The Efficient Intracellular Sequestration of the Insulin-regulatable Glucose Transporter (GLUT-4) Is Conferred by the NH$_2$ Terminus

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Abstract. GLUT-4 is the major facilitative glucose transporter isoform in tissues that exhibit insulin-stimulated glucose transport. Insulin regulates glucose transport by the rapid translocation of GLUT-4 from an intracellular compartment to the plasma membrane. A critical feature of this process is the efficient exclusion of GLUT-4 from the plasma membrane in the absence of insulin. To identify the amino acid domains of GLUT-4 which confer intracellular sequestration, we analyzed the subcellular distribution of chimeric glucose transporters comprised of GLUT-4 and a homologous isoform, GLUT-1, which is found predominantly at the cell surface. These chimeric transporters were transiently expressed in CHO cells using a double subgenomic recombinant Sindbis virus vector. We have found that wild-type GLUT-4 is targeted to an intracellular compartment in CHO cells which is morphologically similar to that observed in adipocytes and muscle cells. Sindbis virus–produced GLUT-1 was predominantly expressed at the cell surface. Substitution of the GLUT-4 amino-terminal region with that of GLUT-1 abolished the efficient intracellular sequestration of GLUT-4. Conversely, substitution of the NH$_2$-terminus of GLUT-1 with that of GLUT-4 resulted in marked intracellular sequestration of GLUT-1. These data indicate that the NH$_2$-terminus of GLUT4 is both necessary and sufficient for intracellular sequestration.

In insulin rapidly stimulates the movement, or "translocation," of glucose transporter proteins from an intracellular compartment to the cell surface (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). This process only occurs in muscle and adipose tissue and plays a central role in the postabsorptive disposal of glucose (Elbrink and Bihler, 1975). Muscle and adipose tissue exclusively express a specialized glucose transporter isoform, GLUT-4 (James et al., 1988). GLUT-4 is one of four mammalian facilitative glucose transporter isoforms which constitute a family of polytopic type II membrane proteins showing an identical predicted secondary structure (Bell et al., 1990). In basal or nonstimulated adipocytes and myocytes, GLUT-4 is located within intracellular tubulo-vesicular structures associated with the trans-Golgi reticulum (TGR) and clustered throughout the cytoplasm (Slot et al., 1990; 1991a,b). After insulin stimulation, GLUT-4 is depleted from these intracellular structures and is increased by as much as 40-fold at the cell surface (Slot et al., 1991a). This effect is adequate to account for the 10–30-fold increase in glucose transport which is observed in insulin-sensitive tissues. Insulin also stimulates the translocation of other proteins such as the transferrin receptor and the IGF II/mannose-6-phosphate receptor to the cell surface (Davis et al., 1986; Oka et al., 1984). However, what distinguishes GLUT-4 from these proteins is its virtual exclusion from the plasma membrane in basal cells (James et al., 1988; Piper et al., 1991; Slot et al., 1991a). It is this efficient intracellular targeting that allows GLUT-4 to be particularly suitable for mediating the regulated entry of glucose into the cell in response to physiological stimuli such as insulin and exercise.

The efficient intracellular targeting of GLUT-4 is quite different from that of other glucose transporter isoforms such as GLUT-1. In 3T3-L1 adipocytes, GLUT-1 is abundantly expressed on the plasma membrane. Furthermore, the intracellular pool of GLUT-1 appears to be partially segregated from the pool of intracellular GLUT-4, suggesting some degree of differential intracellular compartmentation (Piper et al., 1991). The differential targeting of GLUT-4 and GLUT-1 is even more polarized in cell types that do not regulate glucose transport in response to insulin. GLUT-4 expressed in stably transfected HepG2 cells and 3T3 fibroblasts is predominantly intracellular and resides in structures morphologi-
cally similar to those in which GLUT-4 is found within insulin-sensitive cells (Haney et al., 1991; Hudson et al., 1992). In contrast, GLUT-1 is found predominantly at the plasma membrane in these cells (Blok et al., 1988; Haney et al., 1991). Thus, it appears that the GLUT-4 protein itself contains the information required for its efficient sequestration from the plasma membrane and that the cellular machinery involved in regulating this process is endogenous to a variety of cell types.

The purpose of the present study was to define the molecular basis for the unique sequestration of GLUT-4 as a preliminary step toward identifying the cellular machinery involved in insulin-stimulated translocation. Taking advantage of both the differential sorting of GLUT-1 and GLUT-4 and their high degree of structural homology, we have constructed a panel of chimeric glucose transporters in order to define the amino acid domain(s) responsible for the efficient intracellular targeting of GLUT-4. We have expressed this panel of mutant glucose transporters in a variety of cells using a recombinant Sindbis virus expression system (Hahn et al., 1992) and have found that the NH2-terminal hydrophilic region is both necessary and sufficient for intracellular sequestration of the glucose transporter.

**Materials and Methods**

**Cell Culture**

CHO cells (CHO-K1) obtained from the American Type Culture Center (ATCC, Rockville, MD) were cultured in DME supplemented with 10% FCS (Hazelton Systems, Inc., Aberdeen, MD) and nonessential amino acids (Sigma Chemical Co., St. Louis, MO). Confluent monolayers of CHO cells were split 1:3 15 h before viral infection. BHK cells (BHK-21: ATCC clone 13) were cultured in DME containing 7% FCS. CEFs were prepared as described previously (Pierce et al., 1974).

