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GLUT4 Recycles via a *trans*-Golgi Network (TGN) Subdomain Enriched in Syntaxins 6 and 16 But Not TGN38: Involvement of an Acidic Targeting Motif

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Insulin stimulates glucose transport in fat and muscle cells by triggering exocytosis of the glucose transporter GLUT4. To define the intracellular trafficking of GLUT4, we have studied the internalization of an epitope-tagged version of GLUT4 from the cell surface. GLUT4 rapidly traversed the endosomal system en route to a perinuclear location. This perinuclear GLUT4 compartment did not colocalize with endosomal markers (endosomal antigen 1 protein, transferrin) or TGN38, but showed significant overlap with the TGN target (t)-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) Syntaxins 6 and 16. These results were confirmed by vesicle immunoisolation. Consistent with a role for Syntaxins 6 and 16 in GLUT4 trafficking we found that their expression was up-regulated significantly during adipocyte differentiation and insulin stimulated their movement to the cell surface. GLUT4 trafficking between endosomes and *trans*-Golgi network was regulated via an acidic targeting motif in the carboxy terminus of GLUT4, because a mutant lacking this motif was retained in endosomes. We conclude that GLUT4 is rapidly transported from the cell surface to a subdomain of the *trans*-Golgi network that is enriched in the t-SNAREs Syntaxins 6 and 16 and that an acidic targeting motif in the C-terminal tail of GLUT4 plays an important role in this process.

INTRODUCTION

Insulin stimulates glucose uptake in muscle and fat cells by triggering translocation of the glucose transporter GLUT4 from an intracellular compartment to the cell surface (Bryant *et al.*, 2002). The intracellular localization of GLUT4 in adipocytes includes the endosomal system, *trans*-Golgi network (TGN), cytoplasmic tubulovesicular elements and the cell surface, suggesting a complex intracellular trafficking itinerary (Slot *et al.*, 1991b; Martin *et al.*, 2000a). Although previous studies have indicated a role for endosomes in GLUT4 trafficking (Slot *et al.*, 1991b; Livingstone *et al.*, 1996) the precise role of the TGN is not clear. Several observations suggest an important role for the TGN in GLUT4 trafficking. First, there is a significant amount of GLUT4 in the TGN area in insulin-responsive cells (Slot *et al.*, 1991a,b; Ralston and Ploug, 1996; Wang *et al.*, 1996; Slot *et al.*, 1997; Ploug *et al.*, 1998; Martin *et al.*, 2000a). Second, ~60% of the entire GLUT4 pool is localized to atrial natriuretic factor-containing secretory granules in atrial cardiomyocytes and this seems to be due to recycling of GLUT4 through the TGN area (Slot *et al.*, 1997). Third, there is significant overlap between GLUT4 and proteins known to traffic between the TGN and endosomes, including the cation-dependent mannose 6-phosphate receptor (Martin *et al.*, 2000a), the cation-independent mannose 6-phosphate receptor (Kandror and Pilch, 1996), and adaptor-related protein complex-1 (Gillingham *et al.*, 1999; Martin *et al.*, 2000b).

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Abbreviations used: EEA1, early endosomal antigen 1; ER, endoplasmic reticulum; HA, hemagglutinin; HDM, high-density microsome; IRAP, insulin-responsive aminopeptidase; LDM, low-density microsome; PM, plasma membrane; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; Tf, transferrin; TfR, Tf receptor; TGN, *trans*-Golgi network; t-SNARE, target-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

These data suggest that the TGN contributes to the trafficking of GLUT4, adding a further layer of complexity to understanding the insulin-regulated movement of this molecule to the cell surface. In comparison with other recycling proteins, such as the transferrin receptor (TfR), the recycling of GLUT4 via the cell surface, at least in insulin's absence, is relatively slow (Yang and Holman, 1993; Yeh *et al.*, 1995). GLUT4 is localized to AP-2/clathrin-coated pits at the cell surface and is endocytosed via a clathrin-mediated process (Robinson *et al.*, 1992; Kao *et al.*, 1998). This is regulated by two endocytosis motifs in GLUT4; a dileucine motif in the C terminus and an aromatic amino acid-based motif in the N terminus (Piper *et al.*, 1993; Garippa *et al.*, 1994, 1996; Marsh *et al.*, 1995; Verhey *et al.*, 1995). Although there is little data available on the recycling of GLUT4 between intracellular compartments in adipocytes, the presence of GLUT4 in AP-1/clathrin-coated intracellular transport vesicles suggests that GLUT4 is not restricted to a stable storage compartment within the cell (Gillingham *et al.*, 1999; Martin *et al.*, 2000b). A significant proportion of GLUT4 is localized to endosomes where it colocalizes with other recycling proteins such as the TfR (Livingstone *et al.*, 1996). Chemical ablation of endosomes containing the TfR by using a transferrin (Tf)-horseradish peroxidase conjugate demonstrated that ~45% of intracellular GLUT4 is susceptible to ablation (Livingstone *et al.*, 1996). Furthermore, it has been shown that after endocytosis of GLUT4 from the cell surface, GLUT4 is segregated from the TfR in the endosomal system into a separate population of transport vesicles (Sandoval *et al.*, 2000; Lampson *et al.*, 2001; Lim *et al.*, 2001). However, the destination of these vesicles, and the nature of the nonablatable pool of GLUT4, is not clear. Although it is possible that the nonablatable pool corresponds to a type of GLUT4 storage vesicle, the origin of these vesicles has not been identified yet.

Our laboratory has recently described a targeting domain in the C terminus of GLUT4 distal to the dileucine motif (Shewan *et al.*, 2000). Disruption of this domain, consisting of a cluster of acidic amino acids, causes enhanced susceptibility to Tf-horseradish peroxidase-mediated ablation. This acidic domain is therefore likely to be involved in the endocytic sorting of GLUT4. In the present study, we have analyzed the function of this domain and the intracellular trafficking of GLUT4 between endosomes and the TGN by using a more kinetic approach. We have found that GLUT4 traffics rapidly from the cell surface, via the early endosomal system, to a perinuclear compartment that is enriched in the TGN target-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (t-SNAREs) Syntaxins 6 and 16. Both of these molecules have been implicated in the trafficking of cargo between endosomes and the TGN (Mallard *et al.*, 2002). An acidic targeting motif in the C terminus of GLUT4 regulates accumulation of GLUT4 in the perinuclear Syntaxin 6/16-positive compartment. These data implicate an important role for the TGN in GLUT4 trafficking.

MATERIALS AND METHODS

Materials and Antibodies

DMEM, Myoclone-Plus fetal calf serum, and antibiotics were from Invitrogen (Paisley, United Kingdom). Normal sera were from DAKO (Carpinteria, CA). All other materials were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Polyclonal rabbit antibodies were raised against glutathione *S*-transferase-fusion proteins consisting of the cytosolic domain of Syntaxin 6 and insulin-responsive aminopeptidase (IRAP). Monoclonal antibodies raised against Syntaxin 6 were obtained from Transduction Laboratories (Lexington, KY) or were the generous gift of Dr. Jason Bock (Howard Hughes Medical Institute, Stanford University School of Medicine, Palo Alto, CA) (3D10). Rabbit antibodies against Syntaxin 13 were obtained from Dr. Rohan Teasdale (University of Queensland, Australia), anti-TGN38 antibodies were from Dr. Paul Luzio (University of Cambridge, Cambridge, United Kingdom), and anti-human endosomal antigen 1 protein (EEA1) antibodies were from Dr. Marvin Fritzler (University of Calgary, Calgary, Alberta, Canada). The monoclonal antibody 16B12, which recognizes the influenza hemagglutinin (HA) epitope, was purchased from Babco (Richmond, CA). A monoclonal antibody against the TfR was from Zymed Laboratories (South San Francisco, CA). Antibodies against GLUT4 (James *et al.*, 1989), Syntaxin 4 (Tellam *et al.*, 1997), Syntaxin 7 (Wade *et al.*, 2001), Syntaxin 16 (Mallard *et al.*, 2002), and GS15 (Xu *et al.*, 1997) have been described previously.

Cell Culture and Retroviral Transfection

3T3-L1 fibroblasts (American Type Culture Collection, Manassas, VA) were cultured as described previously (Shewan *et al.*, 2000). Briefly, cells were grown in DMEM supplemented with 10% new born calf serum, 2 mM L-glutamine, 100 U/l penicillin and 100 μg/l streptomycin at 37°C in 10% CO_2, and passaged at ~70% confluence. Confluent cells were then differentiated into adipocytes. Cells were used between days 6–10 postdifferentiation and between passages 4 and 12. To establish basal conditions before use, cells were incubated in serum-free DMEM for 2 h unless otherwise indicated.

