INTRODUCTION

The trans-Golgi network (TGN) is the major site where the proteins transported through the Golgi complex are sorted. Machineries operating at this compartment react with transport motifs contained in the arriving proteins and sort them to different destinations (Griffiths and Simons, 1986; Traub and Kornfeld, 1997). Among the membrane proteins sorted at the TGN of adipocytes and muscle cells is the insulin-sensitive glucose transporter GLUT4. In the absence of insulin stimulation the bulk of the intracellular GLUT4 is stored in tubulovesicular structures adjacent to the Golgi complex (GSC, for GLUT4 storage compartment) and to a lesser extent in vesicles scattered throughout the cytoplasm (Slot et al., 1991a,b, 1997; Rodnick et al., 1992; Ralston and Ploug, 1996). It is not clear if the presence of GLUT4 in scattered vesicles reflects its continuous cycling (i.e. transport) between the GSC and the plasma membrane or if these vesicles are used as a separate reservoir (Yeh et al., 1995; Araki et al., 1996). Stimulation of cells with insulin results in translocation of GLUT4 from the intracellular reservoir(s) to the plasma membrane whereas removal of insulin results in its intracellular sequestration.

It is important to note that the intracellular distribution of GLUT4 in non-stimulated adipocytes and muscle cells and in transfected cells of different origin is similar. This similarity suggests the existence of universal mechanisms of transport and distribution (Haney et al., 1991). The nature of these mechanisms is, however, poorly understood. Dileucine and phenylalanine-based transport motifs have been localized to the carboxyl-terminal (Leu489 Leu 490 )-based motif and its release from the GSC involves Tyr 502 and the adjacent carboxyl-terminal Pro 505 AspGluAsnAsp 509 sequence.

MATERIALS AND METHODS

Cell culture

Stably transfected clonal 3T3-L1 fibroblasts were grown on plastic
dishes or glass coverslips and cultured in DMEM supplemented with 10% foetal calf serum (FCS, Biological Industries, Kibbutz Beit Haemek, Israel), 4 mM glutamine, 50 mg/ml gentamicin, 100 mg/ml streptomycin, 100 i.u./ml penicillin, non essential amino acids (normal medium) and 5-7.5 µg/ml puromycin, in a 37°C humidified CO_2 incubator. 3T3-L1 fibroblasts were differentiated into adipocytes as reported (Frost and Lane, 1985). Fusion of L6 rat myoblasts was induced in normal medium supplemented with 1% FCS. COS-7 cells were cultured in normal medium.

DNA constructs
Wild-type rat GLUT4 cDNA cloned into pUC119 was epitope-tagged by introducing the sequence YPYDVPDYADD (HA tag) in its major exofacial loop after Thr78 (Czech et al., 1993). All the mutants were made by site-directed mutagenesis on HA epitope-tagged GLUT4 cDNA cloned into the phage M13mp19 as described (Kunkel, 1985; see Fig. 1A). For transient transfections the cDNAs were cloned into the pcEXV-3 vector (Müller and Germain, 1986). For development of stably transfected clonal 3T3-L1 fibroblasts the cDNAs were cloned into the pPUR vector (Clontech Lab., Inc. USA) modified by cloning the SFFVLTR promoter into the ApaI/EcoRI sites. Clonal 3T3-L1 cells stably expressing GLUT4 were transiently transfected with GLUT4D5 cloned into the pcDNA3 vector (Invitrogen, The Netherlands).

Development of stable transfectants
3T3-L1 fibroblasts were transfected with native GLUT4, or with GLUT4 or GLUT4D5 tagged with the HA epitope and carried in the pPUR vector. The transfections were performed using the calcium phosphate precipitation method (Gorman, 1985). 48 hours after the transfections the selection of the stable transfectants was started with the addition of 5 µg/ml puromycin to the culture medium. Two weeks later the grown clones were isolated, expanded and studied by immunofluorescence (IMF) microscopy. Positive clones were kept thereafter in medium supplemented with 5-7.5 µg/ml puromycin. The studies described here were performed with clones 4, 9 and 7, which expressed GLUT4, HA tagged GLUT4 and HA tagged GLUT4D5, respectively.

