Roles of the N- and C-termini of GLUT4 in endocytosis

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Summary
In insulin target cells, the predominantly expressed glucose transporter isoform GLUT4 recycles between distinct intracellular compartments and the plasma membrane. To characterize putative targeting signals within GLUT4 in a physiologically relevant cell type, we have analyzed the trafficking of hemagglutinin (HA)-epitope-tagged GLUT4 mutants in transiently transfected primary rat adipose cells. Mutation of the C-terminal dileucine motif (LL489/90) did not affect the cell-surface expression of HA-GLUT4. However, mutation of the N-terminal phenylalanine-based targeting sequence (F5) resulted in substantial increases, whereas deletion of 37 or 28 of the 44 C-terminal residues led to substantial decreases in cell-surface HA-GLUT4 in both the basal and insulin-stimulated states. Studies with wortmannin and coexpression of a dominant-negative dynamin GTPase mutant indicate that these effects appear to be primarily due to decreases and increases, respectively, in the rate of endocytosis. Yeast two-hybrid analyses revealed that the N-terminal phenylalanine-based targeting signal in GLUT4 constitutes a binding site for medium chain adaptins \( \mu_1, \mu_2, \) and \( \mu_3A \), implicating a role of this motif in the targeting of GLUT4 to clathrin-coated vesicles.

Key words: Adaptin, Adipose cell, Glucose transporter

Introduction
The GLUT4 glucose transporter isoform is found in adipose and muscle cells, and is responsible for the glucose uptake regulated by insulin (Rea and James, 1997). In adipose cells, GLUT4 are constantly recycling between intracellular compartments and the plasma membrane. To characterize putative targeting signals within GLUT4 in a physiologically relevant cell type, we have analyzed the trafficking of hemagglutinin (HA)-epitope-tagged GLUT4 mutants in transiently transfected primary rat adipose cells. Mutation of the C-terminal dileucine motif (LL489/90) did not affect the cell-surface expression of HA-GLUT4. However, mutation of the N-terminal phenylalanine-based targeting sequence (F5) resulted in substantial increases, whereas deletion of 37 or 28 of the 44 C-terminal residues led to substantial decreases in cell-surface HA-GLUT4 in both the basal and insulin-stimulated states. Studies with wortmannin and coexpression of a dominant-negative dynamin GTPase mutant indicate that these effects appear to be primarily due to decreases and increases, respectively, in the rate of endocytosis. Yeast two-hybrid analyses revealed that the N-terminal phenylalanine-based targeting signal in GLUT4 constitutes a binding site for medium chain adaptins \( \mu_1, \mu_2, \) and \( \mu_3A \), implicating a role of this motif in the targeting of GLUT4 to clathrin-coated vesicles.

Materials and Methods
Antibodies and clones
The cDNA of human GLUT4 was a gift from Graeme I. Bell (University of Chicago, Chicago, IL). The expression vector pCIS2
was a gift from C. Gorman (Genentech Inc., San Francisco, CA). The GAL4 DNA-activation domain (GAL4-AD) plasmids containing cDNAs of the adaptin subunits, pACTII-$\alpha$, pGAD2, pACTII-$\gamma$, pACTII-$\mu_1$, pACTII-$\mu_2$, pACTII-$\mu_3$, pACTII-$\delta_1$, and pACTII-$\delta_2$, were kindly provided by Juan S. Bonifacino (NICHD, NIH, Bethesda, MD). Anti-HA antibody (HA.11) was from Berkeley Antibody Co. (Richmond, CA). Rabbit anti-GAL4 glutathione (1154p) was kindly provided by Hoffmann-La Roche (Nutley, NJ). An affinity-purified polyclonal anti-IRAP antibody against the insulin-responsive aminopeptidase IRAP was a kind gift from Susanna R. Keller (Dartmouth Medical School, Hanover, NH). FITC-conjugated anti-mouse antibody and rhodamine-conjugated anti-rabbit antibody were from Jackson ImmunoResearch (West Grove, PA). 125I-Sheep anti-mouse antibody was from DuPont (Boston, MA).