The construction and generation of retroviral stocks of HA-GLUT4 and HA-TAIL have been described previously (Shewan *et al.*, 2000). Both constructs encode transporters harboring an HA epitope engineered in the large exofacial loop between transmembrane domains 1 and 2 of GLUT4. HA-GLUT4 encodes the full-length GLUT4 protein (Quon *et al.*, 1994). HA-TAIL encodes full-length GLUT4 in which the 12 carboxyl terminal residues have been replaced by the corresponding sequence from GLUT3 (Shewan *et al.*, 2000). HA-EXEY was generated by site-directed mutagenesis of pMEX shuttle HA-GLUT4 (pMS-HA-GLUT4) cDNA by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). To generate this mutant, we took advantage of the unique *Nco*I site present in both human and rat GLUT4 cDNAs. Complimentary oligonucleotides were used to mutagenize $E^{499}LEY^{502}$ to ALAA, using primers 5′-gtgaaacccagtacagcacttgcagccttagggccagatgag-3′ and 5′-ctcatctggccctaaggctgcaagtgctgtactgggtttcac-3′. The *Nco*I-*Eco*RI fragment of pMS-HA-EXEY was fully sequenced before subcloning into pBabepuro for production of retrovirus (Pear *et al.*, 1993). For expression of the hTfR in adipocytes, the *Bam*HI-*Bgl*II fragment coding for hTfR was subcloned from pUC8-hTfR (T.E. McGraw, Cornell University, Ithaca, NY) into pBabepuro and retrovirus expressing pBabe-TfR was produced as described above (Pear *et al.*, 1993).

To generate 3T3-L1 adipocytes expressing each construct, 3T3-L1 fibroblasts (plated at a density of 5×10^5/100-mm plate 16 h prior) were infected with the relevant virus for 5 h in the presence of 4 μg/ml polybrene. After a 48-h recovery period, infected cells were selected in DMEM containing 10% fetal calf serum and supplemented with 2 μg/ml puromycin. Polyclonal pools of 3T3-L1 fibroblasts were then grown to confluence and subsequently differentiated into adipocytes as described above. Puromycin was not included in the differentiation media but was reapplied once the differentiation regime was completed.

Subcellular Fractionation

Subcellular membrane fractions from basal and insulin-treated 3T3-L1 adipocytes were prepared using a previously described

differential centrifugation procedure (Piper *et al.*, 1991; Marsh *et al.*, 1995). Briefly, the plasma membrane fraction was obtained after a 20-min centrifugation at 17,200 × *g* followed by centrifugation through sucrose. The high-density microsomes (HDMs) were obtained by centrifuging the 17,200 × *g* supernatant at 38,700 × *g* for 20 min and the low-density microsomes (LDMs) were obtained by spinning the 38,700 × *g* supernatant at 150,000 × *g* for 75 min. These fractions have previously been characterized in detail (Piper *et al.*, 1991). The HDM fraction contains large endosomal components and endoplasmic reticulum (ER), whereas the LDM fraction contains small vesicles, including those enriched in GLUT4. All fractions were resuspended in HES buffer (20 mM HEPES, 1 mM EDTA, 250 mM sucrose, pH 7.4), protein quantified using the bicinchoninic acid assay (Pierce Chemical, Rockford, IL) and stored at −80°C before use. Total membrane fractions were prepared from 3T3-L1 fibroblasts and adipocytes after homogenization in HES buffer containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 250 μM phenylmethylsulfonyl fluoride). Homogenates were subjected to centrifugation at 50,000 rpm in a Beckman Coulter TLA100–3 rotor for 60 min. The membrane pellet was resuspended in HES buffer and stored at −80°C before use.

Resialylation

Resialylation studies were performed essentially as described by Teuchert *et al.* (1999). Cells were incubated in serum-free medium overnight and insulin (20 nM) was added for 30 min at 37°C. Cells were then washed five times with ice-cold phosphate-buffered saline (PBS) containing 0.1 mM $CaCl_2$, 1 mM $MgCl_2$ (PBS^{++}) and biotinylated twice for 20 min in 2 ml of PBS^{++} containing 0.5 mg/ml sulfo-NHS-biotin (Pierce Chemical). Cells were washed three times with ice-cold PBS^{++} containing 0.1 M glycine to quench free biotin, incubated with neuraminidase *Vibrio cholerae* (80 mU/ml; Roche Diagnostics, Indianapolis, IN) on ice for 1 h and then washed three times with PBS^{++}. Cells were then incubated with prewarmed DMEM containing fetal calf serum (10%) for different times as indicated at 37°C. Cells were washed twice with PBS^{++} at 4°C and incubated with Triton X-100 (1%) containing protease inhibitors (see above) for 20 min at 4°C. Cells were scraped from the dish and centrifuged at 14,000 rpm for 10 min at 4°C. The cell lysate was incubated with streptavidin agarose beads at 4°C for 16 h and washed three times in PBS^{++} containing 1% Triton X-100/0.1% SDS. Samples were then heated to 60°C for 10 min and subjected to SDS-PAGE and immunoblotted with anti-IRAP.

Immunoprecipitation of SNARE Complexes

Basal 3T3-L1 adipocytes were homogenized by passaging twice through a 25-gauge needle followed by passaging twice through a 27-gauge needle in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA containing protease inhibitors. Cell lysates were solubilized using 1% Triton X-100 on ice for 30 min. The solubilized lysate was cleared by centrifugation for 30 min at 4°C in a microcentrifuge. Aliquots of the soluble proteins were incubated overnight with relevant antibodies bound to protein A-Sepharose. Immunoprecipitated proteins were resolved by SDS-PAGE together with aliquots of the supernatant and starting material.

Immunoabsorption of GLUT4 and Syntaxins 6 and 16 Vesicles

Protein G and protein A beads (Pierce Chemical) were incubated with 1% bovine serum albumin (BSA) for 30 min. Beads were then incubated with either monoclonal GLUT4 antibody 1F8, nonspecific mouse IgG, anti-Syntaxin 6, or anti-Syntaxin 16 antibodies. Antibodies were cross-linked to the beads by using 20 mM dimethyl suberimidate (Pierce Chemical) for 30 min at room temperature and cross-linked antibodies were saturated with 1% BSA for 30 min at room temperature. LDMs from noninfected, HA-GLUT4–infected or HA-TAIL–infected 3T3-L1 adipocytes were incubated separately with each of the specific and nonspecific antibody-coupled beads overnight at 4°C. The beads were washed and adsorbed material was eluted with SDS sample buffer and subjected to SDS-PAGE together with aliquots of the starting material.

Electrophoresis and Immunoblotting

Proteins were subjected to electrophoresis on 7.5 or 12% SDS-polyacrylamide gels and transblotted onto polyvinylidene difluoride. Immunolabeled proteins were visualized using horseradish peroxidase-conjugated secondary antibody and either the enhanced chemiluminescence system (Amersham Biosciences, Aylesbury, United Kingdom) or Supersignal (Pierce Chemical). Bands were quantitated by densitometry or by using a Lumi-Imager (Roche Diagnostics, Castle Hill, New South Wales, Australia).

Indirect Immunofluorescence Microscopy

The preparation of plasma membrane (PM) lawns was performed as described in Robinson *et al.* (1992). Briefly, after incubating cells on coverslips with the appropriate treatment, adipocytes were sonicated yielding a lawn of PM fragments attached to the coverslip. Coverslips were then incubated with the relevant antibodies directed against C-terminal domains, followed by fluorescein isothiocyanate-conjugated secondary antibody (Molecular Probes, Eugene, OR). Cells were viewed using either a 63×/1.4 oil immersion objective on an Axiovert fluorescence microscope (Carl Zeiss, Thornwood, NY), equipped with an MRC-600 laser confocal imaging system (Bio-Rad, Hercules, CA), or a 100×/1.4 Plan Apo oil immersion objective on an Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan), equipped with a Radiance 2000 laser confocal imaging system (Bio-Rad).

Endocytosis of HA-GLUT4 and Transferrin in 3T3-L1 Adipocytes

Adipocytes expressing HA-GLUT4, HA-TAIL, HA-EXEY, or hTfR were serum starved for 2 h in Krebs-Ringer phosphate buffer (12.5 mM HEPES, 120 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 0.6 mM Na_2HPO_4, pH 7.4) containing 0.2% BSA (KRP/B) and stimulated with insulin (20 nM) for 20 min to bring a cohort of GLUT4 molecules to the cell surface. Cells were then washed with ice-cold KRP/B and incubated on ice with monoclonal anti-HA for 60 min. To reverse the insulin stimulation, cells were rinsed five times in ice-cold KRP/B, and endocytosis was initiated by transfer to 37°C in prewarmed KRP/B. 3T3-L1 adipocytes expressing hTfR were incubated with Tf-Alexa-488 (Molecular Probes) during the chase at a final concentration of 50 μg/ml. At the times indicated, cells were fixed using 3% paraformaldehyde in PBS for at least 30 min at room temperature. Free aldehyde groups were quenched in 50 mM NH_4Cl in PBS. Cells were permeabilized and labeled in PBS containing 2% BSA and 0.1% saponin by using standard procedures. Cells were double labeled for endocytosed markers (HA or Tf) and either endogenous GLUT4, Syntaxin 6, Syntaxin 16, TGN38, or EEA1, followed by Alexa-488 or Alexa-594–conjugated secondary antibodies (Molecular Probes). Optical sections were analyzed by confocal scanning laser microscopy by using a TCS SP system (Leica Microsystems, Deerfield, IL).