Transient transfections
COS-7 cells and 3T3-L1 fibroblasts were transiently transfected with cDNAs cloned into the pcEXV-3 vector (100-200 ng) using the DEAE-dextran/DMSO procedure (Lopata et al., 1984) and the calcium-phosphate precipitation procedure, respectively. Unless indicated the cells were transfected only for 18 hours, to avoid the overexpression of the foreign proteins and the saturation of the sorting mechanisms. To remove the transiently transfected proteins from the secretory pathway the cells were incubated for the last three hours of the transfection with 0.1 mM cycloheximide.

Antibodies
The mouse monoclonal (mAb) 1F8 (James et al., 1988) and the polyclonal (pAb) OSCR6 antibodies (Camps et al., 1994), both raised against the C-tail of GLUT4 (a gift from Dr A. Zorzano, Universitat de Barcelona) reacted with wild-type GLUT4 but neither raised against synthetic peptides as described (Chavrier et al., 1990).

Immunofluorescence microscopy studies
COS-7 cells and rat L6 myotubes were fixed-permeabilized for 2 minutes with cold (−20°C) methanol. 3T3-L1 fibroblasts and 3T3-L1 adipocytes were fixed with 3% PFA in PBS for 10 minutes at room temperature, and then washed and incubated for 30 minutes at room temperature with 0.1 M NH_4Cl in PBS before their permeabilization with cold (−20°C) methanol. Staining of cells was performed as described before (Barrioconal et al., 1986). Second fluorochrome-conjugated antibodies were from Cappel (Durham, NC). Cells mounted on glass slides using Gelvatol (Montsanto Co., St Louis, MO) were studied with an Axiosvert 135M microscope (Zeiss) and photographed through fluorescence.

GLUT4 metabolic labeling and immunoprecipitation
To label metabolically GLUT4 and GLUT4D5, 3T3-L1 fibroblasts grown to 80% confluence in 60 mm dishes were washed and cultured for 90 minutes with methionine/cysteine-free DMEM supplemented with 2% dialysed serum, then labeled for 1 hour with 0.5 mg/ml methionine/cysteine (specific activity >1000 Ci/mM; PRO-MIX, Amersham Corp.) and the labeled proteins chased for different time periods in regular medium. The cells were extracted with 3 ml of cold 0.1 M Na_2CO_3 for 30 minutes and the membranes collected by centrifugation at 150,000 g at 4°C for 30 minutes and solubilized with 0.4 ml of cold 20 mM Tris-HCl, pH 7.4, 125 mM NaCl, 2 mM EDTA, 1 mM MgCl_2 (buffer B) containing 1% Triton X-100 and 60 mM octylglucoside for 1 hour. After removal of the insoluble material by centrifugation and dilution of the supernatants with one volume of buffer B containing 2% ovalbumin, the solubilized proteins were incubated for 3 hours at 4°C with 1 µl of the monoclonal anti-HA antibody, then for 3 hours at 4°C with 5 µl Protein G-Sepharose (Pharmacia Biotech), and the immunoprecipitates heated for 10 minutes at 37°C in Laemmli buffer (Laemmli, 1970) supplemented with 5% SDS, resolved by 12% SDS-PAGE and the gels treated with 1 M sodium salicylate analyzed by fluorography.

Cell fractionation studies
3T3-L1 fibroblasts grown to 70% confluence on 60 mm plastic dishes were washed twice with PBS, scraped with a rubber policeman in 3 ml of cold HES buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 0.25 M sucrose,) then disrupted by N_2 cavitation (2,400 kPa/10 minutes) and centrifuged at 108,000 g for 20 minutes. All the operations were performed at 4°C. The supernatant was centrifuged at 180,000 g for 90 minutes to yield a fraction enriched in light density microsomes (LDM; Piper et al., 1991) whereas the pellet was resuspended in HES buffer, layered onto a cushion of 35% sucrose prepared in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and centrifuged at 108,000 g for 1 hour using a SW40 rotor to produce a fraction enriched in plasma membrane (PM) at the interface. The PM fraction was then diluted tenfold in 20 mM Tris, 2 mM EDTA, treated with 0.1 mM PMSF, and after removal of the insoluble material by centrifugation at 150,000 g for 30 minutes (Piper et al., 1991). Both LDM and PM pellets were extracted for 1 hour with 100 µl of 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, treated with 0.1 mM PMSF, and after removal of the insoluble material by centrifugation at 150,000 g for 30 minutes, processed and resolved by SDS-PAGE as described above and their content in GLUT4 and GLUT4D5 studied by western blot using the anti-HA 16B12 mAb with the ECL procedure (Amersham Life Science).