Construction of HA-GLUT4 mutants

Site-directed mutagenesis was performed using the polymerase chain reaction (PCR)-based Quick-Change method (Stratagene, La Jolla, CA). Cloned Pfu DNA-polymerase (Stratagene) was used for 16-20 PCR amplification cycles according to the manufacturer's instructions. The cDNA of HA-epitope-tagged human GLUT4, cloned into the expression vector pCIS2, was used as the template. The mutagenesis primers (sense strand; mismatches underlined) were: (HA-GLUT4-F5A) 5'-GAT GTC GCC CGC CCA ACA GAT AGG C-3' and (HA-GLUT4-L1/489/90A) 5'-TTC TAC CCG ACA CCC TCT GCT GCA GAG CAG GTG AAA-3'. For the C-terminal deletion mutants, two unique restriction sites were introduced into the cDNA of HA-GLUT4. A Mutl site was inserted at amino acid position 471-472 (coding for T471 and R472) without changing the amino acid sequence of GLUT4. The primer (sense strand) was 5'-GAC TAC CTG AAA CGC GTG CCC GCA CTG TG-3'. Separately, an Nhel site was introduced at amino acid position 481/482 of GLUT4 (coding for A481 and A482), thereby changing alanine-482 to serine. The primer (sense strand) was 5'-GAC CAG ATC TCA GCT AGC TCC CAG ACA CC-3'. Finally, the HA-GLUT4 deletion mutants, A37 and D28, were generated by ligation of termination codon (TGA)-containing synthetic oligonucleotide adaptors into Mutil/Nool- and Nhel/Nool-digested pCIS2/HA-GLUT4-Mutil and pCIS2/HA-GLUT4-Nhel, respectively. The oligonucleotide adaptors were Mutil (sense strand): 5'-CGC GTC GAC ATA TG-3'; (antisense strand): 5'-GGC CCA TAT GTC AA-3'; and Nhel (sense strand): 5'-CTA GCT GAC ATA TG-3'; (antisense strand): 5'-GGC CCA TAT GTC AG-3'.

The integrity of the mutated GLUT4 sequences was verified by automated DNA sequencing using the dRhodamine Terminator Cycle Sequencing kit (Perkin-Elmer, Norwalk, CT) and an ABI prizm-377 DNA sequencer (Applied Biosystems, Foster City, CA). Double mutants were generated by either subcloning or consecutive mutagenesis rounds using PCR.

Cell culture, transfection of rat adipose cells, and cell-surface antibody binding assay

Preparation of isolated rat epididymal adipose cells from male rats (CD strain, Charles River Laboratories, Wilmington, MA) has been described previously (Weber et al., 1998). The electroporation of rat adipose cells and the cell-surface antibody-binding assay were performed as described (Al-Hasani et al., 1998, 1999). Briefly, isolated primary rat adipose cells ($\approx 10^6$ cells/sample) in Dulbecco's modified Eagle's medium were electroporated (3 square wave pulses of 12 msac, 500 V/cm) in the presence of 0.25 mg/ml carrier DNA (sheared herring sperm DNA, Roche, Mannheim, Germany) and expression plasmid (1.25-12.5 $\mu$g/ml; 0.4 ml/cuvette). After culturing in Dulbecco's modified Eagle's medium for 4-24 hours at 37°C, 5% CO2, the cells were stimulated with insulin (67 nM, 1x10 micromicrons/ml) for 30 minutes at 37°C. Then, subcellular trafficking of GLUT4 was stopped by addition of KCN to a final concentration of 2 mM and the cells were incubated for 1 hour with monoclonal anti-HA antibody. After removal of excess antibody, 0.1 $\mu$Ci of [125I]Sheep anti-mouse antibody was added and the cells were incubated for 1 hour. Finally, the cells were spun through dionynocephalate oil to remove unbound labeled antibodies and the cellsurface-associated radioactivity was counted in a y-counter. The resulting counts were normalized to the lipid weight of the samples. Antibody binding assays were performed in duplicate or quadruplicate. The values obtained for pCIS-transfected cells were subtracted from all other values to correct for non-specific antibody binding.

Kinetic analysis of HA-GLUT4 internalization

Measurement of HA-GLUT4 internalization by reversal of their insulin-stimulated translocation using wortmannin was carried out on the basis of the concept as originally developed by Araki et al. (Araki et al., 1996) and adapted by us for use with transfected rat adipose cells as described previously (Al-Hasani et al., 1998). Briefly, HA-GLUT4-transfected rat adipose cells were cultured for 4 hours. After harvesting, the cells were stimulated with insulin for 30 minutes, and subsequently incubated without or with 100 nM wortmannin (Sigma, St Louis, MO) still in the presence of insulin. At the indicated time points, the cell-surface levels of HA-GLUT4 were determined using the antibody binding assay. The apparent half-times for endocytosis in the presence of insulin and wortmannin were then estimated by curve-fitting using a two-pool model as described previously (Araki et al., 1996). According to the model, the net loss of transporters from the plasma membrane is described by the equation

$$TP = \frac{k_{ex} \cdot (1 - e^{-(k_{ex}+k_{en}) \cdot t})}{(k_{ex} + k_{en})} + TP_0 \cdot e^{-(k_{ex}+k_{en}) \cdot t}$$

where $TP_0$ is the initial fraction of HA-GLUT4 in the plasma membrane, and $k_{ex}$ and $k_{en}$ are the rate constants for endo- and exocytosis, respectively. $TP_0$ was assumed to be 0.5 for wild-type HA-GLUT4, based on extensive biochemical data for endogenous GLUT4 (Satoh et al., 1993). $TP_0$ was adjusted for each of the HA-GLUT4 mutants according to its relative insulin-stimulated steady-state level determined in multiple independent experiments (Fig. 4; Fig. 5).