RESULTS

GLUT4 Traffics from the Cell Surface to a Syntaxin 6/16-Positive Compartment via Early Endosomes

Dissecting the nature of the intracellular compartment(s) through which GLUT4 traverses, and from where it moves to the cell surface with insulin, has been a significant challenge. This has been complicated by the presence of GLUT4

in multiple locations, including early endosomes, recycling endosomes as well as a postendocytic location (Livingstone *et al.*, 1996; Martin *et al.*, 1996; Lampson *et al.*, 2001; Palacios *et al.*, 2001). In an attempt to characterize the communication between these different sites, and in particular to further define the postendocytic compartment, we have established a dynamic method for following the movement of GLUT4 from the cell surface through these various compartments. GLUT4, bearing an HA epitope in the first exofacial loop (Quon *et al.*, 1994), was expressed in 3T3-L1 adipocytes by using a retroviral vector. This generates modest levels of HA-GLUT4 in adipocytes that are lower than the endogenous GLUT4 levels found in these cells (Shewan *et al.*, 2000). To visualize a sufficient number of HA-GLUT4 molecules at the cell surface by immunofluorescence microscopy, adipocytes were stimulated with insulin before incubation of the cells with the anti-HA antibody. Figure 1 shows a representative experiment where we have characterized the kinetics of endocytosis of surface-labeled HA-GLUT4 during insulin reversal and compared this with the distribution of the total cellular pool of GLUT4 by double labeling with an antibody against the GLUT4 C terminus (Figure 1, right). At zero time, the anti-HA labeling was confined to the cell surface (Figure 1, left), whereas endogenous GLUT4 was found both at the surface and in a perinuclear compartment (Figure 1, right). After 5 min at 37°C HA-GLUT4 could be detected in large punctate structures in the cytoplasm. These structures, which were particularly enriched in the basal part of the cell, also contained the EEA1 (Figure 2). In some cells, we also observed HA-GLUT4 in the perinuclear region after 5 min. However, double labeling with the EEA1 antibody revealed that these structures corresponded to perinuclear early endosomes (our unpublished data). Incubation of the cells for longer times (20–60 min) resulted in transport of labeled GLUT4 to a perinuclear compartment concomitant with a decrease in surface staining. There was a high degree of colocalization in this perinuclear compartment between internalized HA-GLUT4 and the steady-state pool of GLUT4, suggesting that antibody labeled GLUT4 molecules had equilibrated with endogenous GLUT4 by this time (Figure 1, bottom). These data are consistent with previous studies with epitope-tagged GLUT4 (Bogan *et al.*, 2001; Lampson *et al.*, 2001; Palacios *et al.*, 2001) and suggest that the HA-GLUT4 molecule has similar trafficking properties to endogenous GLUT4.

To determine whether the perinuclear GLUT4 compartment corresponded to endosomes, we compared the distribution of GLUT4 with that of EEA1, a marker of early endosomes (Mu *et al.*, 1995), and also to endocytosed Tf, which mainly defines recycling endosomes (Mellman, 1996). After 60 min of internalization, most of the HA-GLUT4 was in EEA1-negative peripheral or perinuclear compartments, although there was some overlap between endocytosed anti-HA and EEA1 (Figure 2). Interestingly, HA-GLUT4 was sometimes present in tubular structures emanating from EEA1-positive endosomes. Because Tf uptake in adipocytes was very low, we infected 3T3-L1 cells with a retrovirus expressing the hTfR. As indicated in Figure 2, the recycling endosomal compartment was readily resolved in these cells by following continuous uptake of Tf-Alexa-488 for 60 min. Like GLUT4, the recycling endosomes were concentrated in the perinuclear region of the cell. Despite this, we were able

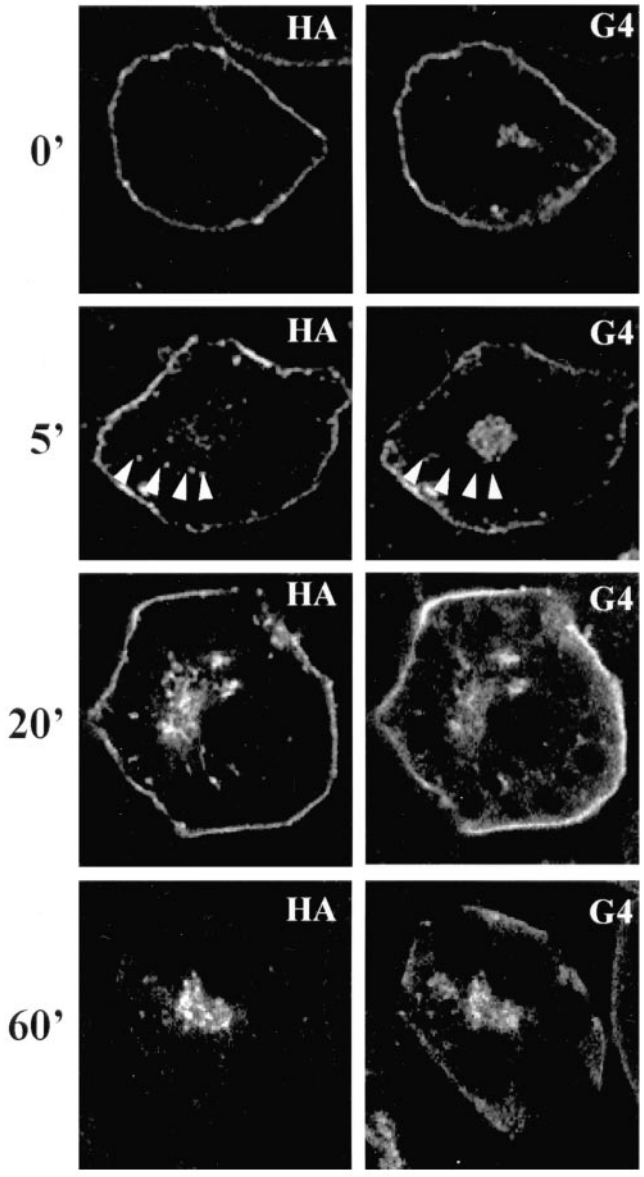

Figure 1. HA-GLUT4 traverses the same organelles as endogenous GLUT4 in 3T3-L1 adipocytes. 3T3-L1 adipocytes expressing HA-GLUT4 were stimulated with insulin, labeled on ice with an anti-HA antibody to label HA-GLUT4 at the cell surface and washed extensively to reverse the effects of insulin as described in MATERIALS AND METHODS. The degree of colocalization of internalized HA-GLUT4 (HA) with endogenous GLUT4 (G4) at various time points after the initiation of endocytosis was determined using confocal microscopy.

to resolve clear differences between GLUT4 and endocytosed Tf in this region. In particular, GLUT4 labeling was much more compact than Tf. To identify additional markers of this perinuclear GLUT4 compartment, we performed colocalization experiments by using antibodies specific for a variety of SNARE proteins. Some SNAREs, such as Syntaxin 7, Syntaxin 13, and GS15, showed poor colocalization with