**Chimera Constructions**

DNA and RNA manipulations were performed according to standard techniques (Maniatis et al., 1982). To construct chimeric cDNAs, base pairs 140-1885 of the rat GLUT-4 clone (James et al., 1989) and base pairs -7-1605 of the human GLUT-1 clone (Mueckler et al., 1985) were used. All transporter cDNAs were subcloned between the XbaI and XhoI sites of Bluescript (Stratagene, La Jolla, CA) in the sense orientation relative to the phage T3 promoter. The previously published alignment GLUT-1 and GLUT-4 (Bell et al., 1990; Biruma, 1989) was used as the basis for domain exchange between these two cDNAs. Chimeras were constructed by fusing portions of each cDNA using restriction sites that were either endogenous or created with polymerase chain reaction (PCR). Hot Tub DNA polymerase (Amersham Corp., Arlington Heights, IL) was used for 30 cycles with 1 min 95°C denaturation, 1 min 50°C hybridization, and 2.5 min polymerization at 65°C. PCR fragments were purified by agarose gel electrophoresis, digested with restriction enzymes, and subcloned into the wild type transporter cDNA in order to minimize the amount of DNA which originated from the PCR. All chimeras left the deduced amino acid sequence of the respective glucose transporter cDNA fragments unaltered except for 4/1-C, 4/1-D, 4/2-A, and 4/4-I/4D/4/2. In these chimeras, the Ser at position 265 of GLUT-4, or the asparagine at position 41 of GLUT-4, was changed to an aspartic acid in order to facilitate the use of a BamHI or EcoR restriction site for recombination. It is unlikely that these amino acid substitutions change the overall structure of the chimeric transporters since GLUT-2 contains an aspartic acid residue at the homologous position (Bell et al., 1990). All regions of the cDNA clones produced by PCR were sequenced (Sanger et al., 1977). Chimeric cDNAs were then excised from Bluescript using XbaI and XhoI and subcloned into the Sindbis virus expression vector plasmid KSind. The amino acid composition of the chimeras is as follows: 4/1-C, GLUT-1, 4-1885; 140-1885; 4/1-D, GLUT-1, 4-1885; 4/2-A, GLUT-1, 4-1885; 4/2-D, GLUT-1, 4-1885; 4/2-A, GLUT-1, 4-1885; 4/4-I, GLUT-1, 4-1885; 4/4-I, 4-1885; 4/4-I/4D/4/2. The restriction sites used to splice 4/1-C, 4/1-D, 4/1-B, 4/1-C, 4/1-D, and 4A/1/4D were BamHI, Bgl II, SacII, BamHI, Bgl II, and EcoRv/BglIII, respectively.

**Sindbis Virus**

Because of the difficulties encountered with obtaining reproducible high-level expression of glucose transporter isoforms in mammalian cells using DNA transfection, we adapted the Sindbis virus expression vector (Hahn et al., 1992; Huang et al., 1989; Xiong et al., 1989) for our studies. The utility of other viral expression systems for studying either protein function (Lucking, 1990) or protein sorting (Martin and Simons 1984; Pfeffer and Rothman, 1987) has been demonstrated. Sindbis virus-based expression vectors offer a number of advantages over these other expression systems for studying glucose transporter targeting and function (Huang et al., 1989). First, manipulation of genomic constructs is simple and rapid (Hahn et al., 1992). Second, the level of glucose transporter expression is high and can be titrated by either adjusting the length of infection time or using various viral mutants (Xiong et al., 1989). The flexibility of infection conditions not only allows transporter expression at physiological levels, appropriate for the study of targeting, but also allows for high-level overexpression suitable for the study of transporter function. Third, Sindbis virus quantitatively infects a wide range of cells, which provides the opportunity to perform studies in a variety of cell types. Fourth, in contrast to stably transfected cell lines, transient expression of glucose transporters avoids the inadvertent isolation of clonal variants with altered transporter targeting. Furthermore, expression of different transporter proteins can easily be coordinated and performed in cells cultured under identical conditions. Finally, it has been proposed that the glucose transporter oligomerizes (Cuoppolo et al., 1989; Jacobs et al., 1991), which raises the possibility that exogenously expressed transporters may form hetero-oligomers with endogenous transporters presumably in the ER (Hultrey and Helenius, 1989). These hetero-oligomers could have ambiguous sorting characteristics. However, the high and rapid expression of transporters by Sindbis virus together with an inhibition of host protein synthesis by Sindbis virus (Straus and Straus, 1986) may allow the exogenous transporter to be synthesized independently of the presence of native transporters and thus minimize the formation of hetero-oligomeric transporters.

The Sindbis virus expression vectors used were based on a full-length Sindbis virus cDNA clone (Rice et al., 1987) containing an additional subgenomic mRNA promoter (Levis et al., 1990). The Sindbis virus expression vector KASind was derived from the vector TotoCATS (Xiong, C., and H. Huang, unpublished data). TotoCATS is comprised of the Totol1 Sindbis virus genomic cDNA (Rice et al., 1987). Between the coding regions for the non-structural and structural proteins was a polylinker (XbaI and Xhol) and subgenomic mRNA promoter (Levis et al., 1990). To produce KASind, a fragment containing the full-length genomic cDNA of TotoCATS, together with the 5′ SP6 RNA polymerase promoter was subcloned by blunt end ligation into the KpnI and Xhol sites of the plasmid Proteus 31 (Huang, H., unpublished data). This resulted in placing Mil and NoI restriction sites immediately 3′ to the viral genomic cDNA. In all experiments, the vector KASind was used to express the transporter isoforms except for the EM localization of GLUT-4 which utilized the vector Totol1. Tox832 is essentially identical to Totol1 except that Totol1 cDNA was utilized (Rice et al., 1987). For Toto32J-GLUT-1, an Ncol-SaPl fragment (base pairs 1-1629) of the human GLUT-1 cDNA was subcloned into the Ncol and BamHI (blunt) site of the plasmid shuttle vector pH2JII (Hahn et al., 1992). The GLUT-1 cDNA, together with subgenomic mRNA promoter sequences contained in pH2JII, was excised with Apal and Xhol and subcloned into Toto32J (Hahn et al., 1992). In order to attach to KASind, Toto32J placed the MspI recognition coding for the structural genes. This has been shown to give somewhat higher expression of foreign genes than KASind and TotoCATS (see Fig. 1 b; Xiong, C., C. Hahn, H. Huang, C. Rice, unpublished observation). However, the foreign gene may be less stable during passage of the virus (Xiong, C. Hahn, H. Huang, C. Rice, unpublished observation).

Virus stocks were produced essentially as previously described (Grakouli et al., 1989). Briefly, capped genômic-length RNA transcripts were synthesized in vitro with SP6 RNA polymerase using the KSind/chimeric plasmids or Toto32J-GLUT-1 linearized with Mil and Xhol, respectively. For transfection, 10 μg of transcript RNA was mixed with 50 μg of Lipofectin reagent (BRL, Bethesda, MD) (Pfeifer et al., 1987), incubated for 10 min in a polystyrene tube, and diluted to 2 ml with PBS. This mixture was then added to BHK cells (109/15 cm plate), incubated for 15 min at 37°C, washed in PBS, and then cultured in DME containing 7% FCS. After 20 h the media was changed and at 32 h the virus-containing culture media was...
collected as viral stock. Stocks were stored in aliquots at −80°C. Thawed aliquots had titers of 10^9−10^10 plaque forming U/ml.

For viral infection CHO cells were washed with PBS containing 0.2% FCS and incubated for 25 min at 37°C with virus diluted with PBS (3 m/15cm plate) at a multiplicity of infection of ∼50. Fresh media was then added and cells were maintained at 5% CO₂ at 37°C. Typically, infection was allowed to proceed for 6 h after which cells were washed with PBS and cultured in DME containing 1% BSA and 20 μg/ml cycloheximide (Sigma Chemical Co.) for 1 h before analysis.

