GLUT-4 NH₂ Terminus Contains a Phenylalanine-Based Targeting Motif that Regulates Intracellular Sequestration

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Abstract. Expression of chimeras, composed of portions of two different glucose transporter isoforms (GLUT-1 and GLUT-4), in CHO cells had indicated that the cytoplasmic NH₂ terminus of GLUT-4 contains important targeting information that mediates intracellular sequestration of this isoform (Piper, R. C., C. Tai, J. W. Slot, C. S. Hahn, C. M. Rice, H. Huang, D. E. James. 1992. J. Cell Biol. 117:729-743). In the present studies, the amino acid constituents of the GLUT-4 NH₂-terminal targeting domain have been identified. GLUT-4 constructs containing NH₂-terminal deletions or alanine substitutions within the NH₂ terminus were expressed in CHO cells using a Sindbis virus expression system. Deletion of eight amino acids from the GLUT-4 NH₂ terminus or substituting alanine for phenylalanine at position 5 in GLUT-4 resulted in a marked accumulation of the transporter at the plasma membrane. Mutations at other amino acids surrounding Phe5 also caused increased cell surface expression of GLUT-4 but not to the same extent as the Phe5 mutation. GLUT-4 was also localized to clathrin lattices and this colocalization was abolished when either the first 13 amino acids were deleted or when Phe5 was changed to alanine. To ascertain whether the targeting information within the GLUT-4 NH₂-terminal targeting domain could function independently of the glucose transporter structure this domain was inserted into the cytoplasmic tail of the H₁ subunit of the asialoglycoprotein receptor. H₁ with the GLUT-4 NH₂ terminus was predominantly localized to an intracellular compartment similar to GLUT-4 and was sequestered more from the cell surface than was the wild-type H₁ protein. It is concluded that the NH₂ terminus of GLUT-4 contains a phenylalanine-based targeting motif that mediates intracellular sequestration at least in part by facilitating interaction of the transporter with endocytic machinery located at the cell surface.

Insulin stimulates glucose transport in muscle and adipose tissue by stimulating the movement of the glucose transporter GLUT-4 from intracellular vesicles to the cell surface (Cushman and Wardzala, 1980; Slot et al., 1991a,b; Suzuki and Kono, 1980). The expression of GLUT-4 as well as the ability to regulate glucose transport in response to insulin is confined to muscle and adipocytes (James et al., 1988, 1989). In nonstimulated muscle and adipose tissue GLUT-4 is efficiently sequestered away from the plasma membrane within intracellular tubulo-vesicular elements, which are either associated with the trans-Golgi reticulum or clustered in the cytoplasm (Slot et al., 1991a,b, 1990).

Transfection studies have shown that the intracellular sequestration of GLUT-4 can occur in a variety of cultured cell types including CHO cells (Piper et al., 1992; Shibasaki et al., 1992), 3T3 fibroblasts (Haney et al., 1991; Hudson et al., 1992), HepG2 cells (Haney et al., 1991), and MDCK cells (D. H. Harris, J. W. Slot, D. James, unpublished data). In contrast, other glucose transporter isoforms such as GLUT-1 (Calderhead et al., 1990; Haney et al., 1991; Piper et al., 1991), GLUT-2 (Orci et al., 1989; Thorens et al., 1990) and GLUT-3 (Harris et al., 1992) are targeted preferentially to the cell surface irrespective of the culture conditions. This unique targeting characteristic of GLUT-4 provides the basis for its specialized function as the insulin-regulatable glucose transporter.

The differential subcellular distribution of the individual glucose transporter isoforms has provided a useful system for studying the molecular regulation of protein targeting because there is considerable amino acid and structural homology between the different isoforms (reviewed in Bell et al., 1990). There have been two separate reports of the subcellular distribution of chimeric transporter proteins, composed of portions of the GLUT-1 and GLUT-4 isoforms (Piper et al., 1992; Asano et al., 1992). Piper et al. (1992) have shown that the GLUT-4 NH₂ terminus contains important targeting information. Replacement of the GLUT-4 NH₂ termin-
nus with that from GLUT-1 resulted in an increase in cell surface expression whereas replacement of the GLUT-1 NH₂ terminus with that from GLUT-4 resulted in an increased accumulation of the chimera in an intracellular compartment (Piper et al., 1992). In contrast, Asano et al. (1992) reported that replacement of the GLUT-4 NH₂ terminus with that from GLUT-1 did not alter the subcellular distribution of the protein. In these studies the dominant targeting domain was localized to a region spanning trans-membrane domains 7 and 8 (Asano et al., 1992). This discrepancy emphasizes the potential pitfalls in studying the targeting of a complex protein such as the glucose transporter which is thought to have 12 membrane spanning domains and three hydrophilic cytosolic domains (Mueckler et al., 1985). In particular, it is possible that chimeras do not preserve essential structural features of the protein. Furthermore, as emphasized by Asano et al. (1992), the glucose transporter is thought to undergo homo-oligomerization which may complicate analysis of chimeras that contain portions of the GLUT-1 isoform if expressed in a host cell that expresses this isoform endogenously. For these reasons, we decided to undertake a different strategy to further study the targeting role of the GLUT-4 NH₂ terminus.

In the present studies, we have analyzed the targeting of a set of GLUT-4 NH₂-terminal deletion mutants. Based on the results from these studies, we have attempted to identify individual amino acids that constitute this targeting domain by performing alanine scanning mutagenesis in a defined region of the NH₂ terminus. Most importantly, in these studies the targeting role of the NH₂ termini of GLUT-4 and GLUT-1 have been studied in the context of a heterologous protein in order to ascertain whether this domain can function outside of the complex structure of the polytopic glucose transporter protein. The results indicate that deletion of eight amino acids from the GLUT-4 NH₂ terminus or mutating a single amino acid in the NH₂ terminus (Phe5→Ala) causes a marked accumulation of the protein at the cell surface. Furthermore, the GLUT-4 NH₂ terminus but not the GLUT-1 NH₂ terminus was capable of directing the intracellular sequestration of the H1 subunit of the asialoglycoprotein receptor.

