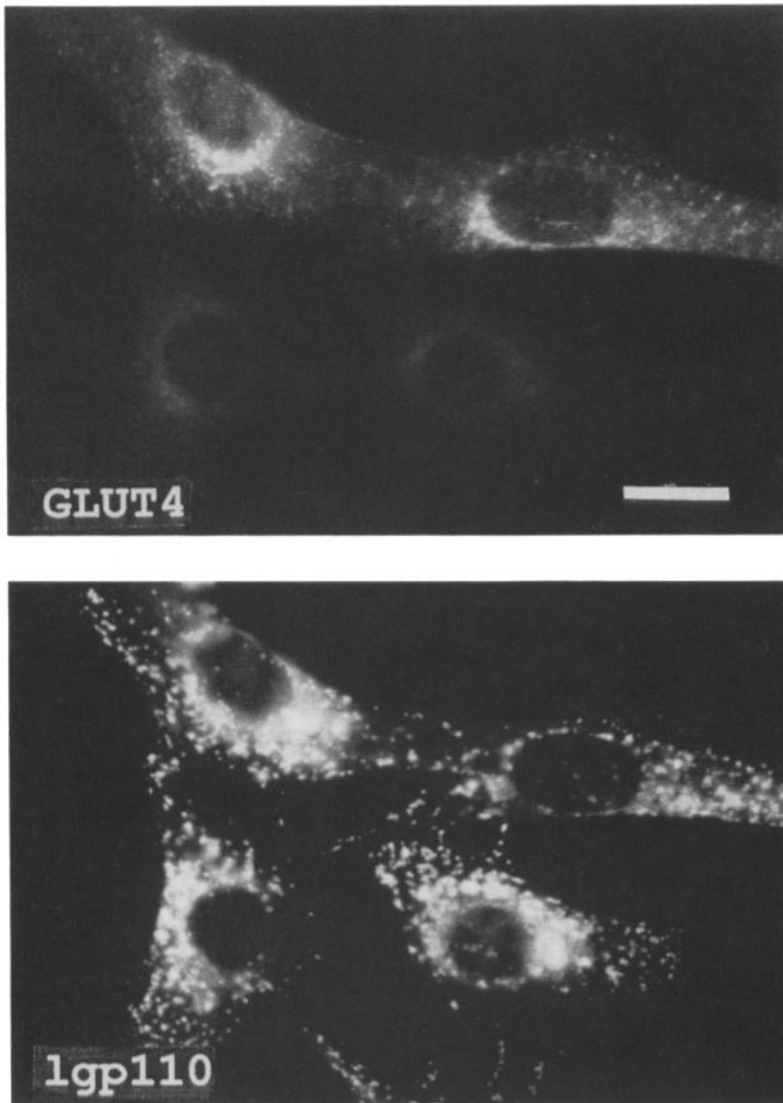


Figure 8. Immuno-localization of lysosomes and GLUT4. Pools of DOJ-SM cells were stained with antisera against GLUT4 (*top*) or Igpl10 (*bottom*). Bar, 20 μ m.

were labeled with α -GLUT4 polyclonal sera and α -Golgi mannosidase mAb, and visualized with rhodamine- and FITC-conjugated secondary antibodies, respectively (Fig. 7). Whereas both antigens were located in the same region of the cell, they clearly resided in distinct subcellular structures. Double-exposure color photomicrographs indicated that the GLUT4 protein was present in punctate structures interspersed amongst the Golgi complex (data not shown).

Another explanation for the differential subcellular distribution of GLUT1 and GLUT4 might have been that the latter was being targeted to a degradative pathway. To address this, immunofluorescent co-localization was performed using Igpl10, a marker for lysosomes (17). As can be seen in Fig. 8, in pools of NIH-3T3 cells infected with DOJ-SM, antisera directed against GLUT4 and Igpl10 detect markedly different labeling patterns in the same cells. Though it is possible that GLUT4 rapidly lost immunoreactivity after entering the lysosomes, it is unlikely since the epitope is directed towards the cytoplasmic surface of the transporter protein.

The data presented thus far indicate that GLUT1 is targeted predominantly to the plasma membrane, but GLUT4 is directed to an intracellular location. A candidate for such

an intracellular structure is the endocytic compartment. To address this possibility, experiments were carried out using antisera directed against the transferrin receptor and the mannose 6-phosphate receptor, markers for early and late endosomes, respectively (38). As shown in Fig. 9 *A*, the intracellular distributions of GLUT4 and transferrin receptor are almost superimposable. Virtually all vesicles containing GLUT4 also react with antisera directed against the transferrin receptor. There is also some overlap in subcellular distribution between GLUT4 and the mannose-6-phosphate receptor, though this is not as striking as the co-localization with the transferrin receptor (Fig. 9 *B*). In particular, the more peripherally located GLUT4 lacked coincident mannose-6-phosphate receptor immunoreactivity.