The concentration of cycloheximide necessary for the inhibition of viral protein synthesis was determined by washing CHO cells (at 6 h postinfection) with PBS before culturing them in methionine and serum-free DME with different concentrations of cycloheximide. After a 10-min incubation, [35S]methionine (100 μCi/ml; ICN Radiochemicals, Irvine, CA) was added and incubation continued for 30 min. Quantitation of TCA precipitable radioactivity showed that treatment with 20 μg/ml cycloheximide inhibited the incorporation of [35S]methionine by >95% in virally infected cells.

**In Vitro Translations**

Translations were performed as detailed previously (James et al., 1989). Plasmids containing cDNAs of chimeric and wild type transporters were linearized with XhoI and used as a template for in vitro synthesis of RNA by phage T7 RNA polymerase. Translations were carried out in 25 μl of reticulocyte lysate (Promega-Biotec, Madison, WI) containing 20 μCi [35S]methionine and 2 μl canine pancreatic microsomes according to the manufacturer's instructions. Microsomes were provided by Dr. R. Mercer (Dept. of Cell Biology, Washington University, St. Louis, MO). Translation reactions were incubated for 60 min at 30°C. Microsomes were centrifuged for 15 min in a microfuge and washed with cold PBS containing 1 mM EDTA. Translation products were subjected to SDS-PAGE and visualized by fluorography using salicylic acid.

**Antibodies**

Antipeptide polyclonal antibodies specific for the COOH terminus of GLUT1 (R493) and GLUT4 (R820) and anti-GLUT-4 mAb IF8 have been described previously (Piper et al., 1991). Rabbit polyclonal anti-Na/K ATPase (α subunit) was provided by Dr. R. Mercer (Dept. Cell Biology, Washington University, St. Louis, MO) and generated against purified rat kidney Na/K ATPase. Rabbit polyclonal anti-IGF II/Mannose-6-Phosphate receptor was provided by Dr. C. Scott (Dept. of Endocrinology, Royal Prince Alfred Hospital, Camperdown, Australia) and was generated against purified bovine IGF II receptor. Rabbit polyclonal anti-β-COP antisera was provided by Dr. T. Kreis (EMBL, Heidelberg, FRG). 125I-labeled goat antirabbit secondary antibody was purchased from ICN.

**Immunofluorescence Microscopy**

Confocal laser scanning microscopy and immunofluorescence labeling of glucose transporters was performed as previously described (Piper et al., 1991). Cells were fixed in 2% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100. Cells were incubated with R820 (40 μg/ml) or R493 (10 μg/ml) overnight at 4°C, washed, and incubated with FITC-conjugated goat antirabbit secondary antibody (Cappel, West Chester, PA). Optical sectioning (2 μm thickness) was performed by scanning laser confocal microscopy.

Immunofluorescence labeling of plasma membrane larvae was performed as detailed (Robinson et al., 1992). Cells were grown on coverslips and incubated at 4°C in KHME buffer (70 mM KCl, 3 mM EGTA, 5 mM MgCl₂, and 30 mM HEPES, pH 7.4) containing 1 mg/ml poly-L-lysine for 10 s. Cells were washed rapidly twice in hypotonic KHME (0.33×) and sonicated with four 0.5-s bursts with a Kontes 115 V Disrupter equipped with a 3.2 x 48 mm tapered probe while immersed in isotonic KHME buffer. This technique produces patches of highly purified plasma membranes attached to the coverslip (Heuser and Anderson, 1989). Plasma membrane fragments were then fixed in KHME buffer containing 2% paraformaldehyde and processed for immunofluorescence labeling as described above except that Triton X-100 was omitted. For quantitation, fluorescently labeled plasma membrane fragments from at least 45 cells in seven different fields of each coverslip were collected with a 63 x objective by confocal microscopy using identical microscope photomultiplier tube settings within an experiment and measured for average pixel intensity using the Bio-Rad image software (Bio-Rad Laboratories, Cambridge, MA). Photomicrography of plasma membrane sheets was performed directly from the microscope monitor using identical settings.

**Electron Microscopy**

Cells were fixed in 100 mM NaPO₄ buffer, pH 7.1, containing 2% paraformaldehyde and 0.25% glutaraldehyde (Sigma Chemical Co.) for 8 h at 22°C. Ultrathin cryosections were labeled with either R820 or R493 and 10-nm gold-conjugated protein A as detailed previously (Slot et al., 1990).

**Subcellular Fractionation by Differential Centrifugation**

All steps were performed at 0–4°C. Cells (2 × 15 cm dishes) were washed three times in 20 ml of HES buffer (20 mM HEPES, pH 7.4, 255 mM sucrose, 1 mM EDTA) containing 1 mM PMSF. Cells were scraped in 10 ml of HES and homogenized by 20 passages with a 15-ml dounce and teflon pestle driven at 720 rpm. The homogenate was centrifuged at 19,000 g for 20 min. The supernatant was centrifuged at 45,000 g for 20 min after which the pellet was discarded and the supernatant centrifuged for 90 min at 180,000 g. The pellet from this high speed spin was designated fraction B. The pellet from the initial 19,000 g spin was resuspended in 15 ml of HES buffer and centrifuged a second time at 19,000 g for 20 min. The resulting pellet was resuspended in 1 ml HES and layered onto a sucrose cushion (1.3 M sucrose containing 20 mM HEPES, pH 7.4, and 1 mM EDTA) and centrifuged at 110,000 g for 90 min in a Beckman SW-41 rotor (Beckman Instruments, Inc., Palo Alto, CA). The membrane band and the interface were collected and designated fraction A.

Equivalent amounts of protein from each fraction were subjected to SDS-PAGE and immunoblotting as previously described (Piper et al., 1991). 2-Deoxyglucose (2DOG) uptake was measured in CHO cells grown in 24-well culture plates. After infection and cycloheximide treatment, cells were washed three times in PBS containing 1% BSA. 2DOG uptake (10 μM 3H 2-deoxyglucose, 2.5 μCi/ml; Sigma Chemical Co.) was performed for 3 min at 37°C. The uptake of 2DOG in CHO cells infected with either GLUT1 or I4-D-producing Sindbis virus was linear for at least 4 min. 2DOG uptake was arrested by three successive washes with HES buffer (4°C).