**Materials and Methods**

**Cell Culture and Recombinant Sindbis Virus**

CHO cells (CHO-K1) and BHK-21 cells (clone 13) obtained from the American Type Culture Center (Rockville, MD) were cultured in DME supplemented with 10% FCS (Hazelton Biologics, Lenexa, KS) and nonessential amino acids (Sigma Chemical Co., St. Louis, MO).

A recombinant Sindbis virus expression system was used as previously described (Piper et al., 1992). All cDNA constructs were subcloned into the XbaI and XhoI sites of pKASind, which encodes the Sindbis genome. CHO cells were infected with recombinant Sindbis virus stocks and incubated for 6 h. Cells were then washed with PBS and cultured in DME containing 0.2% BSA and 20 μg/ml cycloheximide (Sigma Chemical Co.) for 1 h before analysis (Piper et al., 1992).

**cDNA Constructions**

Wild-type rat GLUT-4 cDNA (James et al., 1989) (bp 140-1885) was subcloned between the XbaI and XhoI sites of Bluescript (Stratagene Corp., La Jolla, CA) in the sense orientation relative to the phase T3 promoter. The SacII site within the polylinker of the resulting plasmid was destroyed by cutting with BstXI, blunting the ends with Klenow fragment, and religation to yield pIRGT. This left a single SacII site present at bp 543 in the GLUT-4 cDNA. All NH₂-terminal mutants were constructed by PCR using oligonucleotides which encoded the NH₂-terminal mutation and a 5' XbaI site. The 3' primer in these reactions was derived from the T7 promoter which was located 3' of the GLUT-4 cDNA. pIRGT was used as the template DNA. PCR reactions were performed with Vent polymerase (New England Biolabs, Beverly, MA) by using a 65°C extension, 50°C hybridization for 30 cycles. The XbaI/SacII fragments of these PCR products (>350 bp) were subcloned into the XbaI/SacII sites of pIRGT. All regions of the DNA clones produced by PCR were sequenced (Sanger et al., 1977). The chimeric glucose transporters 1/4-D and 4A/1/4D used in this study have been previously described (Piper et al., 1992). 1/4-D consists of GLUT-1-455-GLUT-431-509; 4A/1/4D consists of GLUT-4-46-AspIle-GLUT-131-455-GLUT-4481-509.

The chimeric cDNAs comprised of the H1 subunit of the asialoglycoprotein receptor and GLUT-4 were constructed by overlapping PCR mutagenesis of two PCR fragments having compatible ends. To construct HUT-4A, a PCR fragment was generated from pIRGT/BssK using a T3 primer and a 5' facing overlapping primer complimentary to bp 192-210 of the rat GLUT-4 cDNA (corresponding to amino acids 14-19). pIRGT/BssK was generated by subcloning the full-length rat GLUT-4 clone (James et al., 1989) into the EcoRI site of Bluescript in the sense orientation relative to the T3 promoter. The 5' portion of the overlapping primer was complimentary to bp 60-78 of the human H1 ASGPR cDNA (Spieß et al., 1985) (kindly provided by Dr. M. Shia, Boston University, Boston, MA). This PCR fragment was purified and used as a template together with the wild type HI cDNA and amplified with a T3 primer and a 3' primer complimentary to bp 879-900 of the HI cDNA. HUT-4B was constructed in a similar way except that the overlapping primer was complimentary to bp 110-128 of the HI cDNA. HUT-B was constructed by direct PCR mutagenesis using a primer containing a 5' XbaI site, bp 1-30 of the human GLUT-1 cDNA (Mueckler et al., 1985) followed by bp 60-78 of the HI cDNA. All PCR generated constructs were subcloned between the XbaI and XhoI sites of Bluescript, sequenced, and subcloned into the Sindbis virus vector pKASind. Wild-type H1 (bp -102-1112) was subcloned into the HindIII and EcoRV sites of Bluescript and then subcloned into the XbaI/XhoI sites of pKASind.

**Antibodies**

The anti-peptide polyclonal antibodies specific for the COOH terminus of GLUT-1 (R493) and GLUT-4 (R820) were used as described (Piper et al., 1992). Anti-H1 (ASGPR) subunit antibody was provided by Dr. R. Fallon (Department of Pediatrics, Washington University, St. Louis, MO). Anti-clathrin heavy chain mAb, X-22, was provided by Dr. F. M. Brodsky (Department of Pharmacy, University of California, San Francisco, CA). Rabbit polyclonal antibody to the α subunit of the Na/K ATPase was provided by Dr. R. Mercer (Department of Cell Biology, Washington University, St. Louis, MO). 125I-labeled goat anti-rabbit secondary antibody was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). FITC conjugated goat anti-rabbit and Texas-red conjugated goat anti-mouse antibodies were purchased from Cappel Laboratories (West Chester, PA).