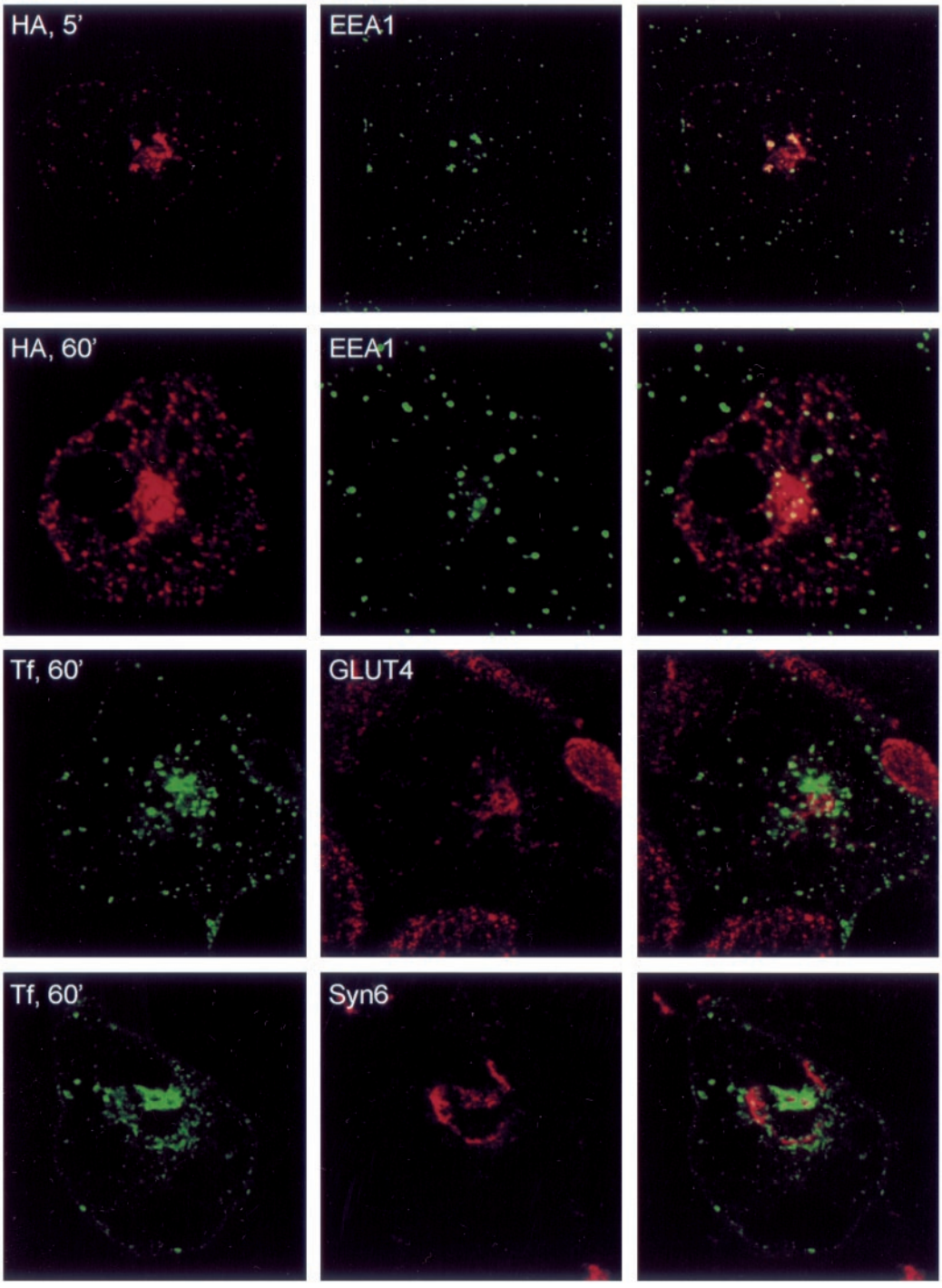

Figure 2. HA-GLUT4 trafficks through early endosomes before reaching a perinuclear compartment. Confluent 3T3-L1 adipocytes expressing either HA-GLUT4 or the hTfR were stimulated with insulin for 20 min. Cells were then either incubated at 4°C with the anti-HA antibody, washed, and chased for either 5 or 60 min at 37°C (HA, 5 min and HA, 60 min, respectively) or incubated with Alexa-488–conjugated transferrin for 60 min at 37°C (Tf, 60 min). Cells were then fixed and double labeled with antibodies specific for either EEA1, endogenous GLUT4, or Syntaxin 6 (Syn6). Note that not all cells in the fields shown were expressing either HA-GLUT4 or the TfR. This confirms the specificity of either HA antibody or transferrin in these experiment.

GLUT4 in this perinuclear region (our unpublished data). Intriguingly, the t-SNARE Syntaxin 6 significantly colocalized with HA-GLUT4 at this location (Figure 3). In addition, like GLUT4, Syntaxin 6 was largely segregated from recycling endosomes and the plasma membrane, as determined by colocalization studies with endocytosed Tf (Figure 2, bottom). These studies are in agreement with previous studies (Watson and Pessin, 2000) showing that Syntaxin 6 is confined to a perinuclear area in adipocytes with low levels on the plasma membrane. Furthermore, HA-GLUT4 also showed a significant level of colocalization with the t-SNARE Syntaxin 16 (Figure 3, bottom). Intriguingly, Syntaxin 16 has been shown to form a complex with Syntaxin 6 in HeLa cells and synaptosomes (Kreykenbohm *et al.*, 2002; Mallard *et al.*, 2002). Interestingly, although Syntaxin 6 has been localized to the TGN in PC12 cells (Bock *et al.*, 1997), we found little overlap between HA-GLUT4 and TGN38, another TGN marker protein (Figure 3). To verify these data, we performed immunoisolation experiments. As expected, IRAP, a protein that shows nearly identical trafficking properties to GLUT4 (Ross *et al.*, 1996), and both Syntaxin 6 and 16 were highly enriched in immunoisolated GLUT4 vesicles, whereas the Golgi SNARE GS15 was not present in GLUT4 containing compartments (Figure 4). These data suggest that GLUT4 constitutively cycles via a perinuclear compartment that is distinct from endosomes and highly enriched in the t-SNAREs Syntaxin 6 and 16. However, these data do not exclude the possibility that GLUT4 transits through recycling endosomes en route to the Syntaxin 6/16-positive compartment.

IRAP Is Transported to the TGN after Internalization at Cell Surface

Syntaxin 6 is highly concentrated in the TGN area in PC12 cells (Bock *et al.*, 1997) and together with the t-SNARE Syntaxin 16 (Mallard *et al.*, 2002) plays an important role in a vesicle transport pathway from endosomes to the TGN. The overlap between GLUT4 and Syntaxins 6 and 16 in adipocytes raised the possibility that the perinuclear GLUT4 compartment may constitute either the TGN or vesicles associated with the TGN. To further explore this possibility we examined the kinetics of IRAP resialylation as an index of the trafficking of GLUT4 via the TGN because this protein has been shown to follow similar trafficking kinetics to GLUT4 (Ross *et al.*, 1996). Cells were treated with insulin to introduce a cohort of IRAP molecules into the surface membrane. Cells were then biotinylated on ice and treated with neuraminidase to remove sialic acid before reincubation for various times at 37°C. The biotinylated molecules were recovered and immunoblotted. IRAP underwent a slight but significant increase in electrophoretic mobility after neuraminidase treatment consistent with a loss of carbohydrate (Figure 5). After 60 min at 37°C, there was a significant reduction in the mobility of IRAP back toward the level observed in cells not incubated with neuraminidase. These data suggest that the cohort of IRAP that was desialylated at the cell surface was resialylated indicative of retrieval back to the TGN. We did not observe significant IRAP resialylation at shorter times (0–30 min), indicating that the kinetics of this process is longer than trafficking of GLUT4 from the cell surface to the Syntaxin 16-positive compartment.

Insulin Stimulates Translocation of Syntaxins 6 and 16 to Cell Surface

The colocalization between GLUT4 and Syntaxin 6 (Figure 3) in the perinuclear region may reflect the presence of both proteins in the intracellular insulin-responsive compartment or in an organelle, such as the TGN, that is involved in the biogenesis of the insulin-responsive compartment. It has previously been shown that not all TGN proteins undergo insulin-responsive movement to the cell surface (Martin *et al.*, 1994). To distinguish between these possibilities, we compared the insulin responsiveness of a variety of t-SNAREs, including Syntaxins 6 and 16, by using a subcellular fractionation approach (Figure 6). As previously shown, we observed a pronounced insulin-dependent movement of GLUT4 to the cell surface. In contrast, insulin did not change the subcellular distribution of TGN38, a protein also enriched in intracellular membranes in the absence of insulin. Strikingly, insulin caused a significant redistribution of Syntaxins 6 and 16 from intracellular membranes to the plasma membrane in adipocytes (Figure 6A). In contrast, we observed no significant effect of insulin on the distribution of Syntaxin 7 (Figure 6A), a late endosomal t-SNARE; Syntaxin 5, an ER-to-Golgi t-SNARE; or GS15, a Golgi t-SNARE (our unpublished data). Insulin caused a slight increase (1.3 $\pm$ 0.3-fold, n = 3) in surface levels of Syntaxin 13, an endosomal t-SNARE involved in recycling of the TfR (Prekeris *et al.*, 1998). However, this effect was quantitatively less than that observed for either Syntaxin 6 or Syntaxin 16 (Figure 6A) and was similar to the 1.5- to 2.0-fold increase reported for the TfR (Hanpeter and James, 1995).