**Results**

**Expression of the Glucose Transporter with Sindbis Virus**

Sindbis virus is an enveloped plus-stranded RNA virus which can be genetically engineered to rapidly express foreign genes at high levels in both mammalian and insect cells (Huang et al., 1989). The types of Sindbis virus expression vectors used in the present study are different from those previously reported (Xiong et al., 1989). The vectors used here produce Sindbis virus recombinants which are capable of infection and propagation independent of a helper virus. This has been accomplished by introducing a subgenomic mRNA promoter into the viral genome which can direct the synthesis of an exogenous gene. This promoter is activated in tandem with a second subgenomic promoter which directs the synthesis of mRNA encoding the viral structural proteins. To test the utility of the Sindbis viral expression vector KASind, recombinant virus stocks containing either the GLUT4 or GLUT1 cDNAs were prepared and used to infect fresh cultures of CHO cells. Immunoblot analysis of membrane samples from CHO cells infected with recombinant GLUT4 virus showed a single band of appropriate molecular mass (∼45 kD) (Fig. 1a). The time-dependence of GLUT4 production in Sindbis-infected CHO cells was also investigated (Fig. 1b). GLUT4 production was followed for 8 h. During the initial stage of viral infection (2 h postinfection) little, if any, GLUT4 was synthesized. Between 2 and 4 h, however, a large amount of GLUT4 had accumulated and by 6 h the level of GLUT4 had begun to plateau. The level of GLUT4 at 6 h postinfection was comparable to the level

Piper et al. Intracellular Sequestration Domain of GLUT-4

731
Glucose Transporters Expressed by Sindbis Virus Are Targeted Appropriately

A potential limitation of the Sindbis expression system is that the viral infection per se may alter cellular targeting functions. Therefore, it was critical for our analysis to demonstrate that the differential sorting of GLUT-1 and GLUT-4 was faithfully preserved in Sindbis virus-infected cells.

In our initial studies, CHO cells were infected for 6 h and the distribution of Sindbis virus–produced glucose transporters was studied using electron microscopic immunogold labeling of ultrathin cryosections of CHO cells infected with recombinant Sindbis virus (KASind) expressing GLUT-4. GLUT-4 was localized by immunogold labeling of ultrathin cryosections of CHO cells infected with recombinant Sindbis virus (KASind) expressing GLUT-4. GLUT-4 was found in rat adipocytes (∼1.5-fold that of rat adipocytes on a per cell basis).

GLUT-1 was also expressed in CHO cells using the Sindbis virus expression system. Since GLUT-1 is the endogenous glucose transporter expressed in CHO cells, distinguishing virally produced GLUT-1 relies on over-expression. The overall level of GLUT-1 production in CHO cells was determined using two different Sindbis viral vectors (KASind and Tot032J). Immunoblot analysis of infected and mock-infected CHO cells with an anti-GLUT-1 COOH-terminal antibody (R493) showed that both vectors were capable of over-expressing GLUT-1 by 7- and 11-fold, respectively (Fig. 1a). Virally produced GLUT-1 was observed as an immunoreactive band of identical molecular weight to the endogenous GLUT-1 transporter. The level of endogenous GLUT-1 was not altered in CHO cells infected for 6 h with either recombinant GLUT-4 Sindbis, or recombinant Sindbis virus producing the chloramphenicol acetyltransferase (CAT) enzyme. Likewise, no change in 2-deoxyglucose uptake was observed in CHO cells (data not shown) or CEF cells infected with the CAT expressing Sindbis recombinant (Fig. 1c). To demonstrate that Sindbis virus-produced GLUT-1 was functional, 2DOG uptake was measured in CEFs infected for various times with Tot032J/GLUT-1 recombinant virus. The increase in 2DOG uptake paralleled the increase in GLUT-1 levels (10-fold) measured by immunoblot analysis (data not shown) as compared to cells infected with parental Sindbis virus (Fig. 1c).

Figure 2. Immunoelectron microscopic localization of GLUT-4 in Sindbis-infected CHO cells. GLUT-4 was localized by immunogold labeling of ultrathin cryosections of CHO cells infected with recombinant Sindbis virus (KASind) expressing GLUT-4. GLUT-4 was labeled with R820 followed by protein A conjugated to 10-nm gold. (A and B) Cells were infected for 6 h and then treated with cycloheximide for 1 h. Labeling near the TGR (arrows) and clustered tubulo/vesicular elements (arrowheads) is shown. (C) Cells were infected for 6 h and fixed immediately without cycloheximide treatment. Labeling of Golgi cisternae (g) and the nuclear envelope (small arrowhead), adjacent to the nucleus (n) is shown. Bar, 200 nm.
Figure 3. Immunoelectron microscopic localization of GLUT-1 in Sindbis-infected CHO cells. CHO cells were infected with GLUT-1 expressing recombinant Sindbis virus (Toto322) and immunolabeled with anti-GLUT-1 COOH-terminal antibody (R493) and protein A-conjugated gold (A and B). Heavy labeling of the cell surface is shown as well as labeling beneath the cell surface (arrowheads). Often dense labeling was observed near the Golgi complex (g) as in A or clustered beneath the cell surface as in B. Labeling is specific for Sindbis virus-produced GLUT-1 because cells not over-producing GLUT-1 (see lower cell in A) were not labeled. Bar, 200 nm.

localization. These studies showed that in some cells there was significant labeling of the biosynthetic pathway, namely the ER and Golgi cisternae (Fig. 2 C). To circumvent this, viral protein synthesis was blocked with cycloheximide for 1 h following a 6-h infection. Under these conditions much less immunogold labeling of transporters was observed in the ER or Golgi cisternae (Fig. 2 B). Thus, to minimize the complexity of our analysis due to the presence of immature transporter molecules en route in the biosynthetic pathway, we adopted these conditions for all experiments.

The differential targeting of GLUT-4 and GLUT-1 in Sindbis-infected CHO cells was examined by immunolocalization at the electron microscopic level. GLUT-4 was localized to intracellular tubulo-vesicular elements that were clustered either in the TGR region of the cell or in the cytoplasm, sometimes quite close to the plasma membrane (Fig. 2 A).
However, little GLUT-4 labeling was observed in the plasma membrane. The intracellular location of GLUT-4 in Sindbis virus–infected CHO cells was very similar to that previously described for endogenous GLUT-4 in brown adipose tissue, white adipose tissue, cardiac muscle, and skeletal muscle isolated from non-insulin-stimulated rats (Rodnick et al., 1992; Slot et al., 1990, 1991a,b). In contrast to the specific intracellular targeting of GLUT-4, Sindbis virus-produced GLUT-1 was labeled prominently at the plasma membrane (Fig. 3, A and B). However, a significant amount of GLUT-1 was also localized to intracellular vesicles. These observations complement previous studies in 3T3-L1 adipocytes which indicate that while the overall subcellular distribution of GLUT-1 and GLUT-4 is distinct, the intracellular pathways of these two transporters do overlap to some extent (Blok et al., 1988; Calderhead et al., 1990; Piper et al., 1991). Fig. 3 a illustrates that viral expression of GLUT-1 was very high such that labeling of endogenous GLUT-1 could almost be neglected. The particular viral stock used in this experiment, which was derived from the vector Toto32Z, did not express GLUT-1 in all cells (Fig. 3 A, bottom) and, thus, provided an internal control for the specificity of virally produced GLUT-1 labeling in comparison with endogenous levels of GLUT-1.