RESULTS

Truncation of the last five carboxyl-residues abrogates the retention of GLUT4 in the GSC
GLUT4 displays an acidic DEND cluster (Bannykh et al., 1998) at the carboxyl-end, fifteen residues downstream the
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Fig. 1. GLUT4 mutants. GLUT4Δ5 was developed by deleting the COOH-Pro505AspGluAsnAsp509 peptide. Mutated residues, named by the one letter code, and the HA epitope-tag inserted into the exofacial loop (black box), are shown in position in diagram of GLUT4 (A). GLUT4 levels in stably transfected 3T3-L1 fibroblasts and adipocytes (A). Western blot analysis of extracts from untransfected 3T3-L1 fibroblasts and adipocytes (A). GLUT4 levels in stably transfected (black box), are shown in position in diagram of GLUT4 and GLUT4Δ5 (Fig. 1A). This was first studied in clonal 3T3-L1 fibroblasts stably expressing GLUT4 or GLUT4Δ5 were grown to 80% confluence in a 50 mm dish then incubated in methionine/cysteine-free DMEM/10% dialyzed FCS for 90 minutes and labeled for 1 hour with 0.5 μCi/ml [35S]methionine/cysteine (specific activity >1000 Ci/mM) before their chase in regular medium for the indicated time periods.

Extracts were prepared as described in Materials and Methods and immunoprecipitated using the mouse anti-HA16B12 mAb. Autoradiograms (left panel) were scanned and the density of the GLUT4 (○) and GLUT4Δ5 (■) bands plotted against the chase periods (right panel) (C).

With regard to the cellular levels of GLUT4 and GLUT4Δ5 in clones 9 and 7, respectively, it is worthwhile to note that the levels of GLUT4 were comparable to those in rat fat pad cells while they were lower than in 3T3-L1 adipocytes differentiated ‘in vitro’, as shown by western blot analysis using the anti-GLUT4 pAb OSCR6 (Fig. 1B). The lack of reactivity of GLUT4Δ5 blotted onto nitrocellulose with the OSCR6 antibody precluded the comparison of its cellular levels with those of GLUT4 by western blot using the same antibody. Their levels were compared by measuring the amount of newly synthesized proteins (Fig. 2C). For these purpose the two proteins labeled for 1 hour with [35S]methionine/cysteine were immunoprecipitated with the anti-HA mAb. Their study showed that clones 9 and 7 synthesized comparable amounts of GLUT4 and GLUT4Δ5 during the 1 hour labeling period (Fig. 1C, left panel). Chasing of the labeled proteins for different time periods revealed, however, that the turnover of GLUT4Δ5 was 2.5 times faster (Fig. 1C, right panel).

To compare the cellular distributions of GLUT4 and GLUT4Δ5, 3T3-L1 fibroblasts from clones 9 and 7 were fixed-permeabilized with methanol and studied by immunofluorescence microscopy. In fibroblasts stained with pAb OSCR6 the bulk of GLUT4 was localized to a reticular structure in the vicinity of the nucleus as well as to numerous punctate structures scattered throughout the cytoplasm (Fig. 2A). Identical patterns of staining were observed with the anti-GLUT4 mAb 1F8 (Fig. 2K) and the anti-HA mAb 16B12 (not shown). Moreover, double-staining of the cells for GLUT4 and the marker of the stack of Golgi cisternae GMPc-1 (Yuan et al., 1987) showed that the two proteins were in juxtaposed but distinct perinuclear structures with similar morphologies (Fig. 2B-D). Henceforth, we shall call the perinuclear compartment that hosts GLUT4, GSC, for GLUT4 storage compartment. The localization and morphology of the GSC in 3T3-L1 adipocytes were similar to those described in fibroblasts (Fig. 2E,F) whereas in transiently transfected COS-7 cells (see below Fig. 4A,B) the GSC adopted a compact donut-shaped morphology and was found at one of the poles of the nucleus. The localization and morphology of the GSC and the TGN were compared in L6 rat myotubes simultaneously stained with the rabbit OSCR6 pAb (Fig. 2G,I) and the mouse monoclonal anti-GMPc-1 mAb (Fig. 2H,I). Their comparison showed that the two reticular structures overlapped almost exactly in the perinuclear area, but there were also numerous peripheral GLUT4 positive structures that did not contain GMT1-1. This result indicated...
that the GLUT4 and GMP$_c$-1 resided either in the same or in closely juxtaposed compartments in the perinuclear area. Furthermore, the GSC was found to contain VAMP2 (Fig. 2K-M), a v-SNARE which is found in vesicles purified by immunoadsorption with anti-GLUT4 antibodies (Cain et al., 1992), appears to be segregated from recycling endosomes (Martin et al., 1996) and is involved in the translocation of GLUT4 to the cell surface (Tamori et al., 1996; Macaulay et al., 1997; Olson et al., 1997; Martin et al., 1998).