**2-Deoxyglucose Uptake**

Uptake of 2-[3H] deoxyglucose (2DOG) into Sindbis-infected CHO cells was performed as described previously (Piper et al., 1992). To compare the surface distribution of each mutant transporter an index of "specific 2DOG uptake" was determined. In each experiment, 2DOG uptake was measured in CHO cells infected with a nonrecombinant virus. This value represented endogenous 2DOG uptake. Uptake of 2DOG above this background level could be attributed to the expression of exogenous glucose transporter. The relative amount of mutant transporter produced was measured in parallel infections by immunoblotting whole cell extracts with R820 antiserum (James et al., 1989). The R820 epitope was common to all constructs used in the analysis. Hence, all 2DOG uptake data were normalized to account for variations in Sindbis-produced transporter expression. The "specific 2DOG uptake" (see Fig. 2) was calculated as follows: 2DOG uptakecontrol - 2DOG uptakebackground/whole cell transporter expression level. To control for interassay variation, specific 2DOG uptake was measured for a chimeric transporter (1/4-D) in each experiment. The specific 2DOG uptake index for this chimera was assigned a value of one and the amount of transport produced by other constructs were expressed relative to this value. The Journal of Cell Biology, Volume 121, 1993
Surface Protease Treatment

A cell surface protease assay was used to determine the proportion of HI or the HUT constructs located at the plasma membrane (Geffen et al., 1989). Cells expressing the HUT constructs were washed three times in PBS (4°C) and treated with or without protease K (1.0 mg/ml) for 30 min at 0°C. Cells were washed three times in 20% bovine calf serum/PBS and then in HES buffer containing 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.). Cell homogenates were prepared by passing the cells through a 22-g needle five times. Whole membranes were collected by centrifugation (100,000 g, 90 min) and immunoblotted with the anti-HI antisera at a dilution of 1/500.

Subcellular Fractionation of CHO Cells

High Gradient Magnetic Chromatography. This method has recently been described in detail (Warnock et al., 1993). Briefly, subconfluent 10-cm culture plates of CHO cells were washed twice in binding buffer (0.14 M NaCl, 5.4 mM KCl, 0.33 mM NaH2PO4, 0.34 mM KH2PO4, 0.8 mM MgSO4, 2.7 mM CaCl2, 20 mM Hepes, pH 7.4). Cells were incubated in binding buffer with 1 mg/ml BSA and WGA conjugated to iron/dextran particles (WGA/FeDex, 1 mg/ml) for 1 h at 4°C, washed twice with binding buffer and scraped into 4 ml of sucrose buffer (0.25 M sucrose, 10 mM Tris, pH 7.4, 20 mg/ml pepstatin, 20 mg/ml antipain, 20 mg/ml leupeptin, 20 mg/ml chymostatin, 25 IU/ml aprotinin A). Cells were homogenized and centrifuged at 400 g to generate a postnuclear supernatant (PNS). The PNS (3 ml) was loaded into a 3 ml syringe packed with stainless steel wool (Brunsonet, 25 mm airlayed web). The syringe was placed between the poles of a 4,000 gauss permanent magnet for 2 h at 4°C. The column was sequential eluted with 20 ml of sucrose buffer (nonretained fraction), 10 ml of 300 mM KCl in sucrose buffer with the magnet in place, and then with 10 ml of sucrose buffer in the absence of the magnet and with agitation of the syringe column (retained fraction). The retained and nonretained fractions were centrifuged at 190,000 g for 1 h at 4°C to obtain membranes. The membrane fractions were resuspended in an equal volume and all analyses were performed using an identical volume for each fraction. The activities of galactosyl transferase and \( \beta \) hexosaminidase activity in the membrane fractions were measured as previously described (Warnock et al., 1993).

Plasma Membrane Lawns. Immunofluorescence labeling of CHO cell plasma membrane lawns using a polyclonal antibody specific for the carboxy terminus of GLUT-4 (RR20) was performed as described previously (Robinson et al., 1992).

Immunofluorescence Microscopy

Immunofluorescence labeling of plasma membrane lawns was performed as described previously (Piper et al., 1992; Robinson et al., 1992). Double labeling for clathrin and GLUT4 was performed by incubating samples with RR20 serum and X-22 anti-clathrin mAb (5 \( \mu \)g/ml). Samples were then washed and incubated with FITC goat anti-rabbit antibody (10 \( \mu \)g/ml) and Texas-red goat anti-mouse antibody (20 \( \mu \)g/ml). Images were collected by confocal microscopy with the laser operating at 515 nm. Samples labeled with only a single antibody were used to compensate for any signal overlap between the two detection channels. Epifluorescence on whole cells was performed as described previously (Piper et al., 1991). Images were photographed using Kodak tungsten 160 slide film.

Immunoelectron Microscopy

CHO cells were fixed for 3 h at 22°C with 1% acrolein (Sigma Chemical Co.) and glutaraldehyde in phosphate buffered saline, pH 7.4. Cryosections were prepared and labeled with RR20 and R493 antiserum followed by Protein A gold (Slot et al., 1991b). Background labeling was measured in CHO cells not expressing Sindbis produced transporters.

Stable Transfection

The GLUT-4 cDNA was excised from pBGT as a Not I and Xba I blunt fragment and subcloned into the Not I and Xba I blunt sites of the vector pCMV-neo (In vitrogene, San Diego, CA). This plasmid was linearized with Pvu II and transfected into CHO-KI cells using lipofectin according to the manufacturer's instructions (Gibco Laboratories, Grand Island, NY).

Results

Piper et al., (1992) previously showed by constructing chimeras between GLUT-I and GLUT-4 that the NH2 terminus of GLUT-4 contained important targeting information that was capable of directing intracellular sequestration. In the present studies we have employed a different strategy to define the constituents of this NH2-terminal targeting motif. The subcellular distribution of a set of NH2-terminal truncation and amino acid substitution mutants of GLUT-4 (Fig. 1) have been assessed in CHO cells. Several different methods have been employed to define the cellular location of these constructs including: (a) 2-deoxyglucose uptake, which provides an estimate of cell surface exposed transporters – this method also provides an indication of the functional integrity of the constructs studied; (b) subcellular fractionation by high gradient magnetic chromatography, which allows measurement of expressed transporters by immunoblotting both in a plasma membrane-enriched fraction and a fraction enriched in intracellular vesicles; (c) subcellular fractionation by isolation of plasma membrane lawns, Neomycin-resistant colonies were selected in media containing 1.5 mg/ml G418 (Gibco Laboratories). Two rounds of subcloning were performed to ensure isolation of a single clone.