The differential targeting of Sindbis virus–produced GLUT-4 and GLUT-1 illustrated by immuno-EM was confirmed by laser scanning confocal immunofluorescence microscopy. GLUT-4 labeling was found entirely intracellularly in a perinuclear pattern characteristic of the TGR and endosomal compartments (Fig. 4 a). This perinuclear distribution was also visualized by confocal cross-sectioning through individual cells (Fig. 4 a). The localization of GLUT-4 observed in CHO cells by immunofluorescence was indistinguishable from the immunofluorescent localization of GLUT-4 expressed either endogenously in 3T3-L1 adipocytes (Garcia de Herreros and Birnbaum, 1990; Piper et al., 1991) or in GLUT-4–transfected L6 myoblasts (Lawrence et al., 1992), 3T3-L1 fibroblasts (Haney et al., 1991), NIH 3T3 fibroblasts (Hudson et al., 1992), and CHO cells (Shibasaki et al., 1992). Localization of over-produced GLUT-1 by confocal immunofluorescence microscopy showed intense labeling of the plasma membrane (Fig. 4, b and b). To ensure that the staining pattern observed was indicative of the plasma membrane, unfixed CHO cells were incubated with FITC-conjugated wheat germ agglutinin (WGA) at 4°C to specifically label the cell surface (data not shown). The fluorescence pattern of WGA–FITC–labeled cells was identical to that of immunolocalized GLUT-1. The GLUT-1 labeling shown in Fig. 4 b was specific for virus-produced GLUT-1 because at the same microscope photomultiplier tube amplification settings, labeling of endogenous GLUT-1 in uninfected cells was not detected (Fig. 4 e). Endogenous GLUT-1 labeling could be detected, however, by increasing the microscope amplification (Fig. 4 f). Importantly, the distribution of the endogenous GLUT-1 was the same as that in cells infected with Sindbis virus expressing GLUT-1.

To test whether the specific targeting of GLUT-4 was both independent of the overall level of GLUT-4 expression and independent of the conditions of viral infection, we localized GLUT-4 at both early and late times of infection. The distribution of GLUT-4 as determined by immunofluorescence was identical in CHO cells infected for either 3 or 7 h (Fig. 4, c and d). GLUT-4 labeling was characteristically intracellular and perinuclear. Even at extended times of infection (13 h), the process of GLUT-4 sequestration was not saturated nor was it perturbed by the pathological effects of the virus which included enlargement of the Golgi as well as significant budding of virus particles from both the plasma membrane and the TGR as observed by immuno-EM (data not shown).

These studies indicate that the differential targeting of GLUT-1 and GLUT-4, previously reported in 3T3-L1 adipocytes (Piper et al., 1991), is also observed with Sindbis virus–produced GLUT-1 and GLUT-4 in CHO cells. GLUT-1 is targeted preferentially to the plasma membrane, while GLUT-4 is accumulated within an intracellular compartment that is morphologically indistinguishable from the intracellular.
lular GLUT-4 compartment observed in vivo (Slot et al., 1991).

Construction of GLUT-1/GLUT-4 Chimeric Transporters

To identify the amino acid domain(s) responsible for the efficient intracellular localization of GLUT-4 in the non-insulin-sensitive CHO cells, we examined the targeting of a panel of chimeric glucose transporters comprised of portions of GLUT-1 and GLUT-4 (Fig. 5). GLUT-1 and GLUT-4 are 65% identical at the amino acid level and share the same predicted secondary structure. The highest degree of amino acid heterogeneity occurs within four hydrophilic domains which include the cytoplasmic amino and carboxy termini, a large cytoplasmic loop between membrane spanning domains 6 and 7, and an exofacial loop between membrane spanning domains 1 and 2. We reasoned that it might be possible to identify the domains which are both necessary and sufficient for sequestration of GLUT-4 in the TGR/endosome by exchanging domains between GLUT-1 and GLUT-4.

The alignment of the deduced amino acid sequence of GLUT-1 and GLUT-4 (Bell et al., 1990; Birnbaum, 1989) was used to identify appropriate sites for recombination. Chimeric cDNAs were assembled using complementary restriction sites. To validate the integrity of each construct, hybrid transporter cDNAs were sequenced and translated in vitro (Fig. 5 b). Translation products of all the chimeric transporters had the predicted molecular weight as determined by SDS-PAGE analysis. For further validation of these constructs, in vitro translation products were immunoprecipitated with antibodies specific for the COOH-terminus (data not shown). Chimeric cDNAs were subcloned into the Sindbis virus vector KASind and viral stocks were produced and used to infect CHO cells. Membranes were prepared from CHO cells infected with the recombinant Sindbis virus stocks and immunoblotted using the appropriate antisera. All chimeric proteins were expressed at high levels following a 6-h infection exhibiting a single band of the appropriate molecular weight by SDS-PAGE and immunoblot analysis (data not shown).

GLUT-4 Contains a Specific Sequestration Domain in the NH2 terminus

The subcellular distribution of chimeric transporters was first determined morphologically using confocal immunofluorescence microscopy. The chimeras 1/4-C and 4/1-C were initially analyzed. These chimeras are spliced at the beginning of membrane spanning domain 7 and are comprised of portions of GLUT-1 and GLUT-4 (Fig. 5). GLUT-1 and GLUT-4 are 65% identical at the amino acid level and share the same predicted secondary structure. The highest degree of amino acid heterogeneity occurs within four hydrophilic domains which include the cytoplasmic amino and carboxy termini, a large cytoplasmic loop between membrane spanning domains 6 and 7, and an exofacial loop between membrane spanning domains 1 and 2. We reasoned that it might be possible to identify the domains which are both necessary and sufficient for sequestration of GLUT-4 in the TGR/endosome by exchanging domains between GLUT-1 and GLUT-4.

The alignment of the deduced amino acid sequence of GLUT-1 and GLUT-4 (Bell et al., 1990; Birnbaum, 1989) was used to identify appropriate sites for recombination. Chimeric cDNAs were assembled using complementary restriction sites. To validate the integrity of each construct, hybrid transporter cDNAs were sequenced and translated in vitro (Fig. 5 b). Translation products of all the chimeric transporters had the predicted molecular weight as determined by SDS-PAGE analysis. For further validation of these constructs, in vitro translation products were immunoprecipitated with antibodies specific for the COOH-terminus (data not shown). Chimeric cDNAs were subcloned into the Sindbis virus vector KASind and viral stocks were produced and used to infect CHO cells. Membranes were prepared from CHO cells infected with the recombinant Sindbis virus stocks and immunoblotted using the appropriate antisera. All chimeric proteins were expressed at high levels following a 6-h infection exhibiting a single band of the appropriate molecular weight by SDS-PAGE and immunoblot analysis (data not shown).