Another experiment was performed to independently confirm the presence of GLUT4 in the endosomal pathway. JSM8 cells were incubated with FITC-WGA at 4°C to label the cell surface. The unbound lectin was removed, and the cells warmed to 37°C for the times indicated in Fig. 10 *A*. The presence of FITC-WGA did not alter the overall steady-state distribution of GLUT4 as ascertained by immunofluorescence microscopy. In the absence of incubation at 37°C,

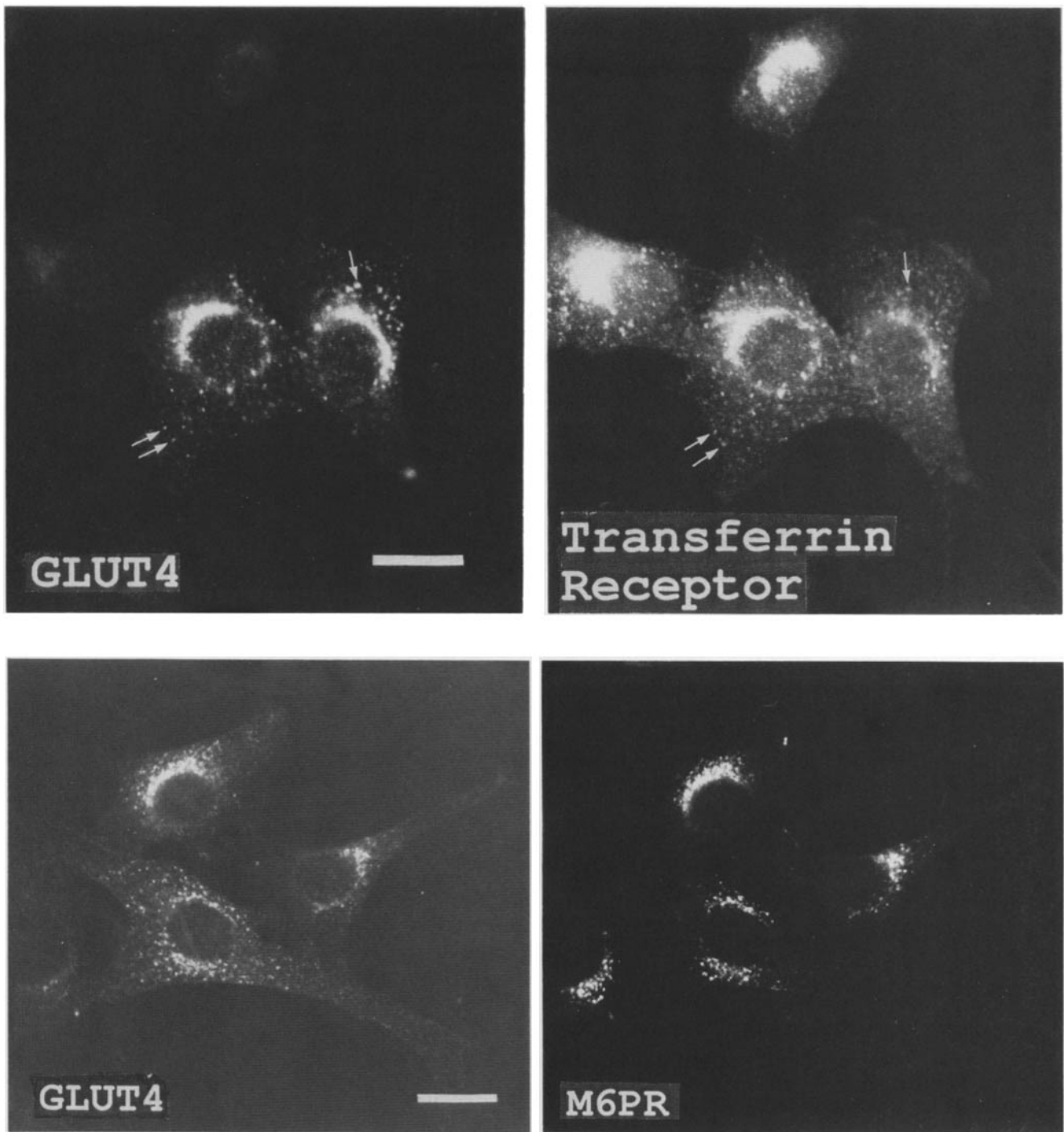


Figure 9. Co-localization of endosomes and GLUT4. Pools of DOJ-SM cells were stained with antisera against GLUT4 (*left*) and transferrin receptor (*upper right*) or the mannose-6-phosphate receptor (*lower right*). Bar, 20 μ m.