To further confirm these results, we performed a similar study with the plasma membrane lawn assay (Robinson *et al.*, 1992). This technique generates highly purified plasma membranes that are devoid of other organelles (Robinson *et al.*, 1992; Parton *et al.*, 2002). As indicated in Figure 6B, little if any GLUT4 could be detected in fragments from basal adipocytes, whereas the addition of insulin resulted in a striking increase in the labeling intensity of GLUT4. Consistent with the data obtained from our subcellular fractionation studies (Figure 6A), there was a significant increase in cell surface labeling for both Syntaxin 6 and 16 after insulin stimulation (Figure 6B). In contrast, neither Syntaxin 4, a t-SNARE enriched on the plasma membrane that is required for efficient GLUT4 translocation to the plasma membrane upon insulin stimulation, nor Syntaxin 13 exhibited an increase in plasma membrane labeling in response to insulin stimulation. The presence of detectable levels of Syntaxin 13 in the plasma membrane is consistent with previous studies (Chao *et al.*, 1999).

The above-mentioned data raise the possibility that GLUT4 and Syntaxin 6 are transported to the cell surface in the same transport vesicles. To test this, we examined the time course of GLUT4 and Syntaxin 6 translocation to the cell surface, reasoning that if different carriers are involved it may be possible to segregate them temporally. We found that the kinetics of Syntaxin 6 translocation to the cell surface were identical to those of GLUT4 (Figure 6C), suggesting that GLUT4 and Syntaxin 6 are transported in the same vesicle to the plasma membrane upon insulin stimulation.

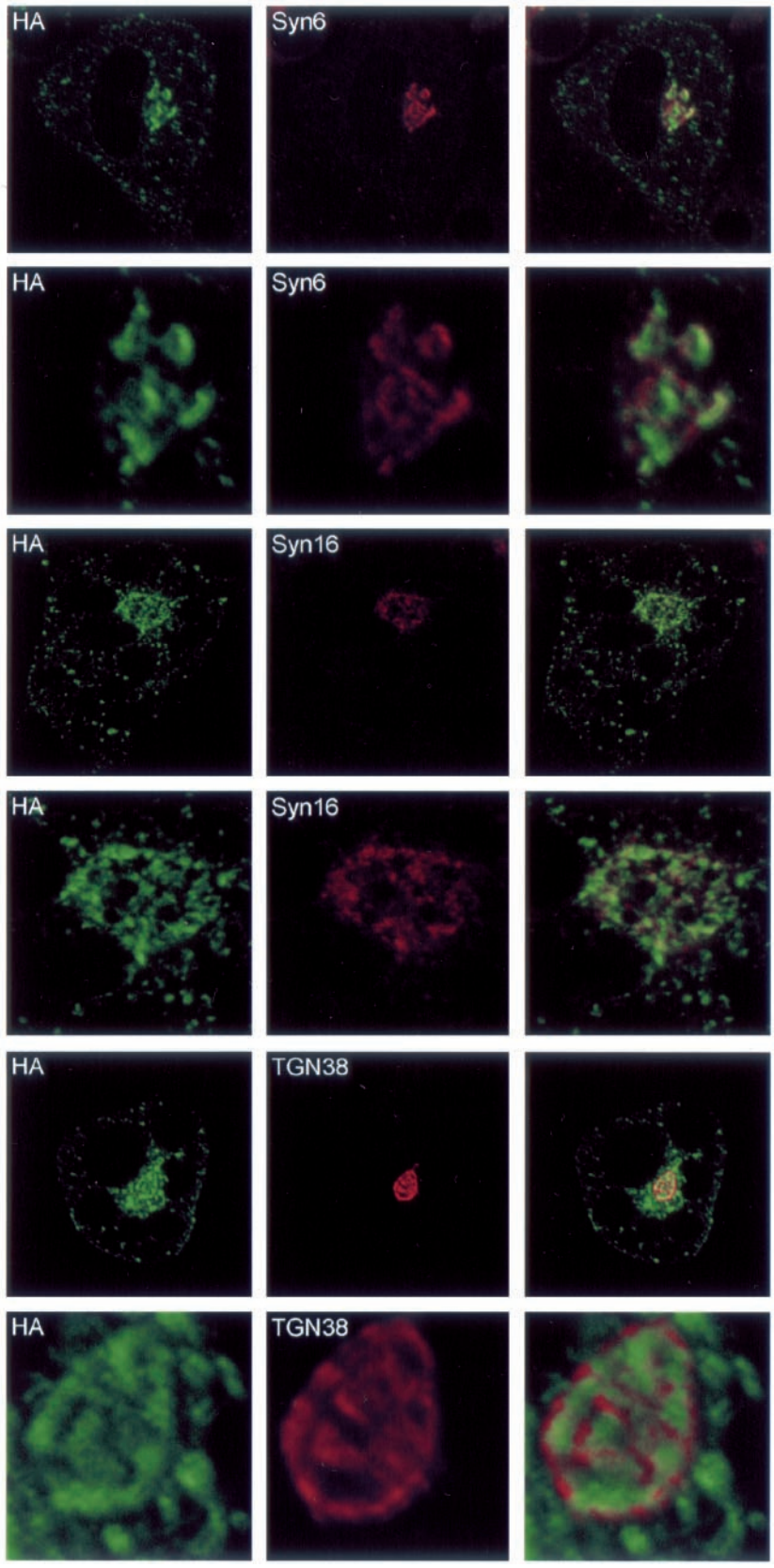

Figure 3. HA-GLUT4 cycles through a perinuclear Syntaxin 6/16-positive compartment. 3T3-L1 adipocytes expressing HA-GLUT4 were stimulated with insulin for 20 min. Cells were then chilled down and incubated with anti-HA antibody, washed, and chased for 60 min at 37°C (HA). Cells were then fixed and double labeled with antibodies specific for either Syntaxin 6 (Syn6), Syntaxin 16 (Syn16), or TGN38.

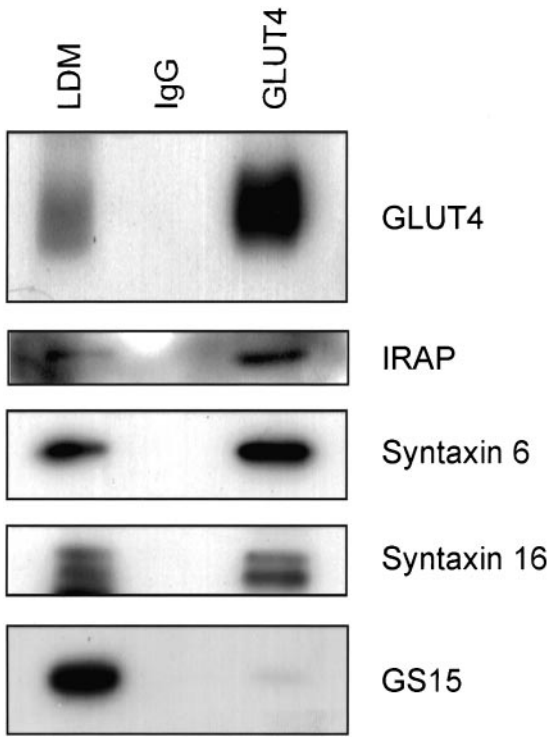

Figure 4. Syntaxins 6 and 16 are enriched in GLUT4 vesicles. GLUT4 antibody and nonspecific IgG-coupled beads were incubated with LDMs from 3T3-L1 adipocytes, washed and eluted with SDS sample buffer. LDMs and bound proteins were immunoblotted with antibodies specific for GLUT4, IRAP, Syntaxin 6, Syntaxin 16, and GS15.

Syntaxin 6 and 16 Interact in Adipocytes and Are Up-regulated during Adipocyte Differentiation

Syntaxin 6 and Syntaxin 16 have recently been shown to form a SNARE complex in HeLa cells and synaptosomes (Kreykenbohm *et al.*, 2002; Mallard *et al.*, 2002). This complex seems to play an important role in trafficking between endosomes and the TGN. As shown in Figure 7, Syntaxin 6 and Syntaxin 16 also form a stable complex in 3T3-L1 adipocytes.

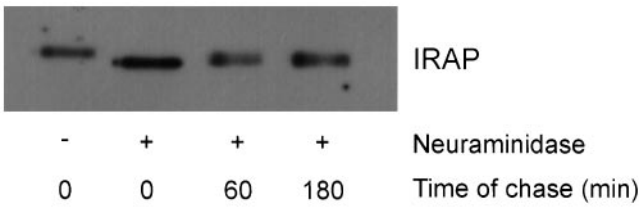

Figure 5. IRAP is transported to the TGN after internalization. Adipocytes were incubated with insulin for 20 min and biotinylated on ice. Cells were then incubated with neuraminidase on ice and reincubated at 37°C for either 60 or 180 min. Solubilized lysates were incubated with streptavidin beads to recover biotinylated proteins which were subjected to SDS-PAGE followed by immunoblotting with antibodies specific for IRAP.