GLUT-4 Contains a Specific Sequestration Domain in the NH2 terminus

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Figure 5. Panel of GLUT-1/GLUT-4 chimeric glucose transporters. (a) GLUT-1 and GLUT-4 cDNAs were recombined at specific restriction enzyme sites (designated alphabetically A–D in a 5'-3', NH2-terminal to COOH-terminal, direction). The nomenclature used to describe the chimeric transporters contains the necessary information to identify the contribution of the GLUT-1 and GLUT-4 isoforms in each construct. The number (I or 4) refers to the contribution of each isoform in an amino to carboxy terminal direction. The letters (A–D) refer to the site of recombination. Shown at the top are the amino acid regions of the transporters used to produce peptide specific antibodies specific for GLUT-4 (R820) and GLUT-1 (R493). TM, transmembrane domain. Shown is a summation of the predominant localization of each chimera; PM, plasma membrane; IC, intracellular. (b) Fluorograph of SDS-PAGE gel showing in vitro translation products from the above chimeric cDNAs.
The amino acid heterogeneity between GLUT-1 and GLUT-4 in the NH₂-terminal region occurs within the exofacial loop and the cytoplasmic NH₂-terminal tail (see Fig. 11). We surmised that the sequestration domain of GLUT-4 might be contained within the cytoplasmic portion of the NH₂ terminus since targeting signals of many other membrane proteins have been localized within their cytoplasmic regions (Ktistakis et al., 1990). To test whether this region was sufficient for the intracellular targeting of GLUT-4 we next constructed a very specific set of chimeric transporters to replace the NH₂ terminus of GLUT-1 with that of GLUT-4. To facilitate the analysis of these chimeras we modified GLUT-1 so that it contained a unique epitope which would distinguish it from the endogenous GLUT-1. This modified GLUT-1 (chimera 1/4-D) was made by replacing the COOH-terminal 25 amino acids of GLUT-1 with those of GLUT-4. Insertion of this 25 amino acid region, which encodes the epitope for the polyclonal antibody R820, offers a number of

Figure 6. Localization of chimeric glucose transporters by confocal immunofluorescence microscopy. Chimeric transporters were expressed in CHO cells with the Sindbis virus vector KASind. Cells were fixed, permeabilized, and immunolabeled as follows: cells expressing chimeras 4/1-C (a) and 4/1-D (b) were labeled with the anti-GLUT-1 antibody (R493); cells expressing chimeras 1/4-B (c), 1/4-C (d), 1/4-D (e), and 4A/1/4D (f) were labeled with the anti-GLUT-4 antibody (R820). For a and b microscope photomultiplier amplification settings were reduced to negate endogenous levels of R493 labeling. Bar, 30 nm.
Figure 8. Subcellular fractionation of Infected CHO cells. CHO cells were infected with recombinant viruses expressing GLUT-4 or chimeric transporters. The cells were homogenized and subjected to differential centrifugation. Membrane fractions enriched in plasma membrane markers (A) or in intracellular markers (B) were isolated. Aliquots (15 μg of protein) of fractions A and B from each plate of infected CHO cells were subjected to SDS-PAGE and immunoblotting using antibodies specific for either the GLUT-4 COOH terminus (R820), the GLUT-1 COOH terminus (R493), the Na/K ATPase α subunit, or the IGF II/mannose 6-phosphate receptor.

Advantages. This chimera could be readily distinguished from the endogenous GLUT-1 transporter. Even though over-expression by Sindbis virus allows for the specific detection of chimeric transporters containing the GLUT-1 COOH terminus by immunofluorescence and by subcellular fractionation, such chimeras cannot be unambiguously detected using other techniques. The presence of the R820 epitope permits the analysis of chimeras with the same COOH-terminal antibody and allows for the quantitative comparison of all chimeric transporters which share the R820 epitope. Based on the immunolocalization and the subcellular fractionation (Fig. 8) of chimeras 4/1-C and 1/4-C, the COOH-terminal portion is neither necessary nor sufficient for GLUT-4 intracellular localization and should not affect targeting. Immunofluorescence localization of Sindbis virus–expressed chimera 1/4-D in CHO cells with R820 showed intense plasma membrane labeling consistent with its predicted targeting to the cell surface (Fig. 6 e). EM immunogold labeling of 1/4-D was prominent at the cell surface (Fig. 7 B). This distribution was indistinguishable from that of chimera 1/4-B (Fig. 7 A). To examine the role of the NH2 terminus, the chimera 4A/1/4D was made which substituted the NH2-terminal 41 amino acids of GLUT-4 in place of the NH2-terminal region derived from GLUT-1 (1/4-B, 1/4-C and 1/4-D). Immunofluorescence localization of chimera 4A/1/4D in CHO cells showed that it was sequestered within the perinuclear region of the cell similar to GLUT-4 (Fig. 6 f). The predominant intracellular targeting of 4A/1/4D was confirmed by immunogold EM localization (Fig. 7 C). By virtue of the plasma membrane localization of chimera 1/4-B and the intracellular localization of chimera 4A/1/4D, these data together indicate that the NH2-terminal 41 amino acids are both necessary and sufficient for the sequestration of GLUT-4.

Table I. Membrane Fractions Were Prepared from CHO Cells and Analyzed by SDS-PAGE and Immunoblotting for the Enrichment of the Na/K ATPase, the IGF II/Mannose-6-Phosphate Receptor, and β-COP

| Fraction          | Total protein | Na/K ATPase | M6PR | β-COP |
|-------------------|---------------|-------------|------|-------|
| Cell homogenate   | 4             | 0.45        | 0.5  | N.D. |
| Whole membranes   | 2             | 1           | 1    | 1     |
| Fraction A        | 0.1           | 4.1         | 3.8  | 0.5   |
| Fraction B        | 0.4           | <0.1        | 4.2  | 3.8   |

The specific activity of each marker protein is expressed in relative units per microgram of protein.