there was a modest amount of detectable FITC-WGA, and all was localized on the extracellular surface of the fibroblasts (Fig. 10 A, 0 min). By 2.5 min, some FITC-WGA was internalized and present in peripherally disposed vesicular structures. Importantly, GLUT4 co-localized with the FITC-WGA. This is shown in more detail in Fig. 10 B, which is a greater magnification of a single JSM8 cell allowed to take up FITC-WGA for 2.5 min. Note the striking overlap between the distribution of GLUT4 and WGA. Allowing the internalization of FITC-WGA to proceed for longer periods of

time resulted in the progressive labeling of more centrally located structures (Fig. 10 A). However, at each time point, some of the GLUT4 co-localized with the WGA.

Discussion

In the experiments described above, we set out to address several questions relevant to an understanding of the regulation of glucose transport by insulin: (a) Do GLUT1 and GLUT4 contain within their primary amino acids sequences

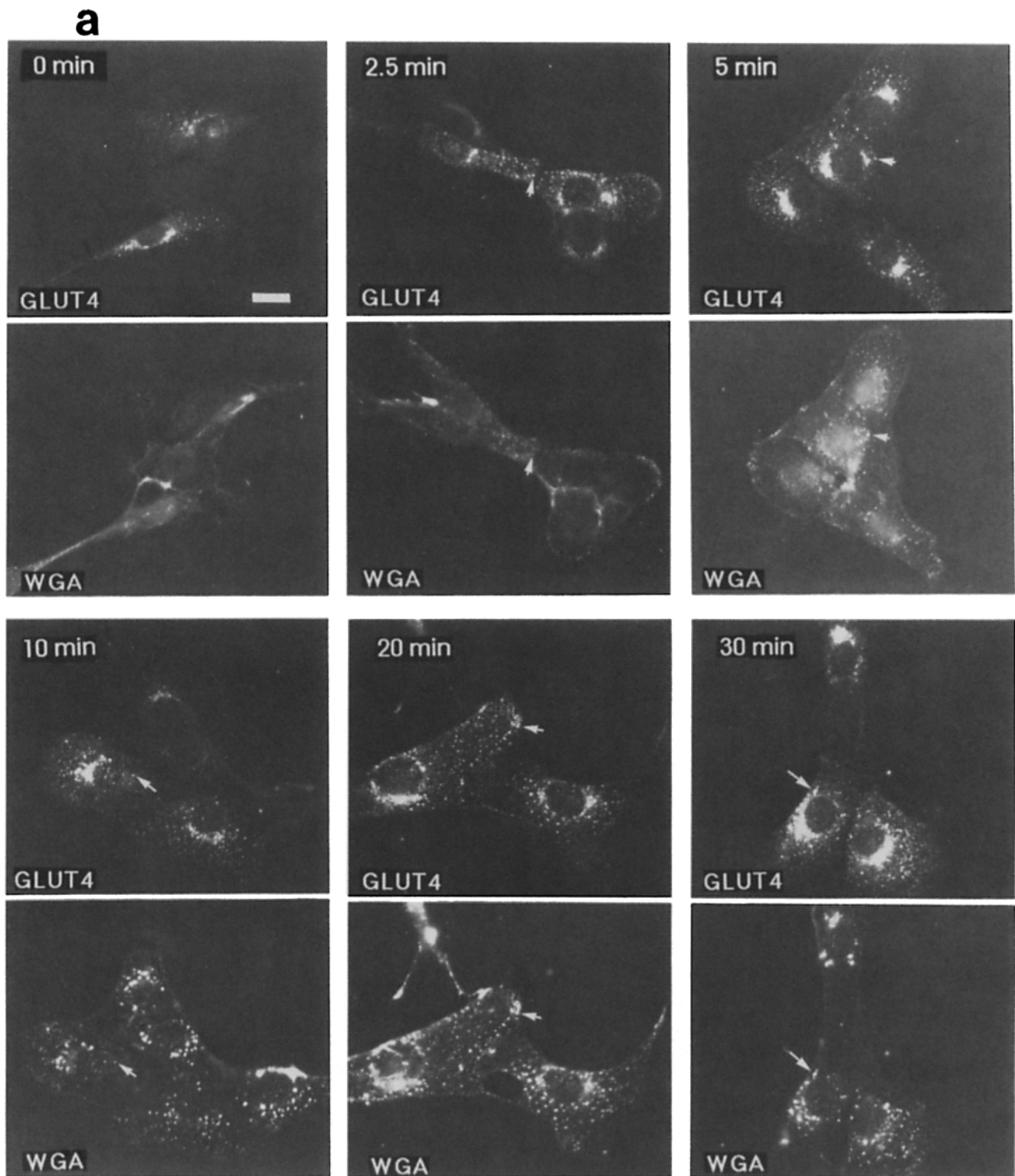


Figure 10. Endocytosis of WGA. JSM8 cells were allowed to bind FITC-WGA at 4°C and then incubated at 37°C in the absence of lectin for the times indicated (*a*) or for 2.5 min (*b*). Immunostaining with α -GLUT4 is shown in the upper panel in *a*, and on the left in *b*. In each pair of photomicrographs, the arrows indicate corresponding locations. Bars: (*a*) 20 μ m; (*b*) 10 μ m.