Western blot analyses of HA-GLUT4

HA-GLUT4-transfected rat adipose cells were harvested after 24 hours of protein expression and total cellular membranes were prepared as described previously (Al-Hasani et al., 1999). The resulting membranes were subjected to SDS-polyacrylamide (12%) gel electrophoresis (25-50 $\mu$g protein/lane) and transferred onto nitrocellulose filters. The filters were probed with monoclonal anti-HA antibody (1:1000), washed, and incubated again with 0.1 $\mu$Ci/ml [125I]Sheep anti-mouse antibody. Quantification of the blots was performed using a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). Protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Yeast two-hybrid analyses

A 70mer oligonucleotide coding for a 17 amino acid glycine/serine-rich linker (GGGSSGSSGSSGSSG) was inserted into the EcoRI and BamHI sites of pAS-2.1 (Clontech, Palo Alto, CA) in frame with the GAL4 DNA-binding domain (BD) to generate pAS-2.1GS. DNA fragments corresponding to the N-terminus of the human GLUT4 (M1-L24) were PCR-amplified to contain a 5' EcoRI site and a 3' BamHI site using Pfu DNA polymerase. The primers (matches underlined) used were (wild-type-sense strand): 5'-CAGATT- CATCGCCGTGGGCTTCCAAACAG-3'; (wild-type-antisense strand): 5'-CAGATTCCAGGGTCCACGTCCGTCG-3'; (P5A-sense strand): 5'-CAGAATTTCATGCCTGGCGCCACAG-3'; and

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Expression of HA-GLUT4 mutants in rat adipose cells

To evaluate the targeting potential of the various motifs of GLUT4 in insulin-responsive cells, the amino acid residues described in Fig. 1 were altered and the resulting mutants were expressed as HA-epitope-tagged GLUT4 in rat adipose cells. These mutants included substitution of F5 and LL489/490 with alanine, deletion of 28 and 37 amino acid residues from the C-terminus, and combinations of N- and C-terminal alterations. Fig. 2 illustrates the relative protein expression levels of the mutant HA-GLUT4’s determined after 24 hours of cell culture by western blot using an anti-HA antibody; under these conditions, HA-GLUT4 expression was ~20-fold that of endogenous GLUT4 in the ~10% of the cells actually transfected (Al-Hasani et al., 1999). As shown in the Fig. 2, the relative protein expression levels of all HA-GLUT4 mutants described in this study were about ±50% of the expression level of the HA-GLUT4 wild-type. Also, as expected, the C-terminal deletion mutants of GLUT4 showed a higher mobility in SDS-PAGE, indicative of a lower apparent molecular mass of the truncated HA-GLUT4.

Immunostaining of isolated rat adipose cells and confocal microscopy

Immunostaining of isolated rat adipose cells was performed as described previously (Malide et al., 1997). Briefly, cells were fixed in suspension for 20 minutes in freshly made 4% paraformaldehyde in PBS at room temperature. Then, the cells were permeabilized and the non-specific binding sites were blocked with 0.1% saponin in PBS containing 1% BSA and 3% normal goat serum (Vector Laboratories, Burlingame, CA) for 45 minutes at room temperature. For immunostaining of HA-GLUT4, the cells were incubated for 2 hours with monoclonal anti-HA antibody (1:1000), followed by incubation with FITC-conjugated secondary antibody (15 µg/ml) for 1 hour at room temperature. For immunostaining of IRAP, the cells were incubated overnight with anti-IRAP antibody (5 µg/ml), followed by an incubation with rhodamine-conjugated secondary antibody (15 µg/ml) for 1 hour at room temperature. In double-labeling experiments, the procedures were conducted sequentially with pairs of primary antibodies and the corresponding secondary antibodies.

Antibody staining was observed with a Nikon Optiphot 2 fluorescence microscope equipped with a Bio-Rad MRC 1024 confocal laser scanning imaging system (Bio-Rad, Hercules, CA). To excite FITC, the 488 nm line of a mixed argon/krypton laser was used at 10% laser energy. To excite rhodamine, the 568 nm line was used. Specimens were viewed using a planapochromat 60x/1.4 NA oil immersion objective. For each cell, 3-20 optical sections were recorded in 0.5 µm steps along the Z axis. All images were taken with Kalman averaging of three frames.