Figure 1. Summary of transporter constructs. NH2-terminal mutations of GLUT-4 are shown schematically. The single letter amino acid code has been used. Shown on the right-hand side is a summary of the relative plasma membrane (PM) distribution of each mutant: (-), low PM distribution, (+), high PM distribution, (+/-), intermediate distribution.
which provides an estimate of transporters present in highly purified plasma membranes: (d) quantitative EM immunogold labeling, which indicates not only the proportion of each construct present at the plasma membrane but also provides a very rigorous assessment of the intracellular distribution of these constructs in the cell. While the absolute differences in subcellular distribution of individual constructs were not identical between each method employed these analyses indicate that the GLUT-4 NH$_2$ terminus contains an important targeting motif encompassing residues 2–8 that regulates the intracellular sequestration of the protein.

### 2-Deoxyglucose Uptake

Because glucose transport is mediated only by those transporters at the cell surface, the level of glucose transport resulting from the expression of various transporter mutants provides an estimate of the relative intracellular distribution of each mutant. This estimate relies on the normalization to the level of each transporter mutant expressed (see Materials and Methods). Deletion of the initial 8, 13, or 16 amino acids in the GLUT-4 NH$_2$ terminus resulted in an increase in specific 2DOG uptake that was fourfold higher than that observed for GLUT-4 and comparable to that observed for the 1/4D chimera (Fig. 2). A high level of specific 2DOG uptake was also observed for a chimeric GLUT-4 construct (Anita), that was comprised of the 13 amino acid NH$_2$-terminal hydrophilic tail of GLUT-1 and the remainder of GLUT-4 (Fig. 1). The mutant, in which phenylalanine at position 5 was changed to alanine, exhibited a very high specific 2DOG uptake index compared to GLUT-4 but that was comparable to the uptake observed for the cell surface distributed trans-

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**Figure 2.** Distribution of transporters determined by measurement of specific 2-deoxyglucose uptake and transporter levels in plasma membrane fragments. Chimeric and mutant transporters were expressed in CHO cells using Sindbis virus. Cells were used for measurement of either specific uptake of [3H]-2-deoxyglucose (2DOG, filled bars) or cell surface levels of the Sindbis-produced transporter (clear bars). Specific 2DOG uptake by the Sindbis-produced transporters was measured by correcting for background uptake measured in cells infected with wild-type Sindbis virus. Values were normalized for differential transporter expression levels measured by immunoblotting extracts from cells infected in parallel. Specific 2DOG uptake due to chimera 1/4D, which was measured in each experiment, was assigned a value of 100 and all other values were expressed relative to this value. The absolute values of 2DOG uptake in cells infected with control virus or with the 1/4-D producing virus were 5 and 15 pmol/mg cell protein/min, respectively. Relative surface expression of transporter mutants was estimated by immunolabeling plasma membrane fragments, which were isolated by sonication. Quantitation was performed by measuring the average pixel intensities from digitized images. Shown are the mean ± SD of three to six separate experiments.

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**Figure 3.** Subcellular fractionation of infected CHO cells. CHO cells were infected with recombinant viruses expressing GLUT-4 or the GLUT-4 mutants and incubated with WGA conjugated to Fe-dextran particles for 4 h at 4°C. Postnuclear supernatants were prepared and bound to magnetized steel wool for 2 h. The nonretained (NR) fraction was eluted with the magnet still in place whereas the retained fraction (R) was eluted subsequent to removal of the magnet. Equal amounts of each fraction vol/vol were subjected to SDS-PAGE and immunoblotted with either an antibody specific for the GLUT-4 COOH terminus (R820), the GLUT-1 COOH terminus (R493) or the alpha subunit of the Na/K ATPase. The immunoreactive bands labeled with anti-GLUT-1 and anti-Na/K ATPase antibodies correspond to the endogenous levels of these proteins in CHO cells.
porters, GLUT-1 and 1/4D (Fig. 2). Mutations at other positions (Pro2Ser3→AlaAla and Gln6Gln:11→AlaAlaAla) also caused an increase in 2DOG uptake that was intermediate between the levels reported for GLUT-4 and chimera 1/4-D. Mutating either Gly4 to Ala or replacing the sequence Ser10Glu11Asp12 with a stretch of three alanine residues was without effect on the level of 2DOG uptake compared to that observed for wild type GLUT-4.

**Subcellular Fractionation**

Two separate methods of subcellular fractionation were employed to study the targeting of the transporter constructs. The first method was based on immunofluorescence labeling of purified plasma membrane fragments isolated from CHO cells by sonication (Fig. 2). The level of immunolabeling was normalized to the level of each transporter mutant produced in a parallel infection. GLUT-4 exhibited low plasma membrane labeling compared to chimera 1/4-D, consistent with our previous studies (Piper et al., 1992). The quantitative differences in plasma membrane labeling between the different transporter constructs was essentially the same as the differences observed by measurement of 2DOG uptake (Fig. 2). High levels of plasma membrane labeling were observed for the deletion mutants and for the Phe5→A point mutant. Labeling for the PS→A, QQI→A, and I→A mutants was intermediate (45–52% of 1/4D) between that observed for GLUT-4 and chimera 1/4-D (Fig. 2).