targeting information which directs each isoform to a distinct subcellular locale?; (*b*) Is the tissue-specific expression of the GLUT4 isoform sufficient to explain the marked disparity among cell types in their ability to respond to insulin with increased hexose uptake?; and (*c*) What is the subcellular

distribution of GLUT4 when expressed in an undifferentiated cell type, and can this pattern be useful in inferring information concerning the normal pathways of GLUT4 trafficking in adipose and muscle cells?

A number of years ago, several groups independently pro-

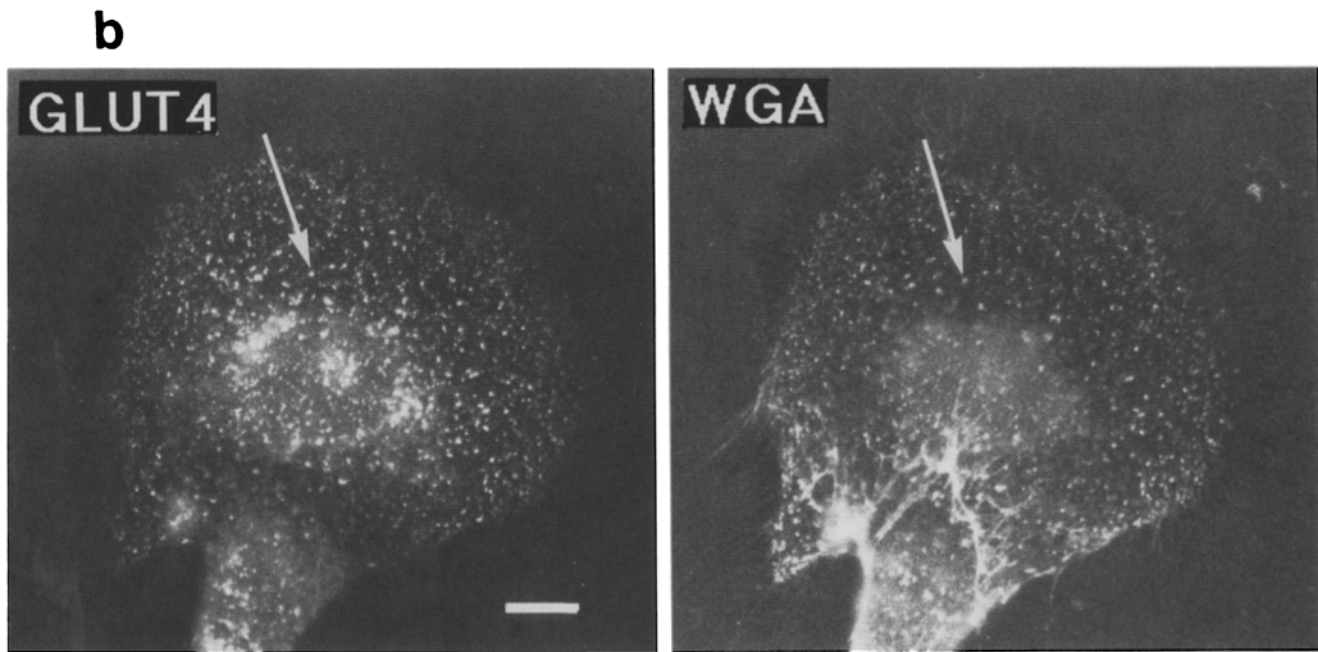


Figure 10.