In sharp contrast, in 3T3-L1 fibroblasts GLUT4$_{5}$ was found confined in vesicles scattered throughout the cytoplasm (Fig. 3A). Moreover, double staining of the fibroblasts for GLUT4$_{5}$ and GMP$_c$-1 (Fig. 3B,C) or VAMP2 (Fig. 3D,E), showed that GLUT4$_{5}$ was completely excluded from the Golgi area and the VAMP2-positive GSC. In contrast to the effect of deleting the last five residues, the removal of the last seventeen residues resulted in deflection of the truncated GLUT4 to the plasma membrane (data not shown), a pattern of distribution often displayed by proteins that lost the motifs involved in their intracellular retention.

Fig. 2. Cellular distribution of GLUT4 in clonal 3T3-L1 fibroblasts, 3T3-L1 adipocytes and L6 myotubes. Clonal 3T3-L1 fibroblasts stably expressing GLUT4 (A-D; K-M), in vitro differentiated 3T3-L1 adipocytes expressing endogenous GLUT4 (E-F), and rat L6 myotubes expressing endogenous GLUT4 (G-J), were single (A) or double stained (B-M) for GLUT4 and the SGC marker GMP$_c$-1, the TGN marker GMP$_c$-1, the TGN marker GMP$_c$-1 or VAMP2, as indicated in the panels. Antibodies used were: anti-GLUT4 rabbit pAb OSCR6 (A,B,D,E,G); anti-GLUT4 mouse mAb 1F8 (K); anti-GMP$_c$-1 mouse mAb 15C8 (C,D) and rabbit pAb (F); anti-GMP$_c$-1 mouse mAb 18B11 (H); anti-VAMP2 rabbit pAb (L) Cells were separately or simultaneously (D,I) photographed through fluorescein and Texas red. The images shown in I and J were produced by superposing the images in G and H. The area marked with an arrow in I was magnified and shown in panel J. GSC, GLUT4 storage compartment; SGC, stack of Golgi cisternae; N, nucleus. Arrows in D mark the cells edges. Bars: 1.9 µM (A,E); 2.9 µm (B,D,G,K); 2.8 µm (G).

GLUT4$_{5}$ is targeted to GLUT4-positive-vesicles and Rab7-positive late endosomes
To further characterize the vesicles to which GLUT4$_{5}$ was targeted, fibroblasts from clone 7 were double-stained for GLUT4$_{5}$ and Rab7 or Rab 5, markers of late and early endosomes, respectively (Chavrier et al., 1990). The study of their distribution by IMF microscopy showed the frequent presence of GLUT4$_{5}$ in Rab7-positive late endosomes (compare Fig. 3F and G, arrows) and, in contrast, its exclusion from Rab5-positive early endosomes (not shown). Furthermore, experiments performed in parallel showed no significant overlapping between the distributions of GLUT4 and the two Rab proteins (not shown).

Next, to know if GLUT4$_{5}$ was targeted to GLUT4 vesicles, clonal 3T3-L1 fibroblasts stably expressing the native GLUT4 molecule, without the HA flag, were transiently transfected with GLUT4$_{5}$ for 18 hours and the distribution of the two proteins studied in cells double stained with the rabbit pAb anti-GLUT4 OSCR6 and the mouse mAb anti-HA 16B12. Their study by IMF microscopy showed that a significant
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number of the GLUT4-positive-vesicles also contained GLUT4Δ5 (Fig. 3H-K).