Plasma membranes were also isolated by affinity chromatography (Warnock et al., 1993). Using this method it is possible to quantitatively recover CHO plasma membranes from a PNS which allows measurement of the amount of transporter both in the plasma membrane fraction (retained or "R") and in the intracellular fraction (nonretained or

![Image of immunolocalization of transporters in CHO cells. GLUT-4 expressed via recombinant Sindbis virus was localized by immunogold labeling in ultrathin cryosections (A). GLUT-4 is located in the trans-Golgi reticulum which is located at one side of the Golgi complex (g), and in separate vesicles throughout the cytoplasm (large arrow). Only a few gold particles are associated with the plasma membrane. GLUT-4 expressed by stable transfection in CHO cells (B) has a similar distribution. In contrast, the F→A GLUT-4 mutant expressed by recombinant Sindbis virus (C) differs by having a much higher level of expression at the cell surface. N, nucleus; small arrows, budding virions. Bar, 500 nm.](image-url)
"NR"). The retained fraction (Fig. 3), contained 60% of the endogenous GLUT-1, 19 ± 3% of galactosyl transferase, 8 ± 2% of the β-hexosaminidase and 85 ± 7% of the Na/K ATPase alpha subunit. Using this procedure 80 and 84% of Sindbis-produced GLUT-4 and chimera 4A/1/4D, respectively, was recovered in the nonretained fraction. In contrast, 60% of GLUT-1 and the NH2-terminal deletion mutant mEP and 50% of the Phe5→Ala substitution mutant were recovered in the "retained" or plasma membrane fraction.

Morphological Localization of Transporter Mutants

To further examine the targeting of the transporter mutants we performed electron microscopic immunogold labeling. Representative micrographs showing the distribution of virally expressed GLUT-4 and the F→A mutant are shown in Fig. 4. Very little labeling of the ER or Golgi cisternae was observed for any of the constructs. The majority of intracellular labeling occurred in small tubules and vesicles clustered either near the trans-Golgi reticulum or in the cytoplasm (Fig. 4). The percentage of total cellular immunogold labeling that was associated with the plasma membrane for the different constructs is shown in Table I. Approximately 60-70% of Sindbis-produced GLUT-1 was located at the plasma membrane in contrast to only 15-20% for GLUT-4. We also examined the distribution of GLUT-4 expressed in CHO cells by stable transfection (Fig. 4 B) and found that its distribution was indistinguishable from that observed for Sindbis virus expressed GLUT-4 by quantitative EM (Table I). These data are in good agreement with our previous studies using the Sindbis expression system (Piper et al., 1992) and with other studies that have employed alternate expression systems (Asano et al., 1992).

The GLUT-4 chimeras containing the NH2-terminal 12 (Anita) or 465 (1/4D) amino acids of GLUT-1 exhibited a significantly higher level of cell surface labeling compared to the parent GLUT-4 molecule (Table I). In contrast, chimeras 4A/1/4D, exhibited a low cell surface distribution similar to GLUT-4. Deletion of the NH2-terminal 8 amino acids in GLUT-4 or substituting alanine for phenylalanine at position 5 in the GLUT-4 NH2 terminus resulted in a marked increase in cell surface labeling (Table I).

Table I. Quantitation of Relative Transporter Concentration on the Cell Surface using Immunogold Labeling

| Experiment     | Percent of total gold particles at cell surface |
|----------------|-------------------------------------------------|
| GLUT-1         | 65.4 ± 1.1                                      |
| GLUT-4         | 20.1 ± 1.6                                      |
| mEP            | 63.3 ± 1.8                                      |
| Anita          | 43.3 ± 1.9                                      |
| G→A            | 19.1 ± 1.6                                      |
| F→A            | 43.1 ± 2.2                                      |
| I→A            | 20.3 ± 1.5                                      |
| 1/4D           | 47.5 ± 2.2                                      |
| 4A/1/4D        | 18.2 ± 1.7                                      |
| GLUT-4stable   | 13.7 ± 0.9                                      |

Cryosections of CHO cells expressing glucose transporter constructs were immunolabeled with the anti-GLUT-4 COOH-terminal antibody (R820) or, in the case of cells expressing GLUT-1, the COOH-terminal GLUT-1 antibody (R493) followed by protein A-conjugated gold. Transporter constructs were expressed in CHO cells using the Sindbis virus expression system or by transfection with the GLUT-4-pCMV plasmid (GLUT-4*top). Gold particles associated with the plasma membrane were expressed as the percentage of total except those over nonrelevant structures such as mitochondria and nuclei. Nonspecific labeling observed in uninfected cells was negligible. Countings represent ± SEM of 10 cell profiles.

Figure 5. Mutagenic scheme for the HUT fusion proteins. HUT fusion proteins were comprised of the transmembrane (shaded box) and the exofacial domains of the HI subunit of the asialoglycoprotein receptor (ASGPR) and the NH2 terminus of either GLUT-1 or GLUT-4. The predicted amino acid sequence of the entire NH2-terminal portions of these constructs is shown below.

GLUT-4 NH2Terminus Contains an Autonomous Sorting Signal

To determine whether the GLUT-4 NH2 terminus encodes an autonomous sorting domain we studied the distribution of heterologous proteins that contained the NH2-terminal tail of GLUT-4. As a host protein for these experiments, the HI subunit of the asialoglycoprotein receptor (ASGPR) was chosen because it spans the membrane only once and, like GLUT-4, it has a cytoplasmic NH2 terminus (Spiess et al., 1985). The composition of the heterologous HI/GLUT proteins (HUT's) is shown in Fig. 5.

The HUT constructs as well as HI, with the exception of HUT-4B-F→Y (see below), migrated as two distinct polypeptide products as determined by SDS-PAGE and immunoblotting (Fig. 6). The higher molecular weight band, which was the more abundant of the two forms, was sensitive to surface protease treatment whereas the lower molecular weight form was protease insensitive. In previous studies, two polypeptide forms were also reported after HI expression in fibroblasts (Shia and Lodish, 1989). The lower molecular weight product represents incompletely glycosylated protein which is probably sequestered in the endoplasmic reticulum (Shia and Lodish, 1989). Hence, only the mature polypeptide is sensitive to cell surface protease treatment. The fact that all constructs, except HUT-4B F→Y, underwent proper glycosylation indicated that the topology of each protein was appropriate.