posed that the mechanism by which insulin increased glucose transport was by effecting the rapid and reversible redistribution of hexose carriers from a latent intracellular pool to the plasma membrane (12, 40). This model, which has gathered increasing support over the past ten years, has placed the problem of hormone-regulated transport squarely within the realm of the control of membrane protein trafficking. Recently, with the recognition of a true gene family of facilitated glucose transporter proteins, it has become clear that insulin-responsive tissues express a distinct hexose carrier, GLUT4 (5, 10, 14, 24, 26). It is thus a very appealing hypothesis that this isoform is uniquely capable of conferring insulin-regulatable glucose transport and, moreover, that a distinctive route of cellular trafficking is responsible for this property. Clearly, in rat adipose cells, a much greater proportion of GLUT1 as compared to GLUT4 resides in plasma membranes relative to intracellular compartments in the basal state, and the fold increase in plasma membrane transporter in response to insulin is greater for GLUT4 (5, 23, 44). These observations provide some evidence for differential cellular trafficking of the two isoforms, but do not address the issue of their precise intracellular compartments. In particular, data regarding the intracellular co-localization of GLUT1 and GLUT4 are a bit confusing. Immunoabsorption of GLUT4-containing vesicles from isolated rat adipocytes has been reported to exclude GLUT1 (44); however, since GLUT4 is expressed at approximately 20-fold higher levels than GLUT1 in rat fat cells, the differential distribution might be related to level of expression. In 3T3-L1 adipocytes, in which GLUT1 is present at levels 3.5- to 5-fold greater than GLUT4, immunopurification of vesicles by immobilized α -GLUT4 has been alternatively reported to result in co-adsorption of some or most of the intracellular GLUT1 (9, 33). Most recently, Piper et al. (1991) have presented immunofluorescent localization data of hexose carriers in 3T3-L1 adipocytes indicating that GLUT1 and GLUT4 reside in distinct subcellular compartments (33).

In the current experiments, we have elected to approach the problem by a different strategy, that is, to use retrovirus-mediated gene transfer to express GLUT1 and GLUT4 under similar conditions, and characterize their subcellular distributions. In this way, differences in cellular trafficking would have to be attributable to information residing in the cloned cDNAs and the isoforms they encode, since expression is driven off the same promoter in identical cellular contexts. Two observations indicate that the transporters are recognized by cellular trafficking pathways in distinct ways: only GLUT1 appreciably alters basal hexose uptake, and the distributions as ascertained by indirect immunofluorescence are distinct (Figs. 2 and 5). The former argument is less compelling, since it assumes that the activity catalyzed by plasma membrane GLUT4 is at least comparable to that of GLUT1. While there are no direct measurements of the turnover number of GLUT4, a number of investigators have argued for greater activity of this isoform at the concentrations of substrate used in these experiments (9, 23, 27, 33). If correct, this would have the effect of making the deoxyglucose uptake assay more sensitive to increased levels of cell surface GLUT4 than GLUT1. Nonetheless, the possibility exists that other cellular proteins interact with and regulate the catalytic activity of facilitated hexose carriers, thus preventing a simple extrapolation of transport activity to plasma membrane carrier number (11). It is of interest that expression of GLUT4 in *Xenopus* oocytes in contrast to fibroblasts leads to increased hexose uptake (Fig. 3). Either the high level of expression in the oocyte system saturates the endogenous sorting pathways, or the oocyte does not completely recognize the differences between the two transporter isoforms.

The immuno-localization data provide considerably more convincing evidence for intrinsic targeting differences between GLUT1 and GLUT4. These are unlikely to be a result of a differential level of expression of the two isoforms. Transfected and endogenous GLUT1 displayed a subcellular distribution distinct from that of GLUT4, in spite of the fact

that non-transfected NIH-3T3 fibroblasts and JGT2 cells express one third and five times, respectively, as much GLUT1 as there was GLUT4 in JSM8 cells. In visually scanning polyclonal pools of GLUT1 and GLUT4 expressing cells, there was a reasonable consistency in the pattern of distribution of each of the isoforms among individual fibroblasts. The exception to this was the rare cell expressing very high levels of GLUT4, in which the isoform was also located on the cell surface. The conclusion from these experiments is that, in spite of the 65% amino acid sequence identity between GLUT1 and GLUT4, the two isoforms contain information dictating distinct cellular trafficking pathways, and that these signals can be recognized in nondifferentiated cell types.

Addition of insulin to fibroblasts expressing GLUT4 protein did not augment hexose uptake to levels different than parental, non-transfected fibroblasts (Fig. 2). Moreover, co-expression of functional insulin receptors in addition to GLUT4 did not reconstitute true hormone-regulatable hexose flux (Fig. 4). The lack of effect of the number of insulin receptors on the transport response is consistent with a previous observation that, in the course of differentiation of 3T3-L1 adipocytes, the acquisition of augmented glucose transport in response to maximal concentrations of insulin precedes the appearance of additional hormone receptors (15). Thus, the most simplistic interpretation of the data in Fig. 4 is that other tissue-specific factors are required to constitute the insulin-responsive differentiated phenotype. This conclusion is strengthened by the fact that the ratio of GLUT4 to GLUT1 in JSM8 fibroblasts, ~3:1, is considerably higher than in authentically insulin-responsive 3T3-L1 adipocytes, in which it is 1:3.5 to 1:5 (9, 33). The difficulty with further interpretation of these experiments is that the development of the hormone-sensitive phenotype is so complex that it is impossible to infer the nature of the deficient function. For example, in the course of differentiation of murine adipocytes, basal hexose uptake decreases substantially (15); obviously, this has not been reproduced in the transfected NIH-3T3 cells. Furthermore, it is unclear at present whether the lack of augmented insulin-responsive deoxyglucose uptake in JSM8 cells is a result of a decreased capacity of GLUT4 to move to the plasma membrane in response to insulin, an inability of GLUT4 to remain on the surface for an appreciable length of time, or an insubstantial contribution of the GLUT4 isoform to the total transport activity. Identification of the defective steps might suggest which adipose-specific function is lacking in the transfected fibroblasts.