Results

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Analysis of subcellular distributions of mutant HA-GLUT4’s by confocal microscopy

To examine the correct subcellular localization of the HA-epitope-tagged GLUT4, we immunofluorescently double-labeled rat adipose cells expressing the wild-type HA-GLUT4 for the HA-epitope and simultaneously for the insulin-responsive aminopeptidase IRAP. Previously, IRAP has been identified as one of the major proteins of the GLUT4-containing vesicles (Keller et al., 1995). Moreover, a large body of biochemical and morphological evidence now exists that both GLUT4 and IRAP share the very same trafficking body of biochemical and morphological evidence now exists that both GLUT4 and IRAP share the very same trafficking body of biochemical and morphological evidence now exists that both GLUT4 and IRAP share the very same trafficking

We have further employed confocal immunofluorescence
independent experiments.

After harvesting, total cellular membranes were prepared and the expression of HA-GLUT4 was determined by western blot using anti-HA antibodies as described in Materials and Methods (inset). The HA-GLUT4 proteins migrated according to their expected molecular masses in SDS-PAGE and appeared as single bands on western blots. Results are the means±s.e.m. of at least three micrographs to determine the in situ localization of the various HA-GLUT4 constructs in transfected rat adipose cells. After harvesting, the cells were fixed and stained under permeabilizing conditions for the HA-epitope tag. Fig. 3B illustrates the subcellular distributions of wild-type HA-GLUT4, and the F5A, Δ37 and F5A-Δ37 mutants. As described previously for the endogenous GLUT4 in rat white adipose cells (Malide et al., 1997), HA-GLUT4 staining revealed a typical punctuate pattern distributed throughout the cytoplasm. No apparent differences in the staining pattern were observed between the wild-type HA-GLUT4 and the mutant constructs in either the basal or insulin-stimulated states (Fig. 3B). Most importantly, the HA-GLUT4s were excluded from the nucleus, as well as from structures resembling ER and Golgi stacks (Fig. 3 and data not shown).

Cell-surface expression of HA-GLUT4 constructs

To analyze the cell-surface expression of the HA-GLUT4 constructs, freshly prepared rat adipose cells were transfected with expression plasmids and the amount of HA-GLUT4 was then determined by an antibody binding assay of intact cells (Al-Hasani et al., 1998). Because the HA-GLUT4 trafficking response to insulin is closely related to the level of HA-GLUT4 expression, with the largest responses occurring at the lowest levels of expression, relatively short expression times (4 hours) and relatively low levels of expression plasmid (generally 1.25 µg DNA/ml) were chosen to examine the insulin-regulated subcellular trafficking of the HA-GLUT4 mutants (Al-Hasani et al., 1999). Under these conditions, the expression of the HA-GLUT4 mutants reached approximately the level of endogenous GLUT4 in the =10% of the cells actually transfected. We have previously determined in cell culture time-course experiments that the levels of expression of HA-GLUT4 measured at 4 hours and 24 hours are proportional (Al-Hasani et al., 1999). Thus, the cell-surface levels of the mutant HA-GLUT4’s assessed after 4 hours of cell culture were normalized to the relative total protein expression levels of the respective mutant HA-GLUT4’s (Fig. 2) and thereby represent relative subcellular distributions (i.e. cell-surface/total HA-GLUT4).

Cell-surface expression of LL489/90AA and F5A HA-GLUT4

As illustrated in Fig. 4A, insulin stimulation of rat adipose cells transfected with wild-type HA-GLUT4 and assayed 4 hours post transfection induced a 6.7±0.8-fold (mean±s.e.m.) increase in cell-surface glucose transporters over basal values. Likewise, the cell-surface levels of the LL489/90AA mutant appeared to be similar to the wild-type HA-GLUT4. This mutant showed no significant change in the insulin response (5.7±0.7-fold). By contrast, the F5A mutant showed markedly elevated basal and insulin-stimulated levels of cell-surface HA-GLUT4 compared with the wild-type. However, because the exchange of alanine for phenylalanine-5 led to a disproportionately larger increase in cell-surface HA-GLUT4 in the basal state (~5-fold) than in the insulin-stimulated state (~2-fold), the insulin response was significantly decreased (2.9±0.2-fold). Likewise, the insulin response of the double mutant F5A-LL489/90AA (2.1±0.1-fold) appeared to be similar to the insulin response of the F5A mutant.