This did not seem to be a nonspecific association, because we could not detect any Syntaxin 4 (Figure 7A) or SNAP-23 (our unpublished data) in the Syntaxin 6-containing complexes. One possibility is that Syntaxins 6 and 16 play an integral role in the intracellular sequestration of GLUT4 in adipocytes. Indeed, Syntaxin 6 and Syntaxin 16 may be involved in the biogenesis of insulin-responsive GLUT4-containing vesicles. The characteristic ability of 3T3-L1 cells to form an insulin responsive GLUT4 compartment is markedly up-regulated soon after adipocyte differentiation (El-Jack *et al.*, 1999), suggesting that the machinery required for the biogenesis of this compartment might be specifically up-regulated in these cells. Strikingly, Syntaxin 6 and Syntaxin 16 levels were increased by 2.7 $\pm$ 0.6- and 4.6 $\pm$ 2.1-fold (n = 4 $\pm$ SEM), respectively, upon differentiation of fibroblasts into adipocytes (Figure 7B). We also noted a slight increase in the levels of Syntaxin 13 after adipocyte differentiation (2.1 $\pm$ 1.1, n = 3). In contrast, the levels of a number of other t-SNAREs was either unchanged (Syntaxin 4, 0.9 $\pm$ 0.1; Syntaxin 5, 0.8 $\pm$ 0.2) or decreased (Syntaxin 7, 0.6 $\pm$ 0.2).

Endosomal Sorting of GLUT4 Is Regulated by a C-Terminal Acidic Motif

We have previously characterized an endosomal targeting motif in the carboxyl-terminal tail of GLUT4 (Shewan *et al.*, 2000). This motif comprises the residues TELEYLGP. We hypothesized that this domain may be involved in the trafficking of GLUT4 between endosomes and the Syntaxin 6/16-positive compartment. To further test this hypothesis, we performed parallel studies to those described in Figures 1, 2, and 3 in cells expressing a HA-tagged GLUT4 molecule in which the C-terminal 12 amino acids had been replaced with those of GLUT3 (HA-TAIL). Consistent with the data shown in Figure 2, after 60 min of endocytosis, HA-GLUT4 accumulated in the perinuclear region and there was limited overlap with the endosomal marker EEA1 (Figure 8). In contrast, HA-TAIL still showed overlap with EEA1, even after 60 min of endocytosis. Endocytosed HA-TAIL was also detected in perinuclear structures that did not overlap with EEA1, however, arguing against a complete block in exit from EEA1-positive early endosomes. To determine whether the perinuclear labeling that was observed for the TAIL mutant represents the bona fide GLUT4 compartment, we assessed the colocalization of HA-TAIL and Syntaxin 16 after a 60-min endocytosis regime. Surprisingly, although HA-GLUT4 showed considerable overlap with Syntaxin 16 in the perinuclear region (Figure 9, top, and 3 middle), HA-TAIL showed very little colocalization in the perinuclear region with Syntaxin 16 (Figure 9, middle) or Syntaxin 6 (our unpublished data).

We have previously mapped the essential residues in the C-terminal GLUT4 targeting motif to amino acids 498–505 (TELEYLGP). Herein, we have undertaken a more detailed analysis of this region. A mutant, in which residues E_{499}, E_{501}, and Y_{502} were mutated to A (HA-EXEY), had a similar phenotype to HA-TAIL in that it showed increased retention in early endosomes subsequent to its endocytosis for 60 min (Figure 8). More importantly, like TAIL, there was little overlap between HA-EXEY, after a 60-min uptake, and Syntaxin 16 (Figure 9, bottom). These data suggest that the acidic motif in the GLUT4 C terminus regulates transport between endosomes and the TGN.

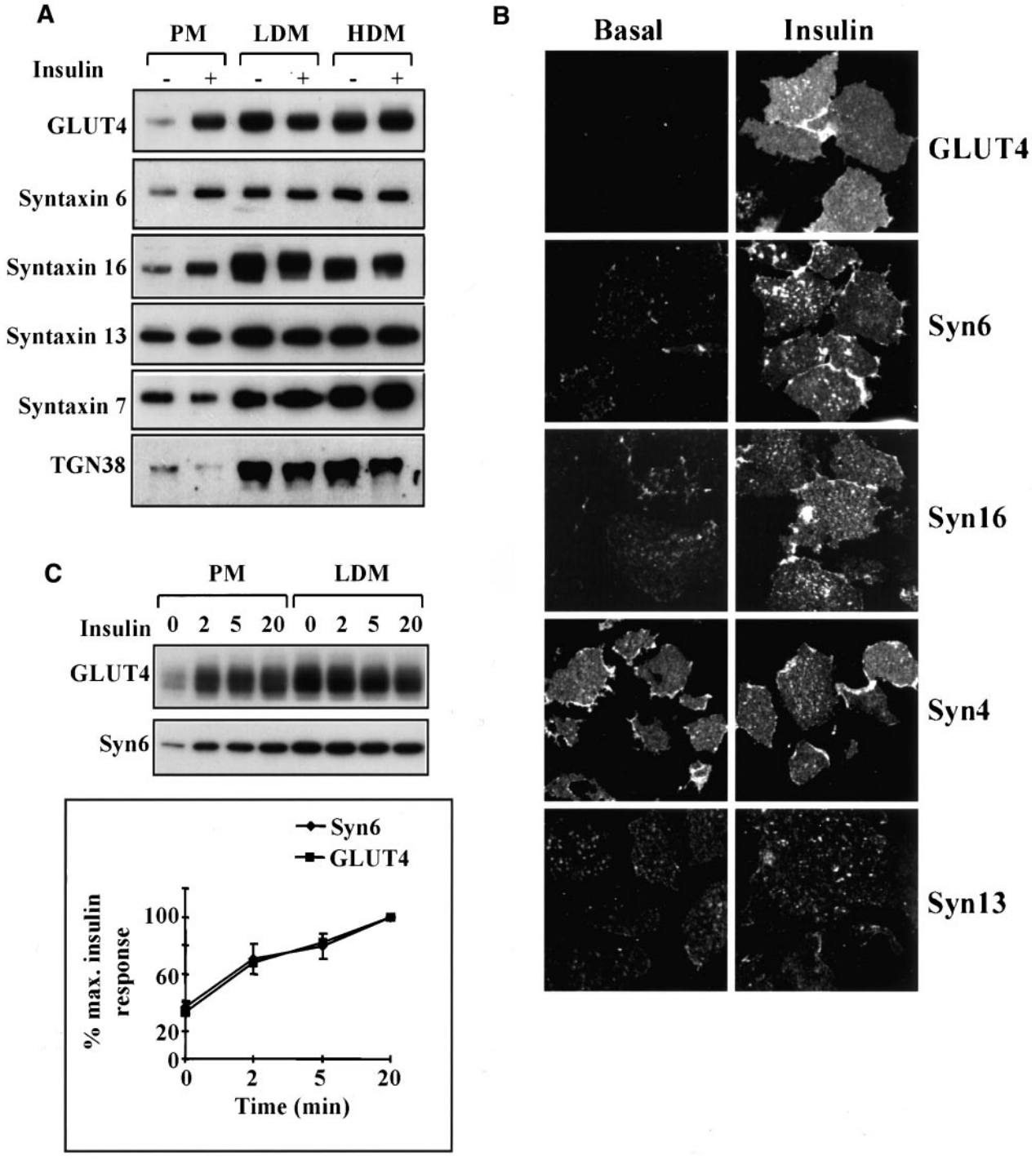

Figure 6. Insulin stimulates the translocation of Syntaxin 6 and Syntaxin 16 to the cell surface in 3T3-L1 adipocytes. (A) 3T3-L1 adipocytes were incubated in the absence (−) or presence of insulin (+) for 20 min. Cells were then fractionated into PMs, LDMs, and HDMs as described in MATERIALS AND METHODS. Aliquots of each fraction (10 μg of protein) were then immunoblotted with antibodies specific for GLUT4; TGN38; and Syntaxins 6, 7, 13, and 16. (B) Adipocytes were grown on glass coverslips and incubated with or without insulin for 20 min. Cells were then sonicated as described in MATERIALS AND METHODS and fixed yielding a lawn of plasma membrane fragments attached to the glass coverslip. These fragments were then labeled with antibodies specific for GLUT4 or Syntaxins 4, 6, 13, or 16. Labeling was visualized by confocal immunofluorescence microscopy. (C) Adipocytes were incubated with insulin for different periods of time at 37°C (0–20 min) and fractionated into PMs and intracellular membranes (LDMs). Fractions (10 μg of protein) were immunoblotted with antibodies specific for either GLUT4 or Syntaxin 6. Immunoblots were quantified using densitometry. Data are mean ± SEM, n = 3.