Having established that upon expression in fibroblasts GLUT4 was targeted to a subcellular site distinct from GLUT1, we sought to define the nature of the compartment in which the former resided. Immuno-localization of endocytic pathways by several independent strategies revealed the presence of GLUT4. The transferrin receptor, which was chosen as a marker for early endosomes and to a lesser extent late endosomes and *trans*-Golgi network (38, 39), demonstrated the most striking coincidence of labeling with GLUT4. The mannose-6-phosphate receptor, which served to identify late endosomal structures (18), also co-localized with GLUT4 found in the perinuclear region. Taken together, these data indicate that GLUT4 is directed to all components of the endocytic pathways. To obtain further support

for this conclusion using an independent, dynamic marker of endocytosis, cell surface glycoproteins were labeled by the lectin, FITC-WGA. The uptake of WGA into CHO fibroblasts has been studied in some detail, and shown to be mediated by a subpopulation of membrane proteins, the high molecular weight acidic glycoproteins (34). WGA was rapidly internalized in CHO cells, entering the tubulo-vesicular structures of the early and late endosomal pathway, as well as recycling back to the cell surface (34). FITC-WGA appeared to behave in a similar manner in the experiments reported above. At 2.5 min, it labeled peripheral structures also detected by α -GLUT4 (Fig. 10). At later times, the FITC-WGA moved to more centrally located structures, but again co-localized with some of the GLUT4. Again, these data are most consistent with the targeting of GLUT4 to endosomal tubulovesicular structures. It is unlikely that FITC-WGA influenced the trafficking of GLUT4, since the general pattern of transporter immunoreactivity was unchanged. The data presented herein do not distinguish whether GLUT4 is transiently expressed on the cell surface or is targeted directly to an early endosomal compartment. Though the former seems more likely, proof awaits a method of labeling cell surface GLUT4.

The distribution of GLUT4 in fibroblasts is reminiscent of that of synaptophysin, a polytopic membrane-spanning protein residing in the small synaptic vesicles (SSV) of neuroendocrine cells (13). The recycling of synaptophysin in neurons in many ways resembles that of GLUT4 in adipocytes: both are recruited to the plasma membrane in response to agonist, and then follow an early endosomal pathway on their way back to the "storage" compartment (13, 37). Thus, it is striking that synaptophysin expressed in non-neuroendocrine cells localized in a distribution very much resembling that of GLUT4, concentrating in transferrin receptor-positive vesicles (25, 28). A major question now outstanding is whether the cellular location for GLUT4 in adipocytes is an extension of the ubiquitous endosomal system, or a distinct, tissue-specific organelle, perhaps analogous to small synaptic vesicles. Interestingly, when GLUT4 was expressed in the neuronal-like cell line PC12, it co-localized with synaptophysin in endosomes, in which most of the synaptophysin resided, but was excluded from small synaptic vesicles (Hudson, A. W., D. Fingar, and M. J. Birnbaum, unpublished observations).

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Note Added in Proof. A recent publication (Haney, P. M., J. W. Slot, R. C. Piper, D. E. James, and M. Mueckler. 1991. *J. Cell Biol.* 114:689-699) describing the subcellular localization of GLUT1 and GLUT4 expressed in fibroblasts agrees in substance with the results reported herein.

References

1. Andersson, S., D. L. Davis, H. Dahlback, H. Jornvall, and D. W. Russell. 1989. Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic en-