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To provide a quantitative comparison of the subcellular distribution of the chimeric transporters, membrane fractions enriched in plasma membrane markers (fraction A) or intracellular markers (fraction B) were prepared from Sindbis virus–infected CHO cells by differential centrifugation and analyzed by SDS-PAGE and immunoblotting (Fig. 8). In comparison to whole cell membranes, fraction B was enriched 3.5-fold in the IGF II/Mannose-6-phosphate receptor (Kornfeld and Mellman, 1989), 3.2-fold in the Golgi marker β-COP (Allan and Kreis, 1986), and depleted for the plasma membrane marker protein, and Na/K ATPase (Glynn, 1985). Fraction A was enriched fourfold in the Na/K ATPase (Glynn, 1985) over whole cell membranes (Table I). Fraction A also contained significant amounts of the IGF II/Mannose-6-phosphate receptor and most likely represents contamination from intracellular compartments. Thus, while this fractionation procedure is crude, it can be used to distinguish the overall distribution of transporter proteins as shown by the differential distribution of GLUT-1 and GLUT-4 within these fractions (Fig. 8).

GLUT-4 (per unit protein) was distributed primarily within the intracellular fraction B (Fig. 8). The amount of endogenous GLUT-1 (per unit protein), however, was highest in fraction A. The ratio of glucose transporter immunoreactivity in fraction A versus fraction B was 4.5:1 for endogenous GLUT-1 and 1:3.1 for Sindbis produced GLUT-4. Sindbis virus–produced GLUT-1 also showed a ratio of 4.1:1 (data not shown), indicating that the distribution of Sindbis virus–produced GLUT-1 was the same as the endogenous GLUT-1. These fractionation data are consistent with the morphological data (Figs. 2 and 4). All chimeras containing the NH2-terminal region derived from GLUT-1 (1/4-B, 1/4-C, and 1/4-D) were enriched in fraction A with a distribution similar to that of endogenous GLUT-1. In contrast, chimeras which contained the NH2-terminal portion of GLUT-4 (4/1-C and 4A/1/4D) were enriched in fraction B. Chimera 4/1-C...
was detected using the anti-GLUT-1 COOH-terminal R493 antibody. 4/1-C was distinguished from endogenous GLUT-1 by its over-expression; the additional immunoreactivity in fractions from 4/1-C recombinant virus–infected cells was recovered in fraction B.

To further demonstrate the differential targeting of the chimeric transporters, subcellular fractionation was performed using a sonication technique which yields highly purified plasma membrane fragments. Infected CHO cells were grown on coverslips, immersed in hypotonic intracellular buffer, and subjected to sonication. This treatment produces a lawn of plasma membranes which remains attached to the coverslip (Heuser and Anderson, 1989). Plasma membrane sheets were fixed and immunofluorescently labeled and digital images were collected by confocal microscopy (Fig. 9). Very low plasma membrane labeling was observed in cells infected with either GLUT-4 or 4A/1/4D Sindbis viruses. However, all of the chimeras which contained the NH2-terminal region of GLUT-1 gave intense plasma membrane labeling. For quantitative comparison of plasma membrane labeling, several images were collected and quantitated for average pixel intensity. Whole membrane fractions prepared from parallel infections were immunoblotted to determine the amount of each chimeric transporter produced. All chimeras in this experiment contained the R820 epitope to allow for normalization. All chimeras were made at similar abundance and gave plasma membrane labeling consistent with their localization by immunofluorescence. The amount of each chimeric transporter in isolated plasma membrane per unit of total transporter was seven- to eightfold higher for chimeras containing the NH2-terminal portion of GLUT-4 compared with those containing the NH2-terminal portion of GLUT-4 (Fig. 9f).

**Discussion**

We have found that GLUT-4 expressed in CHO cells using a Sindbis virus recombinant expression vector is targeted to intracellular structures which are morphologically and biochemically similar to those in which GLUT-4 is found in vivo. In both insulin-sensitive tissues which naturally express GLUT-4 (Slot et al., 1991a) or cell lines transfected with the GLUT-4 cDNA (Haney et al., 1991; Hudson et al., 1992; Lawrence et al., 1992; Shibasaki et al., 1992), GLUT-4 is efficiently excluded from the plasma membrane and is localized to intracellular tubulo-vesicular elements distributed throughout the cytoplasm. We have found that the subcellular distribution of GLUT-4 produced by Sindbis virus in CHO cells was very similar to that previously observed in 3T3-L1 adipocytes, brown adipocytes, and cardiac myocytes (Piper et al., 1991; Slot et al., 1991a,b). The localization of GLUT-4 to these structures was unaffected by the Sindbis virus infection itself. These data indicate that the large quantities of Sindbis structural protein, which is concentrated throughout the synthetic pathway (Strauss and Strauss, 1986), did not affect the cells ability to sequester GLUT-4. Likewise, the over-production of GLUT-4 by Sindbis was unable to saturate the ability of the cell to sequester GLUT-4.

We have used Sindbis virus to express GLUT-4 in a variety of cell types including chicken embryonic fibroblasts, baby hamster kidney cells, L6 myoblasts, L929 fibroblasts, 3T3-L1 fibroblasts, and L9 rat fibroblasts (Piper, R. C., and D. E. James, unpublished observation). In each case, GLUT-4 had the same characteristic perinuclear distribution. These data demonstrate that the cellular machinery responsible for the distinct intracellular sorting of GLUT-4 is present in a variety of cells. Furthermore, because of the transient nature of GLUT-4 expression by Sindbis virus, no long term adaptation is required in order to direct the appropriate targeting of GLUT-4, implying that GLUT-4 is targeted to a preexisting compartment. Alternatively, the GLUT-4 molecule itself may induce the formation of its own compartment in a manner analogous to that proposed for certain secreted proteins (Burgess and Kelly, 1987). Little is known about the functional characteristics of this compartment or about other proteins which may reside in this compartment. Analysis of the
intracellular GLUT-4 vesicles in 3T3-L1 adipocytes has shown that at least part of this compartment overlaps with the sorting pathways of the transferrin, the Mannose-6-Phosphate receptor, GLUT-1, and the constitutively secreted protein adipsin (Kitagawa et al., 1989; Piper et al., 1991; Tanner and Lienhard, 1989). We have recently found that the polypeptide composition of intracellular GLUT-4 vesicles immunoincubation of infected CHO cells is very similar to that of GLUT-4 vesicles isolated from adipocytes and skeletal muscle. SDS-PAGE analysis of immuno-isolated intracellular GLUT-4 containing vesicles has revealed that many of the proteins enriched in vesicles isolated from adipose and muscle tissue are also present in GLUT-4 vesicles isolated from CHO cells (Hanpeter, D., and D. E. James, manuscript in preparation). Identifying these proteins will represent a major step in understanding the biogenesis of this compartment and perhaps will help elucidate the mechanism of the intracellular targeting of GLUT-4.