To assess the subcellular distribution of these heterologous proteins in CHO cells we employed a cell surface protease digestion assay (Geffen et al., 1989). The relative distribution of each mutant at the plasma membrane was scored by the loss of immuno-reactive protein after surface protease
These data indicate that the GLUT-4 NH2 terminus of aromatic amino acids (usually tyrosine) in regulation of the plasma membrane concentration of glucose transporters. The previous studies indicated the importance of tyrosine substitutions at this position. In addition, the HUT-4A and HUT-4B proteins were digested by surface protease treatment (Fig. 6). This is consistent with the previous findings (Moore et al., 1987). A high level of tyrosine substitution in both GLUT-4A and GLUT-4B. Mutating Phe5 to alanine in either GLUT-4A or GLUT-4B resulted in a significant accumulation of the protein at the cell surface (Fig. 6). This is consistent with the previous findings in GLUT-4 suggesting that this NH2-terminal domain operates in a similar fashion in both proteins. In contrast, GLUT-4A with Phe5 changed to tyrosine was very efficiently sequestered from the plasma membrane as there was no detectable loss after surface protease treatment (Fig. 6). However, tyrosine substitution of Phe5 in the GLUT-4B protein resulted in retention of the protein in the ER as judged both by SDS-PAGE (Fig. 6) and immunofluorescence microscopy (Fig. 7). The cellular distribution of each of the HUT constructs was compared to GLUT-4 using immunofluorescence microscopy (Fig. 7). GLUT-4 exhibited a very focused staining pattern in the peri-nuclear region and was also distributed in punctate structures scattered throughout the cytoplasm (Fig. 7A). Labeling of wild-type H1 was also concentrated in the same region and in punctate structures throughout the cytoplasm (Fig. 7D). However, significant amounts of H1 were evident at the cell surface consistent with the surface protease assay (Fig. 6A). HUT-1B was found primarily at the plasma membrane (Fig. 7G) similar to GLUT-1 (data not shown). In contrast, both HUT-4A and HUT-4B showed substantial intracellular labeling similar to that observed for wild-type GLUT-4. Most notably, the labeling of GLUT-4B (Fig. 7C) was predominantly intracellular within a very focused perinuclear region. Mutation of Phe5 to Ala in both GLUT-4A (Fig. 7E) and GLUT-4B (Fig. 7F) resulted in an increase in cell surface labeling. In addition, these constructs did not exhibit the pronounced focused staining in the perinuclear region that was observed for either GLUT-4, GLUT-4A or GLUT-4B in that the residual intracellular staining was much more diffuse. The propensity to form this very discrete intracellular labeling was clearly observed for GLUT-4B(Fig. 7H). Interestingly, the targeting of this protein was clearly different than that of wild-type H1 suggesting that while the intracellular targeting information present in the GLUT-4 NH2 terminus can be enhanced by a tyrosine residue, it is clearly distinguishable from the sorting information within the H1 NH2 terminus.

Localization to Cell Surface Clathrin Lattices Is Dependent on the GLUT-4 NH2 Terminus

Previously, we have observed that GLUT-4 is localized to cell surface clathrin-coated pits in 3T3-L1 adipocytes (Robinson et al., 1992). To determine if the GLUT-4 NH2 terminus mediates this interaction we performed double label confocal immunofluorescence microscopy using antibodies specific for GLUT-4 and clathrin heavy chain on plasma membrane fragments isolated from CHO cells that were expressing either GLUT-4, the mEP deletion or the Phe5 to Ala substitution mutants. Clathrin was located in discrete punctate arrays throughout the plasma membrane consistent with previous findings (Moore et al., 1987). A high level of colocalization was observed between GLUT-4 and clathrin in the plasma membranes. About 5–10% of the clathrin...
Figure 7. Immunofluorescence localization of HUT chimeras and GLUT-4. CHO cells were infected with KASind recombinant virus expressing GLUT-4 (A), HUT-4A (B), HUT-4B (C), wild-type H1 (D), HUT-4A F→A (E), HUT-4B F→A (F), HUT-1B (G), HUT-4A F→Y (H), and HUT-4B F→Y (I). Cells were fixed, permeabilized, and labeled with antibodies specific for the GLUT-4 COOH terminus (A) or the exofacial domain of the H1 subunit of the ASGPR (B-I). Bar, 10 μm.

Practice areas were enriched in GLUT-4 (Fig. 8 A). However, there was very little GLUT-4 labeling outside of these areas. In contrast, the GLUT-4 mutant in which Phe5 was changed to Ala or the NH2-terminal deletion mutant mEP showed very little colocalization with clathrin lattices. In fact, these mutants appeared to be excluded from clathrin-enriched domains in the membrane.

Discussion

A major functional feature of the insulin-regulatable glucose transporter, GLUT-4, is its exclusion from the cell surface in the absence of insulin (James et al., 1988, 1989; Slot et al., 1991b; Zorzano et al., 1989). This property is not shared by other glucose transporter isoforms, such as GLUT-1, which are constitutively targeted to the plasma membrane (Calderhead et al., 1990; Haney et al., 1991; Harris et al., 1992; Hudson et al., 1992; Piper et al., 1991; Shibasaki et al., 1992). We have previously shown by constructing GLUT-1/GLUT-4 chimeras that the cytoplasmic NH2 terminus of GLUT-4 can confer intracellular sequestration upon GLUT-1 suggesting that this domain may contain important
targeting information (Piper et al., 1992). In the present study, the specific amino acids within the GLUT-4 NH$_2$ terminus that comprise this targeting signal have been identified. Phe$_3$ was a critical constituent of this signal because mutating this residue to alanine resulted in a large increase in the surface expression of GLUT-4. Other surrounding amino acids including Pro$_2$ and/or Ser$_3$, as well as Ile$_8$, also contributed to GLUT-4 targeting, albeit to a lesser extent than Phe$_3$.