- zyme. *J. Biol. Chem.* 264:8222-8229.
2. Asano, T., Y. Shibasaki, S. Ohno, H. Taira, J. L. Lin, M. Kasuga, Y. Kanazawa, Y. Akanuma, F. Takaku, and Y. Oka. 1989. Rabbit brain glucose transporter responds to insulin when expressed in insulin-sensitive Chinese hamster ovary cells. *J. Biol. Chem.* 264:3416-3420.
 3. Baron, M. D., and H. Garoff. 1990. Mannosidase II and the 135-kDa Golgi-specific antigen recognized monoclonal antibody 53FC3 are the same dimeric protein. *J. Biol. Chem.* 265:19928-19931.
 4. Bell, G. I., T. Kayano, J. B. Buse, C. F. Burant, J. Takeda, D. Lin, H. Fukumoto, and S. Seino. 1990. Molecular biology of mammalian glucose transporters. *Diabetes Care.* 13:198-208.
 5. Birnbaum, M. J. 1989. Identification of a novel gene encoding an insulin-responsive glucose transporter protein. *Cell.* 57:305-315.
 6. Birnbaum, M. J., H. C. Haspel, and O. M. Rosen. 1986. Cloning and characterization of a cDNA encoding the rat brain glucose-transporter protein. *Proc. Natl. Acad. Sci. USA.* 83:5784-5788.
 7. Burke, B., G. Griffiths, H. Reggio, D. Louvard, and G. Warren. 1982. A monoclonal antibody against a 135-K Golgi membrane protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:1621-1628.
 8. Cahill, G. F. 1971. Physiology of insulin in man. *Diabetes.* 20:785-799.
 9. Calderhead, D. M., K. Kitagawa, L. I. Tanner, G. D. Holman, and G. E. Lienhard. 1990. Insulin regulation of the two glucose transporters in 3T3-L1 adipocytes. *J. Biol. Chem.* 265:13801-13808.
 10. Charron, M. J., F. C. Brosius, S. L. Alper, and H. F. Lodish. 1989. A glucose transport protein expressed predominantly in insulin-responsive tissues. *Proc. Natl. Acad. Sci. USA.* 86:2535-2539.
 11. Clancy, B. M., S. A. Harrison, J. M. Buxton, and M. P. Czech. 1991. Protein synthesis inhibitors activate glucose transport without increasing plasma membrane glucose transporters in 3T3-L1 adipocytes. *J. Biol. Chem.* 266:10122-10130.
 12. Cushman, S. W., and L. J. Wardzala. 1980. Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell. *J. Biol. Chem.* 255:4758-4762.
 13. De Camilli, P., and R. Jahn. 1990. Pathways to regulated exocytosis in neurons. *Annu. Rev. Physiol.* 52:625-645.
 14. Fukumoto, H., T. Kayano, J. B. Buse, Y. Edwards, P. F. Pilch, G. I. Bell, and S. Seino. 1989. Cloning and characterization of the major insulin-responsive glucose transporter expressed in human skeletal muscle and other insulin-responsive tissues. *J. Biol. Chem.* 264:7776-7779.
 15. Garcia de Herreros, A., and M. J. Birnbaum. 1989. The acquisition of increased insulin-responsive glucose transport in 3T3-L1 adipocytes correlates with expression of a novel transporter gene. *J. Biol. Chem.* 264:19994-19999.
 16. Gould, G. W., and G. E. Lienhard. 1989. Expression of a Functional Transporter in *Xenopus* oocytes. *Biochemistry.* 28:9447-9452.
 17. Granger, B. L., S. A. Green, C. A. Gabel, C. L. Howe, I. Mellman, and A. Helenius. 1990. Characterization and cloning of Igpl10, a lysosomal membrane glycoprotein from mouse and rat cells. *J. Biol. Chem.* 265:12036-12043.
 18. Griffiths, G., B. Hofflack, K. Simons, I. Mellman, and S. Kornfeld. 1988. The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell.* 52:329-341.
 19. Harrison, S. A., J. M. Buxton, B. M. Clancy, and M. P. Czech. 1990. Insulin regulation of hexose transport in mouse 3T3-L1 cells expressing the human HepG2 glucose transporter. *J. Biol. Chem.* 265:20106-20116.
 20. Harrison, S. A., J. M. Buxton, A. L. Helgerson, R. G. MacDonald, F. J. Chlapowski, A. Carruthers, and M. P. Czech. 1990. Insulin action on activity and cell surface disposition of human HepG2 glucose transporters expressed in Chinese hamster ovary cells. *J. Biol. Chem.* 265:5793-5801.
 21. Hartman, S. C., and R. C. Mulligan. 1988. Two dominant-acting selectable markers for gene transfer studies in mammalian cells. *Proc. Natl. Acad. Sci. USA.* 85:8047-8051.
 22. Haspel, H. C., M. J. Birnbaum, E. W. Wilk, and O. M. Rosen. 1985. Biosynthetic precursors and *in vitro* translation products of the glucose transporter of human hepatocarcinoma cells, human fibroblasts, and murine preadipocytes. *J. Biol. Chem.* 260:7219-7225.