Because it has been reported previously that mutation of the dileucine motif affects GLUT4 trafficking only at high levels of overexpression in 3T3-L1 adipocytes (Marsh et al., 1995), rat adipose cells were transfected and assayed for cell-surface HA-GLUT4 after 24 hours, rather than the usual 4 hours, of protein expression, as illustrated in Fig. 4B. As reported previously (Shepherd et al., 1993; Al-Hasani et al., 1999), high expression levels of GLUT4 in adipose cells resulted in a reduction in the insulin fold-response (compare Fig. 4A with Fig. 4B). However, prolonged protein expression did not change the cell-surface levels of any of the constructs examined: F5A, LL489/90AA and the double mutant F5A-LL489/90AA. In addition, the same results were obtained when adipose cells were transfected with either low (1.25 µg/ml) or high (12.5 µg/ml) amounts of expression plasmid and assayed after 4 hours of protein expression (data not shown).

Cell-surface expression of C-terminal truncation mutants of HA-GLUT4

We then looked for other potential targeting signals in the GLUT4 C-terminus by expressing two different mutants of HA-GLUT4 where the distal 37 and 28 C-terminal amino acids of GLUT4 were deleted, respectively (Fig. 1). Fig. 5 shows the steady-state cell-surface levels of the C-terminal mutants in rat adipose cells. Surprisingly, both deletion mutants, Δ37 and Δ28, displayed lower basal levels (1.5-2-fold) of cell-surface
HA-GLUT4 compared with the wild-type HA-GLUT4. Furthermore, the insulin-stimulated cell-surface levels were also reduced, to 48±13% and 61±11%, respectively. However, the insulin fold-responses (6.5±0.9 and 7.4±2.5, respectively) appeared to be unchanged. Compared with the wild-type, the F5A-D37 double mutant showed an =2.6-fold increase in basal cell-surface levels, whereas the insulin-stimulated cell-surface levels were not significantly changed. This resulted in a decreased insulin fold-response (2.2±0.2) similar to that observed for the F5A single mutant (Fig. 4).

Kinetic analysis of the endocytosis of mutant HA-GLUT4s

To obtain kinetic evidence related to the altered cell-surface distributions of the GLUT4 mutants, we measured their rates of endocytosis in the insulin-stimulated state using wortmannin (Araki et al., 1996; Al-Hasani et al., 1998). In essence, wortmannin is used to block the stimulation of exocytosis of GLUT4 by insulin thereby allowing to monitor the ongoing removal of the glucose transporters from the plasma membrane by endocytosis. When added either before or together with insulin, wortmannin completely abolished the insulin-stimulated translocation of the HA-GLUT4 constructs to the cell surface (data not shown). Fig. 6 shows the clearance of the cell-surface HA-GLUT4 mutants from the plasma membrane. Wild-type HA-GLUT4 was internalized with a half-time of =16 minutes as reported previously (Al-Hasani et al., 1998). By contrast, the F5A mutant showed impaired internalization, with a half-time of =30 minutes, whereas the Δ37 deletion mutant displayed accelerated internalization, with a half-time of =6 minutes. Likewise, the Δ28 deletion mutant showed a reduced half-time of =10 minutes compared with the control (data not shown). In all cases, however, the cell-surface levels reached after 60 minutes were approximately the same as those observed in the basal state (Fig. 4; Fig. 5).

Coexpression of HA-GLUT4 mutants with dominant-negative dynamin

To confirm the endocytosis kinetic data for the mutant HA-GLUT4’s, we used the following molecular approach: instead of blocking the exocytosis of GLUT4 with wortmannin (Fig. 6), we analyzed cell-surface HA-GLUT4 under conditions where endocytosis is inhibited. Previously, we (Al-Hasani et al., 1998) and others (Kao et al., 1998) have shown in adipose cells that the 100 kDa GTPase dynamin is involved in the endocytosis of GLUT4. In particular, expression of a dominant-negative dynamin mutant, dynamin-K44A, has been shown to lead to an accumulation of GLUT4 on the plasma membrane due to an inhibition of GLUT4 endocytosis. As illustrated in Fig. 7,
The N-terminal F5QQ1 motif in GLUT4 appears to resemble a tyrosine-based sorting signal found in the cytoplasmic domains of many membrane proteins (Bonifacino and Dell’Angelica, 1999). Most tyrosine-based signals conform to the consensus sequence YXXØ, where the tyrosine residue is followed by multiple hydrophobic residues. Interaction of the GLUT4 N-terminus with adaptins in the yeast two-hybrid system