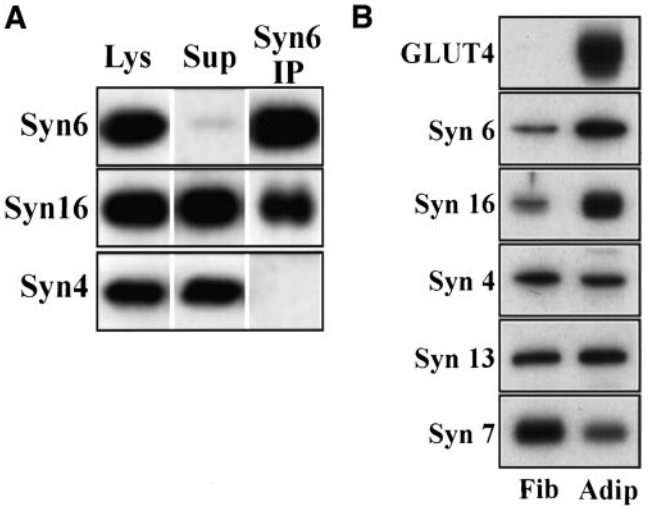

Figure 7. Syntaxin 6 and 16 form a complex in adipocytes and are up-regulated during adipocyte differentiation. (A) 3T3-L1 adipocyte lysates were solubilized in 1% TX-100. Antibodies specific for Syntaxin 6 were used to immunoprecipitate protein complexes from the soluble fraction. After incubation at 95°C for 5 min, immunoblot analysis was used to determine the amount of Syntaxins 4, 6, and 16 present in the lysate (Lys), unbound fraction (Sup), and in the bound fraction (Syn6 IP). (B) Total membrane fractions were prepared from 3T3-L1 fibroblasts (80% confluent) (Fib) and 3T3-L1 adipocytes (day 8 postdifferentiated) (Adip), and immunoblot analysis was used to determine the levels of GLUT4 and Syntaxins 4, 6, 7, 13, and 16 in equivalent amounts of these fractions (5 μg of protein).

DISCUSSION

In this study, we have made several novel observations pertinent to the insulin-regulated trafficking of GLUT4 in adipocytes. First, we have shown that subsequent to its endocytosis and entry into the endosomal system, GLUT4 diverges from other recycling molecules, such as Tf, and is selectively transported back to a subdomain of the TGN that is enriched in the t-SNAREs Syntaxins 16 and 6. Second, we have shown that the transport of GLUT4 between endosomes and the TGN is regulated via an acidic targeting motif in the carboxyl tail of GLUT4. Third, we provide indirect evidence implicating a role for Syntaxins 6 and 16 in GLUT4 trafficking. These t-SNAREs, unlike other t-SNAREs, are translocated to the cell surface in response to insulin, their expression is markedly increased upon adipocyte differentiation, they colocalize with GLUT4, and they form a stable complex in adipocytes.

GLUT4 Recycles via a Subdomain of TGN

Although the majority of GLUT4 is found in small tubulovesicular elements in muscle and adipocytes, a finite pool (10–15%) is also located in the TGN (Bryant *et al.*, 2002). This definition is based upon immunoelectron microscopy localization studies, which find a significant pool of GLUT4 in a tubulovesicular compartment adjacent to the Golgi. In the present study, we provide evidence that GLUT4 recycles via a perinuclear compartment that has a number of characteristics consistent with it being a subdomain of the TGN. Although this compartment is morphologically distinct from the highly tubular TGN38 compartment, it is often immediately adjacent to the latter compartment (Figure 3). This is in agreement with previous studies showing that there is little colocalization between TGN38 and GLUT4 in adipocytes (Martin *et al.*, 1994). In addition, both Syntaxins 6 and 16 are thought to function in the TGN acting as t-SNAREs for transport vesicles arriving from the endosomal system (Mallard *et al.*, 1998). Thus, the significant overlap between GLUT4 and these t-SNAREs in the perinuclear region is consistent with this representing the TGN. Although the TGN was originally defined as the sorting and exit site of the Golgi, its structure of cisternae and tubulovesicular elements has been poorly defined. Three-dimensional electron microscopic analysis of the Golgi has recently led to the formulation of a model in which molecules are sorted in the secretory pathway in multiple cisternae of the TGN, each of which served as an exit site (Ladinsky *et al.*, 2002). This suggests that *trans*-cisternae and tubulovesicular elements may be separate but interconnected. Likewise, entry sites into different subdomains of the TGN are feasible and may explain the discrepancy we find in kinetics between entry of HA-GLUT4 in the Syntaxin 16-positive compartment and resialylation of IRAP. The majority of TGN38 and sialyl transferase is located in the *trans*-cisternae of the Golgi in other cell types (Bennett and O'Shaughnessy, 1981; Roth *et al.*, 1985; Ladinsky and Howell, 1992; Ladinsky *et al.*, 2002), whereas a significant pool of GLUT4 in the TGN area colocalizes with other TGN recycling proteins, such as the cation-dependent mannose 6-phosphate receptor (Martin *et al.*, 2000a). It may thus be that the Syntaxin 6/16-positive compartment is a specialized compartment that has arisen from the TGN. Consistent with such a model are our previous studies in atrial cardiomyocytes (Slot *et al.*, 1997). This is an unusual cell that possesses both a regulated secretory pathway and an insulin-responsive glucose transport system. A considerable proportion (~60%) of GLUT4 is localized to secretory granules in these cells (Slot *et al.*, 1997). The GLUT4 trafficking and the regulated secretory pathways seem to merge in the TGN, again suggesting that the flux of GLUT4 through this pathway is considerable. Intriguingly, Syntaxin 6 is also found in regulated secretory granules where it plays a role in granule maturation (Wendler *et al.*, 2001). Conversely, it has been reported that during granule maturation Syntaxin 6 is removed from immature granules by AP-1/clathrin-coated vesicles and then delivered to endosomes (Klumperman *et al.*, 1998). Interestingly, GLUT4 is also found in clathrin-coated AP-1–positive vesicles within the TGN area (Gillingham *et al.*, 1999; Martin *et al.*, 2000b), suggesting that GLUT4 is rapidly cycling between the TGN and endosomes in basal adipocytes, which may call into question the idea of a stable intracellular storage compartment. Based on this evidence, we speculate that GLUT4 may be retained in a compartment that is generated from the TGN and that is possibly analogous to immature secretory granules in secretory cells. The t-SNARES Syntaxin 6 and 16 may play an integral role in the maturation of this compartment in adipocytes and we are currently in the process of testing this hypothesis.

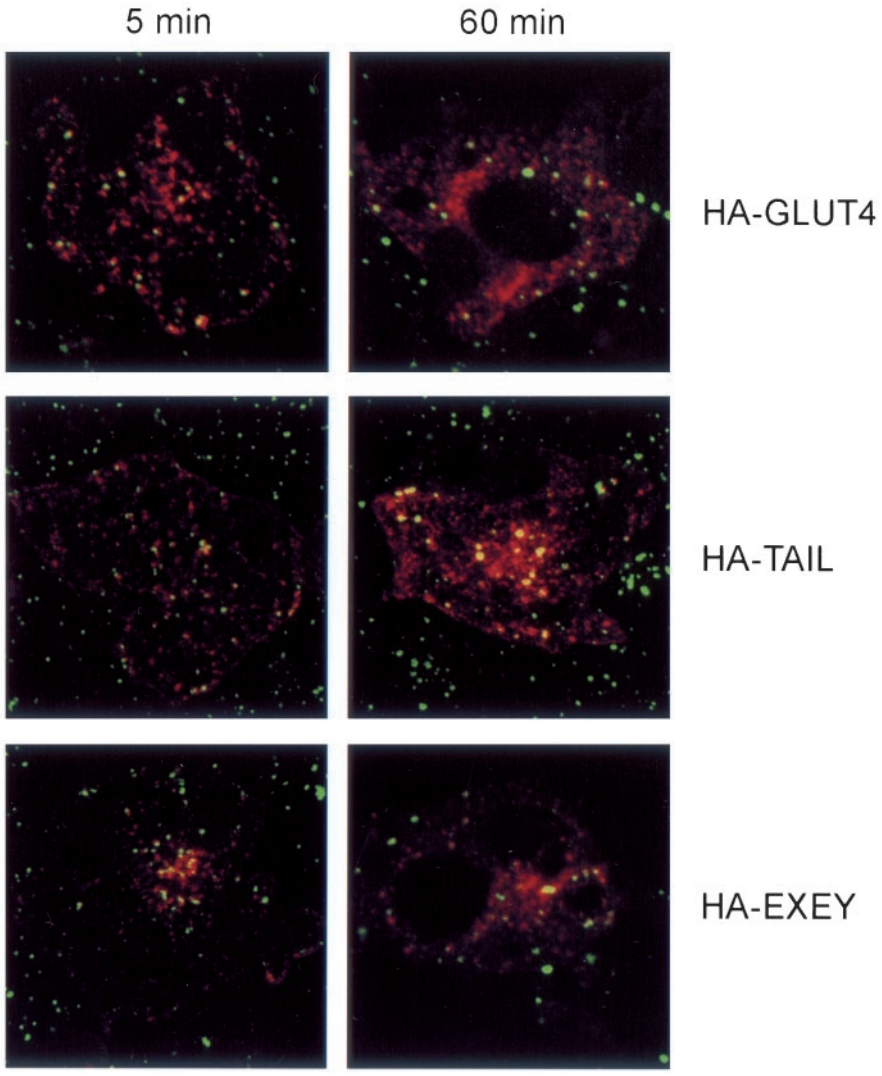

Figure 8. An acidic targeting motif is required for endosomal trafficking of GLUT4. 3T3-L1 adipocytes expressing either HA-GLUT4 or C-terminal mutants, HA-TAIL and HA-EXEY, were treated with insulin before labeling surface accessible recombinant transporter on ice with anti-HA. After either 5 or 60 min of endocytosis cells were fixed and double-labeled with an antibody specific for EEA1 to label early endosomes. HA was visualized by anti-mouse-Alexa-594 (red), EEA1 was visualized by anti-human-ALEXA488 (green). Merged images are shown.