GLUT-4 is unique compared to other facilitated glucose transporter isoforms because of its efficient exclusion from the cell surface and sequestration in intracellular structures (Blok et al., 1988; Orci et al., 1989). To elucidate the amino acid domains responsible for this unique targeting we constructed a panel of chimeric transporters comprised of portions of GLUT-4 and GLUT-1 as a means of identifying the intracellular targeting domain of GLUT-4. This conservative strategy was attractive because the predicted primary and secondary structures of GLUT-1 and GLUT-4 are highly homologous. Thus, identification of putative targeting domains should be confined to discrete regions of heterogeneity within the transporters. Also, exchanging domains between these molecules maintains the overall structural integrity of the glucose transporter. This is important not only to avoid misfolding and mistargeting of proteins but also to preserve the necessary structural requirements for the function of the sorting domain(s).

Our analysis has excluded the COOH-terminal region of GLUT-4 from playing a role in the specific targeting of GLUT-4 since replacement of the COOH-terminal 1/3 of GLUT-4 with the corresponding region of GLUT-1 does not alter the intracellular distribution of GLUT-4 (Chimera 4/1C). This finding is important because the COOH-terminal tail of GLUT-4 is quite distinct from that of other glucose transporter isoforms and yet it is conserved among species. This is in contrast to the nonconserved COOH termini of GLUT-2, GLUT-3, and GLUT-5 transporter isoforms. Thus, it is likely that the COOH terminus plays a different role, rather than targeting, in the function of GLUT-4. Of particular interest is the role of the phosphorylation of the COOH terminus of GLUT-4 by CAMP-dependent protein kinase in view of the interaction between insulin and intracellular CAMP levels with regard to glucose transport (Lawrence et al., 1990). Instead, our targeting analysis has revealed the importance of the GLUT-4 NH2 terminus. This region is necessary for intracellular sequestration since its substitution in chimera 4A/1/4D resulted in a predominant plasma membrane distribution. This region was also sufficient for intracellular targeting demonstrated by the chimera 4A/1/4D which replaced the NH2 terminus of an epitope-tagged GLUT-1 with the NH2-terminal 41 amino acids of GLUT-4. Localization of this chimera was predominantly intracellular and very similar to that of GLUT-4.

The NH2-terminal regions of GLUT-1 (amino acids 1-30) and GLUT-4 (amino acids 1-41) which were recombined in the chimera 4A/1/4D are shown in Fig. 10. Based upon the high degree of identity between GLUT-1 and GLUT-4 within the membrane spanning domain, it is unlikely that this region contributes to the sorting function of these proteins. Therefore, we propose that the specific sorting function of the NH2-terminal portion of GLUT-4 is encoded within the first 19 unique amino acids located on the cytoplasmic side of the membrane. Additional studies are required to identify the critical amino acids in this domain which contribute to the sequestration of GLUT-4. One feature of the GLUT-4 sequestration domain which may be involved in its targeting function is the presence of an aromatic amino acid (Phe) at position 5. It has been shown for a number of membrane proteins that an aromatic amino acid, particularly tyrosine, in the cytoplasmic tail can direct efficient internalization and confer an overall intracellular distribution (Davis et al., 1988; Fuhrer et al., 1991; Jing et al., 1990; Kiistakis et al., 1990).

Two models by which the NH2-terminal cytoplasmic domain may direct the intracellular sequestration of GLUT-4 are likely. First, GLUT-4 may be excluded from the plasma membrane by a very efficient endocytic process. Thus, in a manner similar to the targeting domains described for other membrane proteins (Davis et al., 1987; Fuhrer et al., 1991; Jing et al., 1990; Kiistakis et al., 1990), the NH2-terminal cytoplasmic domain of GLUT-4 may direct targeting to clathrin-coated pits, possibly by a strong interaction with plasma membrane adaptors (Pearse and Robinson, 1990). In support of this model, GLUT-4 has been shown to recycle between the plasma membrane and intracellular tubulo/vesicular elements via coated pits (Slot et al., 1992) and GLUT-4 has been co-localized with markers specific for the endocytic pathway (Ezaki et al., 1989; James and Pilch, 1988; Hudson et al., 1992). Furthermore, localization of GLUT-4 in 3T3-L1 adipocytes by quick freeze deep etch EM has shown that GLUT-4 in the plasma membrane of 3T3-L1 adipocytes is concentrated in flat clathrin lattices (Robinson et al., 1992). A second model is that the N-terminal GLUT-4 sequestration domain may act as a specific retention signal for the TGR/endosomal compartments. Thus, GLUT-4 could be sorted in a manner analogous to the retention of KDEL containing ER proteins (Pelham, 1989) or perhaps in a manner analogous to the diversion of proteins into the regulated secretory pathway (Burgess and Kelly, 1987). Such a TGR retention signal may be responsible for the highly reproduc-

Figure 10. NH2-terminal region of GLUT-1 and GLUT-4. Deduced amino acid sequence of the NH2-terminal region of GLUT-1 and GLUT-4. Identical amino acids are connected by vertical line. The recombinant site (A) used for the construction of the chimera 4A/1/4D is shown (arrow).
ible and specialized intracellular compartmentation of GLUT-4 to what may be specific regions of the TGR (Piper et al., 1991; Slot et al., 1990, 199a,b).

To distinguish between these possibilities and to further analyze this NH2-terminal domain it will be informative to graft this domain onto a heterologous protein. It will be important not only to determine if this domain can exclude plasma membrane proteins from the cell surface independent of glucose transporter structure, but also to determine if this domain also can specifically direct a heterologous protein into the same intracellular compartment in which GLUT-4 resides. It will also be important to determine the role of this sequestration domain in the process of insulin-stimulated translocation (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). Interestingly, in 3T3-L1 fibroblasts (Blok et al., 1988), 3T3-L1 adipocytes (Piper et al., 1991), rat adipocytes (Ezaki, 1990), and CHO cells (Fig. 2), there is a significant intracellular pool of GLUT-1. The amount of GLUT-1 within this compartment appears to vary relative to the cell type. Thus, GLUT-1 may possess a lower efficiency sequestration domain compared to GLUT-4. This partial sequestration of GLUT-1 raises an important question. Given that GLUT-1 can also undergo regulated movement to the cell type. Thus, GLUT-1 may possess a lower efficiency sequestration domain compared to GLUT-4. This partial sequestration of GLUT-1 raises an important question. Given that GLUT-1 can also undergo regulated movement to the plasma membrane, how does insulin regulate the movement of proteins to the cell surface? We contend that insulin may accelerate the movement of all proteins which participate in the recycling pathway and what allows GLUT-4 to have a particularly insulin-sensitive subcellular distribution is its efficient sequestration in basal cells. Thus, in the future, it will be critical to determine the role of this NH2-terminal targeting domain in the insulin-stimulated translocation of GLUT-4 to the cell surface.

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The Journal of Cell Biology, Volume 117, 1992

742
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