Interaction of the GLUT4 N-terminus with adaptins in the yeast two-hybrid system

The N-terminal F5QQ1 motif in GLUT4 appears to resemble a tyrosine-based sorting signal found in the cytoplasmic domains of many membrane proteins (Bonifacino and Dell’Angelica, 1999). Most tyrosine-based signals conform to the consensus sequence YXXØ, where the tyrosine residue is followed by multiple hydrophobic residues.
two non-conserved amino acids (X) and an amino acid with a bulky hydrophobic side chain. YXXØ motifs are known to bind to the medium chain subunits (µ-adaptins) of heterotetrameric adaptor proteins (AP's). Consequently, the membrane protein/AP complex is incorporated into clathrin-coated vesicles and sorted into a different compartment of the cell. Therefore, we have tested whether the N-terminus of GLUT4 might interact with adaptin subunits by employing the yeast two-hybrid system (Fields and Song, 1989). The entire N-terminus of GLUT4 (GLUT4-NT) was fused to the GAL4 DNA-binding domain (GAL4-BD) and served as the bait. Because in the yeast two-hybrid system the spacing between the putative binding site (i.e. the YXXØ motif) and the GAL4-BD has been reported to be crucial for the resulting signal strength (Ohno et al., 1996), we have placed a 17-amino acid glycine/serine-rich spacer between GAL4-BD and GLUT4-NT (see Materials and Methods; preliminary analyses showed that this spacer insertion resulted in an >5-fold increase in β-galactosidase activity without affecting the background signal; data not shown). As prey, we used several adaptin subunits of AP-1 (α, β2, γ, μ1, μ2, μ3A, σ1, and σ2) fused to the GAL4 DNA-transcription activation domain (GAL4-AD). Saccharomyces cerevisiae strains HF7c and SFY526 were cotransformed with bait and prey, and assayed for reporter gene activation. In both strains, an interaction between bait and prey results in expression of β-galactosidase (LacZ).

In addition, strain HF7c contains the HIS3 reporter gene under the control of the GAL4 promoter, enabling the cells to grow on medium lacking histidine.

No detectable reporter gene activation was observed in cells coexpressing GLUT4-NT and the adaptin subunits α, β2, γ, σ1,
and σ2 (data not shown). However, cotransformation of yeast with GLUT4-NT and the µ-adaptins μ1, μ2, and μ3A leads to activation of both the HIS3 and LacZ reporter genes (Fig. 8).

Quantitation of the corresponding β-galactosidase activity reveals a preference of GLUT4-NT for the adaptins in the order of μ1>μ3A>μ2 (Fig. 8B). Interestingly, a tyrosine in position 5 in GLUT4-NT (F5Y) increases the interaction with the μ1 adaptin, and to a much lesser extent with the μ2 adaptin (Fig. 8B). By contrast, mutation of phenylalanine-5 to alanine (F5A) in GLUT4-NT completely abolishes both HIS3 and LacZ reporter gene activation.

**Discussion**

In the present study, we have evaluated the potential roles in subcellular trafficking of two motifs found in GLUT4, F5 and

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**Fig. 7.** Coexpression of HA-GLUT4s and dominant negative dynamin. Isolated cells were cotransfected with 0 (−) or 12.5 (+) μg/ml dynamin-K44A plasmid/cuvette and 2.5 μg/ml of expression plasmid for HA-GLUT4s and cultured for 24 hours. After harvesting, the cells were incubated without (basal, solid bars) or with (open bars) 67 nM insulin for 30 minutes, and the cell-surface levels of HA-GLUT4s were determined using an antibody binding assay as described in Materials and Methods. The cell surface-associated HA-GLUT4s were determined using an antibody binding assay as described in Materials and Methods. The cell surface-associated radioactivity was normalized to the relative protein expression level of each respective mutant. Results are the means of the mean values (Δ, Y) obtained from at least duplicate determinations in two independent experiments.

**Fig. 8.** Yeast two-hybrid interaction of the N-terminus of GLUT4 with µ-adaptins. (A) Yeast cells (strain HF7c) cotransformed with plasmids for GAL4-BD/GLUT4-NTs and GAL4-AD/µ-adaptins were plated onto drop-out agar plates lacking tryptophane and leucine with (+) and without (−) histidine and incubated for 2 days at 30°C. (B) Quantification of β-galactosidase activity. Yeast cells (strain SFY526) cotransformed with plasmids for GAL4-BD/GLUT4-NTs and GAL4-AD/µ-adaptins were grown into mid-log phase, harvested and assayed for β-galactosidase activity using CPRG as substrate (see Materials and Methods).
LL489/90, and the entire C-terminus of GLUT4 by analyzing the trafficking of mutant HA-epitope-tagged GLUT4 in rat adipose cells. Therefore, we have employed physiological expression levels of recombinant reporter-GLUT4 in a physiological target cell for insulin-stimulated glucose transport. As judged by confocal immunofluorescence microscopy (Malide et al., 1997) (Fig. 3), both glucose transporters, HA-GLUT4 and endogenous GLUT4, are highly colocalized with a specific marker of the insulin-sensitive storage compartment (Gsv) in rat adipose cells, the insulin-responsive aminopeptidase IRAP. Since the translocation of endogenous GLUT4 and HA-GLUT4 to the plasma membrane show identical insulin dose-response curves (Quon et al., 1994), the time-courses of insulin action on the two glucose transporter species are the same (C.S.H. and S.W.C., unpublished), and the kinetics of endocytosis of HA-GLUT4 and endogenous GLUT4 in the insulin-stimulated state are similar (Al-Hasani et al., 1998) (Fig. 6), it is reasonable to assume that both the subcellular trafficking, and localization of HA-GLUT4 and endogenous GLUT4 are identical.