GLUT4Δ5 is transported to the TGN for further transport to vesicles

To compare the pathways of transport involved in the targeting of GLUT4 and GLUT4Δ5 to the GSC and vesicles, respectively, the two proteins were transiently transfected into COS-7 cells. The experimental conditions were carefully designed to avoid the overexpression of the proteins as well as to clean the secretory pathway, for which purpose the cells were transfected only for 18 hours and were incubated for the last three hours of the transfection with 0.1 mM cycloheximide. Under these conditions, the bulk of GLUT4 was localized to the GSC which, again, as in 3T3-L1 fibroblasts was localized in the vicinity of the nucleus and adopted the morphology of the Golgi complex, as shown in cells double-stained for GLUT4 and the Golgi marker GMPC-1 (Fig. 4A,B). It is important to note that whereas the incubation with cycloheximide removed from the Golgi the plasma and lysosomal membrane proteins transported through this (not shown), the incubation with the antibiotic did not affect the retention of GLUT4 in the perinuclear GSC. This resistance was helpful to distinguish the GSC from the Golgi, given the almost identical localization and morphology of these two organelles.

In contrast with the distribution of GLUT4 in transiently transfected COS-7 cells (Fig. 4A), GLUT4Δ5 was localized exclusively to vesicles which often contained Rab7 (Fig. 4C and D); this result as well as those from similar experiments performed in NRK and CHO cells (not shown), confirmed the universality of the GLUT4Δ5 targeting to late endosomes.

To further investigate the pathway of GLUT4Δ5 transport, we studied the effect of low temperature on its distribution. For this purpose, cells transfected with GLUT4Δ5 for 15 hours were incubated at 20°C with 0.1 mM cycloheximide for 3 hours. Previous, studies have shown that at 20°C the proteins transported through the secretory pathway are arrested at the TGN (Saraste et al., 1986). We observed that the 20°C incubation provoked a dramatic change in the distribution of GLUT4Δ5. The localization of the protein, which in cells incubated at 37°C was found in vesicles (Fig. 4G), was now indistinguishable from that of the GLUT4 retained in the GSC, whose morphology was not affected by the low temperature (compare Fig. 4E and F). Furthermore, release of the blockage by shifting the temperature to 37°C for 5 hours in the presence of cycloheximide, resulted in redistribution of the protein to vesicles (Fig. 4G). These results indicated that GLUT4Δ5 was
transported through the Golgi. Therefore, the removal of the Pro$^{505}$AspGluAsnAsp$^{509}$ sequence was not causing its retention in the ER, as previously described for membrane proteins with DEXD motifs (Bannykh et al., 1998). They also showed that GLUT4$_D^{5}$ could not be directly targeted from the ER to the vesicles in which it is found, as shown previously for secretory proteins which upon their retention in the ER were directly targeted from this to lysosomes (Noda and Farquhar, 1992).

GLUT4$_D^{5}$ is excluded from the plasma membrane

To further study the trafficking of GLUT4 and GLUT4$_D^{5}$, we compared their distribution in transiently transfected COS-7 cells under overexpression conditions (24 hours transfection in the absence of cycloheximide). Under these conditions GLUT4 was detected in the GSC and in 80-90% of the cells also in the plasma membrane, whereas GLUT4$_D^{5}$ was exclusively found in vesicles (not shown). This difference probably reflected both the inability of GLUT4$_D^{5}$ to reach the plasma membrane from the vesicles to which is targeted and the saturation of the mechanisms that retain GLUT4 in the GSC. Further evidence supporting the complete exclusion of GLUT4$_D^{5}$ from the plasma membrane was provided by the analysis of cellular fractions and by the study of its resistance to trypsin in intact cells. Comparison of the levels of GLUT4 and GLUT4$_D^{5}$ in fractions enriched in low density microsomes and plasma membrane (Piper et al., 1991), revealed that whereas their levels in the microsomal fraction were comparable, only GLUT4 was detected in the fraction enriched in plasma membrane (Fig. 4H). Furthermore, the sensitivity of GLUT4 and the resistance of GLUT4$_D^{5}$ to trypsin when KCN poisoned fibroblasts stably expressing GLUT4 and GLUT4$_D^{5}$ (H). The blots were probed with the anti-HA mouse mAb 16B12. The experiment shown is one of three separate experiments.