Mutations which resulted in loss of intracellular sequestration correlated with loss of colocalization with cell surface clathrin lattices. These data are consistent with the localization of GLUT-4 to clathrin coated pits and vesicles within insulin-sensitive cells (Robinson et al., 1992; Slot et al., 1991b) and indicate that the endocytic machinery may play a major role in the sequestration of GLUT-4. It remains to be determined, however, as to whether this domain regulates the targeting of the protein elsewhere in the cell.

The analysis of targeting domains within the glucose transporter is complicated by its complex predicted secondary structure. Hence, the subcellular distribution of chimeric transporters or even the NH$_2$-terminal mutants may be determined by a composite of competing targeting signals located in different regions of the protein. To overcome this
problem in the present studies the role of the GLUT-4 NH2 terminus in intracellular sequestration has been studied in
the context of a heterologous protein, the HI subunit of the
ASGPR. These data clearly show that the GLUT-4 NH2
terminus can function as an autonomous sorting domain
confirming our previous studies with chimeric glucose trans-
porters (Piper et al., 1992).

The HI subunit is efficiently internalized via clathrin
coated pits and ~50% of the mature polypeptide is found at the
plasma membrane in both stably transfected fibroblasts
(Fuhrer et al., 1991; Geffen et al., 1989) and in CHO cells
infected with recombinant Sindbis virus (Figs. 6 and 7). The
 tyrosine residue within the cytoplasmic NH2-terminal tail of
HI is required for its intracellular targeting (Fuhrer et al.,
1991). Replacement of the cytosolic tail of HI with the NH2
terminus of GLUT-1 (HUT-1B) caused a marked distribution
at the plasma membrane (Figs. 6 and 7). In contrast, HI pro-
teins containing the GLUT-4 NH2 terminus were predomin-
antly located in a peri-nuclear location within the cell. The
positional requirement of the GLUT-4 NH2 terminus was
assessed by either replacing the entire 40 amino acid HI tail
with the 23 amino acid GLUT-4 tail (HUT-4B) or by substi-
tuting the first 23 amino acids in HI with that from GLUT-
4 (HUT-4A). Both HUT-4 proteins showed a distribution like
that of GLUT-4 and were located intracellularly within a
perinuclear compartment. There was no significant differ-
ence in the sequestration of either construct indicating that
there may be no strict positional requirement for this motif.

Deletional and amino acid substitution mutagenesis of the
GLUT-4 NH2 terminus revealed that the targeting informa-
tion is found within residues 2-8. This sequence bears some
similarity to previously described tyrosine-containing mo-
tifs found in the cytoplasmic tails of many recycling recep-
tors such as those for transferrin (Collawn et al., 1990),
LDL (Davis et al., 1986, 1987) and mannose-6-phosphate/IGF
II (Canfield et al., 1991; Jadot et al., 1992). These mo-
tifs are responsible for the efficient endocytosis of these
receptors and are thought to promote the association of these
proteins with clathrin coated pits via an interaction with
plasma membrane adaptors (Pearse and Robinson, 1990).
The essential features of these internalization signals are an
aromatic amino acid, usually a tyrosine, and a bulky hydro-
phobic residue separated by two amino acids from the aro-
matic residue (Fig. 9). Phenylalanine can functionally sub-
stitute for tyrosine but it is generally less efficient (Jadot
et al., 1992; Kistia kis et al., 1990; Peters et al., 1990).

The domain FQQI within the GLUT-4 NH2 terminus
fulfills this consensus and the functionally critical amino
acids within this domain bear homology to the constituents
of tyrosine-containing internalization signals. Mutation of
either Phes or, to a lesser extent, Iles to alanine within
GLUT-4 or HUT-4 resulted in a significant distribution at the
plasma membrane. Furthermore, an alternate bulky hydro-
phobic group, leucine, could functionally substitute for
isoleucine as shown by the intracellular targeting of the
EQLP mutant.2 However, Iles2 played a much less dominant
role than Phes because the increase in surface expression
when this residue was changed to Ala was relatively small
(Figs. 2 and 3). In fact, by EM immunogold labeling this mu-
tation had very little effect (Fig. 4). Tyrosine was able to
functionally substitute for Phes in the GLUT-4 NH2 ter-
mminus in the context of HI. However, this complementation
was only evident in HUT-4A in which the GLUT-4 NH2-
terminal tail was located in an extended position from the
membrane (Fig. 5). The same substitution in HUT-4B, in
which the GLUT-4 NH2 terminus was in its natural position
from the membrane, resulted in retention of the protein in
the ER (Figs. 6 and 7). Furthermore, we were unable to ex-
press a GLUT-4 mutant, in which Phes was changed to
 tyrosine, in CHO cells using recombinant Sindbis virus
(data not shown). These data suggest that it is only
phenylalanine which can properly function in this targeting
domain. Thus, the phenylalanine-based motif present in
the GLUT-4 NH2 terminus may represent a class of targeting
motifs different from those previously described for recy-
cling receptors.