According to western blot analyses (Fig. 2) and confocal immunofluorescence microscopy (Fig. 3), all of the mutant HA-GLUT4's studied here appear to be expressed properly and targeted to a post-Golgi compartment in rat adipose cells. Because the relative protein expression levels of all the HA-GLUT4 mutants were similar to the expression level of the HA-GLUT4 wild-type (Fig. 2), all of the cell-surface binding values in this study were normalized to the expression levels of the respective HA-GLUT4 constructs. Thus the normalized cell-surface binding values actually represent relative subcellular distributions between the plasma membrane and intracellular compartments.

Our results for the F5A mutant clearly show an involvement of the N-terminal phenylalanine-5 in the internalization of GLUT4 from the plasma membrane. Compared with the wild-type HA-GLUT4, the cell-surface levels of the F5A mutant are increased in both the basal and insulin-stimulated states (Fig. 4). In the basal state, wortmannin does not influence the cell-surface level of this HA-GLUT4 mutant (data not shown). However, kinetic measurements of the clearance of this construct from the plasma membrane in the insulin-stimulated state in the presence of wortmannin demonstrate an extended half-time (=30 minutes), indicating a reduced rate of endocytosis (Fig. 6). While at best the four-point time courses reported here can yield only rough estimates of the kinetic rate constants, the observed increase in the half-time of endocytosis is consistent with the increased cell-surface levels observed at steady-state (=2-fold and greater).

The 100 kDa GTPase dynamin is an essential component of vesicle formation in clathrin-mediated endocytosis (Hinshaw, 2000), and expression of a dominant-negative mutant (dynamin-K44A) inhibits the internalization of GLUT4 from the plasma membrane in both primary adipose cells and cultured adipocytes (Al-Hasani et al., 1998; Kao et al., 1998).

As a result, GLUT4 not only accumulates on the plasma membrane in cells expressing dynamin-K44A (Fig. 7), but also the cell-surface levels observed under these conditions represent the maximum possible cell-surface values, roughly equivalent to total cellular expression (Al-Hasani et al., 1998).

With the F5A mutant, similar values are observed in the insulin-stimulated state without the mutant dynamin, and in the basal and insulin-stimulated states in dynamin-K44A-cotransfected cells. Thus, consistent with the kinetic data, coexpression of dynamin-K44A with the F5A mutant results in a smaller fold increase in glucose transporters accumulating on the cell surface compared with the effect of the mutant dynamin on wild-type HA-GLUT4 because the subcellular distribution of the former is already shifted towards the plasma membrane. Nevertheless, since the F5A mutant displays a reduced but still significant internalization, it is likely that additional residues of GLUT4 are involved in their endocytosis.

The C-terminal dileucine motif (LL489/90) has been reported to function in both the internalization and sequestration of GLUT4 dependent upon the cell type where studied and the level of construct expression (Marsh et al., 1995; Verhey et al., 1995; Araki et al., 1996; Garippa et al., 1996). By contrast, we could not detect an involvement of the LL489/90 motif in GLUT4 trafficking in rat adipose cells at either low or high levels of protein expression (Fig. 4A,B). Furthermore, in the double mutant F5A-LL489/90AA, where both the phenylalanine-5 and the two leucines (LL489/90) were exchanged for alanines, only mutagenesis of the phenylalanine residue in the N-terminus of GLUT4 affects the trafficking of GLUT4 (Fig. 4).

To identify other potential targeting signals in the C-terminus of GLUT4, we generated two mutants where parts of the C-terminus was deleted. As indicated in Fig. 1, roughly half of the GLUT4 C-terminus is highly homologous to the C-terminus of GLUT1. Since GLUT4 and GLUT1 recycle along different pathways within the cell (Al-Hasani, et al., 1999; Millar et al., 1999), it is tempting to speculate that the targeting motifs responsible for the specific targeting of GLUT4 in adipose cells are located in the C-terminal half of that domain. To our surprise, deletion of the distal 28 and 37 amino acids of the C-terminus, including the dileucine motif, reduced by ≈50% the cell-surface GLUT4 distributions in both the basal and insulin-stimulated states in rat adipose cells. Thus, the insulin fold-responses of Δ37 and Δ28 were unchanged compared with the wild-type. Kinetic measurements of the internalization of the Δ37 mutant revealed a significant increase in the rate of endocytosis (Fig. 6), and coexpression of mutant dynamin results in a dramatic increase in cell-surface levels of the truncation mutants (Fig. 7), indicating that the truncation mutants shift the steady-state distribution towards intracellular compartments. Thus, it appears that the C-terminus of GLUT4 has an inhibitory function in internalization, perhaps by masking targeting signals somewhere else in the molecule that are important for endocytosis.