GLUT4 Contains an Acidic C-Terminal Targeting Motif

Several studies have shown that GLUT4 is segregated from constitutively recycling proteins such as the TfR in early endosomes into a separate compartment(s) (Martin *et al.*, 1996; Lampson *et al.*, 2001; Lim *et al.*, 2001; Palacios *et al.*, 2001). The entry of GLUT4 into endosomal carrier vesicles, that exclude the TfR, is facilitated at least in part by the carboxy terminus of GLUT4 (Lim *et al.*, 2001). This is in line with the present study where we have shown that mutation of two acidic residues in the carboxy terminus of GLUT4 perturbs the trafficking of GLUT4 between endosomes and the perinuclear Syntaxin 6/16-positive compartment. The morphological data presented herein (Figures 7 and 8) together with previous studies with endosomal ablation (Shewan *et al.*, 2000) suggest that the GLUT4 TAIL mutant is predominantly found in endosomes. Hence, these data suggest that the C-terminal acidic targeting motif either regulates retention in the Syntaxin 6/16-positive compartment or exit of GLUT4 from endosomes. Future studies will be required to distinguish between these possibilities.

This acidic targeting motif seems to have no role in GLUT4 endocytosis. The rate of uptake of surface labeled HA-GLUT4 and HA-TAIL into the cell interior seemed indistinguishable by using immunofluorescence (Figure 8). We have obtained similar data by using a biochemical internalization assay (Govers and James, unpublished data). GLUT4 also contains two additional targeting signals; an aromatic amino acid based motif (FQQI) in the N terminus, and a dileucine in the C terminus. Both of these signals have been shown to play a role in regulating GLUT4 endocytosis (Piper *et al.*, 1993; Garippa *et al.*, 1994, 1996; Marsh *et al.*, 1995; Verhey *et al.*, 1995). The FQQI motif has also been shown to regulate trafficking of GLUT4 to the perinuclear GLUT4 storage compartment in fibroblasts (Palacios *et al.*, 2001). In our hands, a GLUT4 mutant in which F_5 is mutated into A is significantly delayed in its internalization from the cell surface but eventually this mutant does reach the Syntaxin

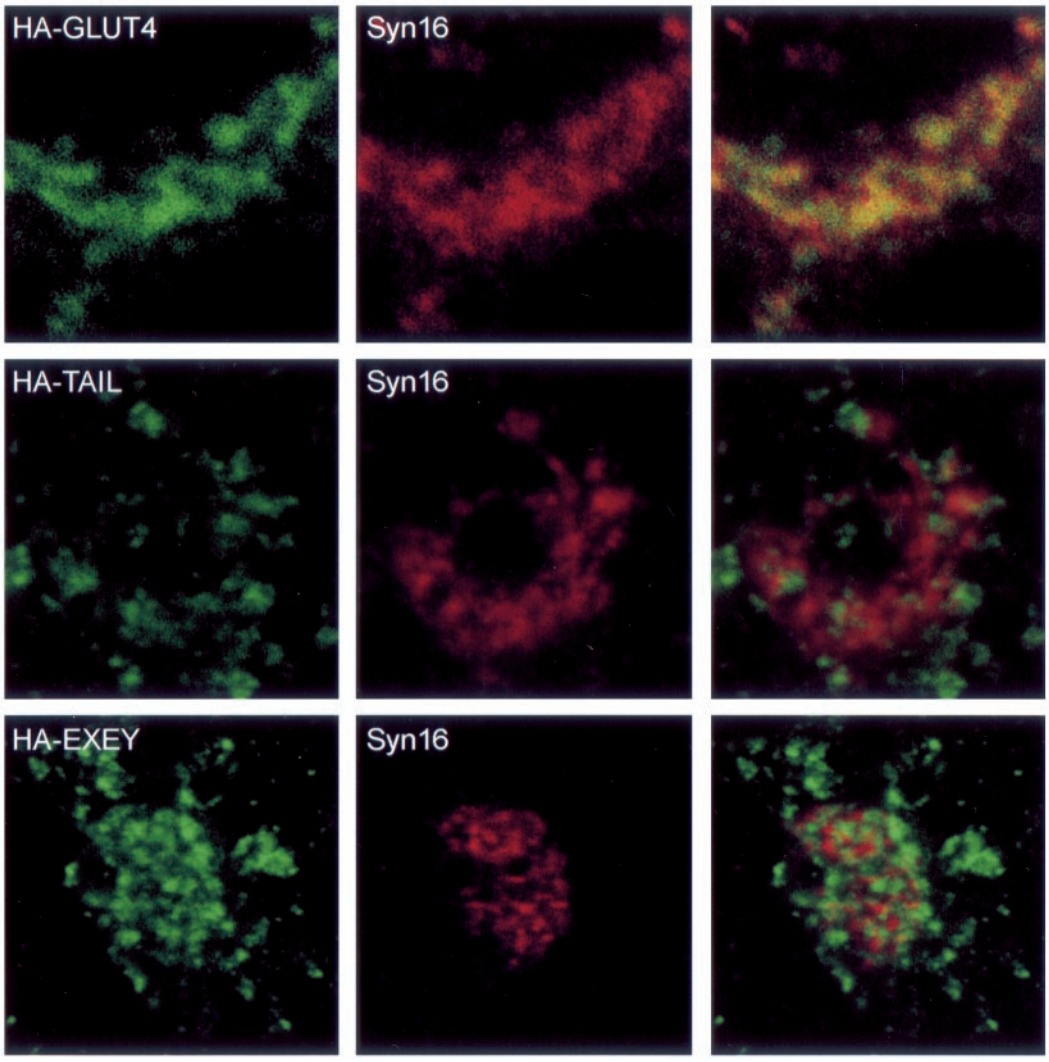

Figure 9. Targeting of GLUT4 to the Syntaxin 16 compartment is mediated by an acidic targeting domain in its C-terminal tail. 3T3-L1 adipocytes expressing either HA-GLUT4 or C terminal mutants, HA-TAIL and HA-EXEY, were treated with insulin before labeling surface accessible recombinant transporter on ice with anti-HA. After 60 min of endocytosis cells were fixed and double labeled with an antibody specific for Syntaxin 16. High magnification images of the perinuclear area are shown.

6-positive perinuclear compartment (Shewan and James, unpublished data). Moreover, we have not observed any evidence for the mistargeting of either HA-TAIL or HA-EXEY to lysosomes in the present studies (our unpublished data).

Role of TGN in GLUT4 Recycling

A model has been proposed in which GLUT4 may constitutively cycle between endosomes and the TGN in the basal state (Bryant *et al.*, 2002). A major question that stems from the present studies is what is the function of the endosome to TGN trafficking pathway for GLUT4? One possibility is that the TGN is involved in the biogenesis of the insulin-responsive exocytic GLUT4 vesicles. If this is the case, then it is unclear why the GLUT4 TAIL mutant, which is defective in endosome to TGN transport, retains insulin responsiveness (Shewan *et al.*, 2000). One possibility is that at steady state sufficient HA-TAIL may traffic into the insulin-responsive compartment to generate the acute insulin response. Alternatively, the major function of the TGN/endosome pathway may be to prevent recycling of GLUT4 via the cell surface. In this instance, the major insulin effect may be in endosomes. It is noteworthy that McGraw and colleagues have described two unique transport features for GLUT4 and IRAP: endosomal retention and sorting into a separate compartment (Zeigerer *et al.*, 2002). The latter may represent TGN recycling as reported herein. In this regard, mutagenesis of the C-terminal targeting motif EXEY may not disrupt endosomal retention and this may compensate for loss of TGN recycling. Detailed studies involving the GLUT4

TAIL mutant and Syntaxin 6/16 mutants should be instructive in distinguishing between these and other possibilities.

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