Because HI also contains an efficient tyrosine-containing
internalization motif (YQDL) (Fuhrer et al., 1991) it was
possible to compare the efficiency of sequestration conferred
by the HI internalization domain with that from the GLUT-4
NH2 terminus. Two observations indicate that the GLUT-4
NH2 terminus contains additional sorting information not
present in tyrosine-containing internalization motifs. First,
the heterologous protein containing the GLUT-4 NH2 ter-
mminus (HUT-4B) was more efficiently excluded from the
plasma membrane than wild type HI (Figs. 6 and 7). Fur-
thermore, the intracellular distribution of HUT-4B closely
resembled that of GLUT-4 in that it was very focused around
the nucleus, while this was not the case for wild-type HI.
The marked intracellular distribution of both chimeric glu-

2. The EQLP mutant (Fig. 1) was originally made on the basis of the homol-
logy between the GLUT4 NH2 terminus and the putative intracellular tar-
getting domain of the invariant chain (the NH2-terminal retention signal of
IL-1). EQLP, bears some homology with the GLUT4-NH2 terminus, QQIG.
Our results from alanine mutagenesis, however, are inconsistent with the
hypothesis that these domains are functionally homologous.

Figure 9. Comparison between cytoplasmic domains that are in-
volved in targeting to the endocytic/lysosomal system and the puta-
tive sequestration domain of GLUT-4. Regions of significance are
aligned in the amino to carboxy-terminal direction. Amino acids
that are critical are in bold or boxed in the case of GLUT-4. Target-
ing domains are shown for: two lysosomal Type I membrane pro-
teins-LAMP-1 (reviewed in Fukuda, 1991) and lysosomal acid
phosphatase (LAP) (Peters et al., 1990); two Type II membrane pro-
teins that undergo efficient endocytosis-mannose 6-phosphate/IGF
II receptor (MPR/IGF IIR) (Canfield et al., 1991) and the low den-
sity lipoprotein receptor (LDLR) (Davis et al., 1987); two Type II
membrane proteins that are efficiently endocytosed-transferrin
receptor (TfR) (Collawn et al., 1990) and the HI subunit of the
asialoglycoprotein receptor (ASGPR) (Piper et al., 1991).
Glucose transporters and heterologous proteins containing the GLUT-4 NH₂ terminus clearly indicates that this targeting is not due solely to the presence of a weak internalization signal. Such a weak internalization signal would be expected in GLUT-4 because phenylalanine constitutes an inferior internalization signal compared with tyrosine within a number of cytosolic receptor tails (Jadot et al., 1992; Kitakaki et al., 1990; Peters et al., 1990). Secondly, the HUT-4A F→Y protein was also distributed very much like GLUT-4 and was not detectable at the plasma membrane. The distribution of HUT-4A F→Y was very different than the wild-type H1 despite the fact that both proteins contain a consensus internalization motif (YQQI vs YQDL, respectively) in an identical position with respect to the transmembrane domain. This latter observation also suggests that the additional targeting information in the GLUT-4 NH₂ terminus is not encoded merely by the presence of phenylalanine at position 5 instead of tyrosine. Thus, the GLUT-4 NH₂ terminus must contain additional sorting information which could act either to configure the phenylalanine-containing internalization motif into a very efficient internalization signal, or it could act as a retention signal which could sort GLUT-4 into a specific intracellular compartment.

It has recently been shown that GLUT-4 constitutively recycles between its intracellular location and the cell surface (Jhu et al., 1992; Yang et al., 1992). Hence, this would implicate an important role for an efficient internalization domain to ensure intracellular sequestration of GLUT-4 under nonstimulated conditions. However, it is not clear if an internalization signal alone is sufficient to account for the intracellular distribution of GLUT-4. That GLUT-4 contains a specific “retention” signal is supported by immunofluorescence and biochemical localization studies which have shown that GLUT-4 occupies a unique intracellular compartment in adipocytes (Piper et al., 1991; Robinson and James, 1992; Zorzano et al., 1989). This compartment may contain specialized proteins such as synaptobrevin; a synaptic vesicle membrane protein which has been colocalized with GLUT-4 in rat adipocytes (Cain et al., 1992). Thus, the phenylalanine within the GLUT-4 NH₂ terminus may have dual sorting functions. Other studies have recently shown that aromatic amino acids may have pleiotropic effects on the targeting of proteins. It has been shown that the tyrosine in the cytoplasmic tail of lpgl20 can mediate both internalization and direct targeting from the TGR to lysosomes (Harter and Mellman, 1992). The relative effects of each of these targeting functions is determined by other surrounding residues, in particular a glycine at position i-1. Matter and colleagues have identified two tyrosine-dependent targeting motifs in the cytoplasmic tail of the LDL receptor that are sufficient for basolateral targeting in MDCK cells (Matter et al., 1992). However, these motifs were distinct from the signal that was required to mediate clathrin-mediated endocytosis. Furthermore, Kornfeld and colleagues have shown that the YSKV internalization motif in the mannose 6-phosphate/IGF II receptor also contributes to the sorting of the receptor to late endosomes from the TGR (Johnson and Kornfeld, 1992).

The present studies indicate that the NH₂ terminus of GLUT-4 contains important targeting information that functions at least in part like tyrosine-containing internalization motifs that have been described for a number of recycling receptors. However, the essential aromatic amino acid in the GLUT-4 tail is a phenylalanine and this motif appears to be more effective in mediating intracellular sequestration than the tyrosine-containing motif in the H1 subunit of the ASGPR. Other studies suggest that there may be other portions of GLUT-4 which regulate targeting (Asano et al., 1992). Because the present studies examined only the NH₂ terminus, we cannot exclude the potential role of other putative targeting domains. EM immunogold labeling showed that there was a high level of surface expression for the mEP deletion mutant (which shares the same length and the same first three amino acid residues with the GLUT-1 NH₂ terminus) near the levels achieved by GLUT-1. However, mutation of the Phe5 to alanine or a chimera of GLUT-4 containing the cytoplasmic tail of GLUT-1 had high surface distribution but not to the same extent as GLUT-1. Thus, it will be of critical interest to determine the role of the GLUT-4 NH₂ terminus with respect to the insulin-stimulated movement of GLUT-4 to the cell surface in insulin-sensitive adipocytes.

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