It could be argued that deletion of the C-terminal 38 amino acids in GLUT4 might alter the structural integrity of the molecule and/or remove some important targeting signals, resulting in a redirection of the mutant GLUT4 to a compartment in rat adipose cells different from the GSC. This way, the level of mutant HA-GLUT4 in the GSC, as well as the levels on the cell surface in the steady-state, would be reduced. However, in Xenopus oocytes, the same Δ37 deletion mutant of GLUT4 has been demonstrated to be fully active in glucose transport, indicating structural integrity of the molecule (Dauterive et al., 1996). Also, the observed normal insulin responses of the mutants (Fig. 5), together with the
dynamin-K44A-induced increases in the cell-surface levels of the Δ37 and Δ28 mutants (Fig. 7), clearly show that these mutants are indeed recycling within the adipose cells, again indicating a proper folding and expression of the proteins. Lastly, the observed reductions in the half-times of endocytosis for the deletion mutants (=2-fold) are entirely consistent with the reduced cell-surface levels observed at steady-state (=2-fold). Thus, the C-terminus of GLUT4 does not appear to contain signal sequences essential for the targeting of the molecule to the GSC, but rather is indirectly related to the rate of clearance of GLUT4 from the plasma membrane.

These conflicting data concerning the role of the C-terminus and the dileucine motif could reflect the use of different DNA constructs, different transfection methods, different cell types, and/or different levels of expression, although we could not demonstrate significant effects of protein expression level. Perhaps most importantly, however, different cell types appear to display preferences for different targeting motifs, as has been shown for the targeting potential of the dileucine motif in 3T3-L1 fibroblasts versus 3T3-L1 adipocytes (Verhey et al., 1995).

Heterotetrameric adaptor proteins (APs) are known to be involved in the targeting of integral membrane proteins (Kirchhausen et al., 1997). To date, four adaptor complexes, known as AP-1, AP-2, AP-3 and AP-4, have been identified. In several recent studies it has been demonstrated that the heterotetrameric adaptor proteins (Bonifacino and Dell’Angelica, 1999). It is believed that binding of AP-1 and AP-2 recruits these proteins to the clathrin-coats of nascent transport vesicles. The N-terminal F3QQI motif in GLUT4 resembles such a YXXØ motif, thus implying a possible interaction of the N-terminus of GLUT4 with the medium chains of the heterotetrameric adaptor proteins. In the present preliminary study, we show that the N-terminus of GLUT4 does indeed bind to all of the three tested μ-adaptons μ1, μ2, and μ3A (the non-neuronal isofrom of μ3) in the yeast two-hybrid system. Indicative of the specificity of this interaction, no binding is observed when the phenylalanine-5 in GLUT4 is replaced by alanine. By contrast, the binding to μ1, and to a lesser extent to μ2, is enhanced when F5 is replaced by a tyrosine. These observations are in line with the recently described in vivo association of GLUT4 and the adaptor complexes AP-1, AP-2 and AP-3, containing μ1, μ2 and μ3A, respectively (Gillingham et al., 1999). Furthermore, it has been shown that in CHO cells, the endocytosis of TfR/GLUT4 chimera is modulated by phenylalanine-5 in the N-terminus of GLUT4: the corresponding F5A mutant showed decreased internalization, whereas the rate of internalization of the corresponding F5Y mutant was increased (Garippa et al., 1994). Presumably, these differences in the rates of internalization of TfR/GLUT4-F5 chimera in CHO cells and HA-GLUT4-F5 mutants in adipose cells reflect different affinities for μ2-adapton, which has been implicated in recruiting membrane proteins to clathrin-coated pits. Thus, while further biochemical analyses are required, our preliminary data are consistent with the proposed binding of the FQXI motif of GLUT4 to the μ-adaptons in vivo. Because this interaction occurs with several μ-adaptons, the N-terminus may be involved in the targeting of GLUT4 at multiple sites in the trafficking pathway.

In summary, none of the HA-GLUT4 constructs examined here resulted in extensive targeting of the protein to the plasma membrane as would be expected for the removal of a signal responsible for intracellular retention. Nevertheless, both the phenylalanine-5 in the N-terminus as well as the last 37 amino acids of the C-terminus clearly appear to be involved in the general endocytosis of GLUT4. Thus, further studies are required to locate the targeting signals within GLUT4 responsible for their unique subcellular distribution and insulin-stimulated translocation in insulin target cells.

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