 23. Holman, G. D., I. J. Kozka, A. E. Clark, C. J. Flower, J. Saltis, A. D. Habberfield, I. A. Simpson, and S. W. Cushman. 1990. Cell surface labeling of glucose transporter isoform GLUT4 by bis-mannose photolabel. Correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester. *J. Biol. Chem.* 265:18172-18179.
 24. James, D. E., M. Strube, and M. Mueckler. 1989. Molecular cloning and characterization of an insulin-regulatable glucose transporter. *Nature (Lond.).* 338:83-87.
 25. Johnston, P. A., P. L. Cameron, H. Stukenbrok, R. Jahn, P. de Camilli, and T. C. Sudhof. 1989. Synaptophysin is targeted to similar microvesicles in CHO and PC12 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2863-2872.
 26. Kaestner, K. H., R. J. Christy, J. C. McLenithan, L. T. Braiterman, P. Cornelius, P. H. Pekala, and M. D. Lane. 1989. Sequence, tissue distribution, and differential expression of mRNA for a putative insulin-responsive glucose transporter in mouse 3T3-L1 adipocytes. *Proc. Natl. Acad. Sci. USA.* 86:3150-3154.
 27. Keller, K., M. Strube, and M. Mueckler. 1989. Functional expression of the human HepG2 rat adipocyte glucose transporters in *Xenopus* oocytes. *J. Biol. Chem.* 264:18884-18889.
 28. Leube, R. E., B. Wiedenmann, and W. W. Franke. 1989. Topogenesis and sorting of synaptophysin: synthesis of a synaptic vesicle protein from a gene transfected into nonneuroendocrine cells. *Cell.* 59:433-446.
 29. Mann, R., R. C. Mulligan, and D. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell.* 33:153-159.
 30. Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035-7056.
 31. Morgenstern, J. P., and H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* 18:3587-3596.
 32. Mueckler, M., C. Caruso, S. A. Baldwin, M. Panico, I. Blench, H. R. Morris, W. J. Allard, G. E. Lienhard, and H. F. Lodish. 1985. Sequence and structure of a human glucose transporter. *Science (Wash. DC).* 229:941-945.
 33. Piper, R. C., L. J. Hess, and D. E. James. 1991. Differential sorting of two glucose transporters expressed in insulin-sensitive cells. *Am. J. Physiol.* 260:C570-580.
 34. Raub, T. J., M. J. Koroly, and R. M. Roberts. 1990. Endocytosis of wheat germ agglutinin binding sites from the cell surface into a tubular endosomal network. *J. Cell Physiol.* 143:1-12.
 35. Ruther, U., and B. Muller-Hill. 1983. Easy identification of cDNA clones. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1791-1794.
 36. Simpson, I. A., and S. W. Cushman. 1986. Hormonal regulation of mammalian glucose transport. *Annu. Rev. Biochem.* 55:1059-1089.
 37. Slot, J. W., H. J. Geuze, S. Gigengack, G. E. Lienhard, and D. E. James. 1991. Immuno-localization of the insulin-regulatable glucose transporter in brown adipose tissue of the rat. *J. Cell Biol.* 113:123-135.
 38. Stoorvogel, W., H. J. Geuze, J. M. Griffith, A. L. Schwartz, and G. J. Strous. 1989. Relations between the intracellular pathways of the receptors for transferrin, asialoglycoprotein, and mannose 6-phosphate in human hepatoma cells. *J. Cell Biol.* 108:2137-2148.
 39. Stoorvogel, W., H. J. Geuze, J. M. Griffith, and G. J. Strous. 1988. The pathways of endocytosed transferrin and secretory protein are connected in the trans-Golgi reticulum. *J. Cell Biol.* 106:1821-1829.
 40. Suzuki, K., and T. Kono. 1980. Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. *Proc. Natl. Acad. Sci. USA.* 77:2542-2545.
 41. Vera, J. C., and O. M. Rosen. 1989. Functional expression of mammalian glucose transporter in *Xenopus laevis* oocytes: evidence for cell-dependent insulin sensitivity. *Mol. Cell Biol.* 9:4187-4195.
 42. White, M. F., J. N. Livingston, J. M. Backer, V. Lauris, T. J. Dull, A. Ullrich, and C. R. Kahn. 1988. Mutation of the insulin receptor at tyrosine 960 inhibits signal transmission but does not affect its tyrosine kinase activity. *Cell.* 54:641-649.
 43. Whittaker, J., A. K. Okamoto, R. Thys, G. I. Bell, D. F. Steiner, and C. A. Hoffman. 1987. High-level expression of human insulin receptor cDNA in mouse NIH 3T3 cells. *Proc. Natl. Acad. Sci. USA.* 84:5237-5241.
 44. Zorzano, A., W. Wilkinson, N. Kotliar, G. Thoidis, B. E. Wadzinski, A. E. Ruoho, and P. F. Pilch. 1989. Insulin-regulated glucose uptake in rat adipocytes is mediated by two transporter isoforms present in at least two vesicle populations. *J. Biol. Chem.* 264:12358-12363.