GLUT4$_D^{5}$ is arrested at the TGN in COS-7 cells incubated at 20°C and moves into vesicles after raising the temperature to 37°C. COS-7 cells transfected for 15 hours with GLUT4 (A,B,F) or with GLUT4$_D^{5}$ (C,D,E,G), carried in the pCEXV plasmid, were incubated for 3 hours at 37°C (A-D) or at 20°C (E-F) with 0.1 mM cyclohexamide prior incubation for 5 hours at 37°C with the antibiotic (G). The cells were either double-stained for GLUT4 (A) and GMP$_{c-1}$ (B) or for GLUT4$_D^{5}$ and Rab7 (C,D), and were single-stained for GLUT4$_D^{5}$ (E,G) or GLUT4 (F) as indicated in the panels. Antibodies used were: anti-GLUT4 mouse mAb 1F8 (A,F); anti-HA mouse mAb 16B12 (C,E,G); anti-GMP$_{c-1}$ rabbit pAb (B) and anti-Rab7 rabbit pAb (D). N, nucleus. Arrows in C and D mark GLUT4$_D^{5}$/Rab7-positive late endosomes. Bars: 2.12 μm (A,E-G); 2.13 μm (C). Distribution of GLUT4 and GLUT4$_D^{5}$ between fractions enriched in plasma membrane (PM) and low density microsomes (LDM) purified from clonal 3T3-L1 fibroblasts stably expressing GLUT4 and GLUT4$_D^{5}$ (H). The blots were probed with the anti-HA mouse mAb 16B12. The experiment shown is one of three separate experiments.
its targeting to vesicles, GLUT4Δ5 is unable to reach the plasma membrane.

The Pro505AspGluAsnAsp509 sequence does not contain the information required to target and/or retain GLUT4 in the GSC

To further investigate if the Pro505AspGluAsnAsp509 sequence contained the information required to target and/or retain GLUT4 in the GSC, we developed the GLUT4(Ala)505-509 mutant by replacing the Pro505AspGluAsnAsp509 sequence by five alanines. The distribution of GLUT4(Ala)505-509 was studied in COS-7 cells transfected for 18 hours and treated the last 3 hours of the transfection with 0.1 mM cycloheximide. The experiment showed that, like GLUT4, GLUT4(Ala)505-509 was retained in the GSC (Fig. 5B). This result excluded, therefore, the possibility that the Pro505AspGluAsnAsp509 sequence contained the information necessary to retain GLUT4 in the cycloheximide resistant GSC, and suggested that instead it could regulate a motif(s) with those activities.

Substitution of Ala or Phe for Tyr502 provokes the retention of GLUT4Δ5 in the GSC. The tyrosine kinase inhibitor genistein inhibits the targeting of GLUT4Δ5 to vesicles

To characterize the motif regulated by the Pro505AspGluAsnAsp509 sequence, we individually replaced in the GLUT4Δ5 molecule the residues contiguous to Pro505 by alanine residues. The study of the resulting mutants, performed in COS-7 cells transfected for 18 hours and incubated for the last three hours of the transfection with cycloheximide, showed that the replacement of Ala for Tyr502 caused the retention of GLUT4Δ5 in the cycloheximide insensitive GSC (compare Fig. 5A and C).

Since the analysis of the surrounding sequence indicated that Tyr502 was a potential substrate for tyrosine kinases and phosphatases (Songyang and Cantley, 1995), we studied the possibility that the activity of Tyr502 could require its phosphorylation. For this purpose, we first investigated whether the replacement of Phe for Tyr502 impeded the targeting of GLUT4Δ5 to vesicles. The answer was yes. The
The Leu₄₈⁹Leu₄₉₀-based motif regulates the transport of GLUT₄Δ₅, GLUT₄Δ₅Phe⁵⁰² and GLUT₄ from the TGN to the GSC

Surface internalization studies have involved the Leu₄₈⁹Leu₄₉₀-based motif in GLUT4 endocytosis as well as in its access to a slow recycling compartment (Piper et al., 1993; Verhey and Birbaum, 1994; Corvera et al., 1994; Garippa et al., 1994; Haney et al., 1995; Araki et al., 1996). In addition, dileucine motifs have been involved in the intracellular sorting of membrane proteins at the TGN (Letourneur and Klausner, 1992; Sandoval et al., 1994; for a review see Sandoval and Bakke, 1994).

To further investigate the role of the dileucine motif in the transport of GLUT4Δ₅ we replaced the first Leu by a Ser residue. We noted that the GLUT4Δ₅Ser⁴₈⁹ mutant transiently expressed in COS cells accumulated both in the perinuclear area and the plasma membrane (Fig. 6A). Moreover, incubation of these cells for the last three hours of the transfection with 0.1 mM cycloheximide, resulted in removal of the mutant protein from both sites and in its concentration in peripheral vesicles (Fig. 6B, arrowheads). These results suggested that the inactivation of the dileucine motif could hinder the targeting of GLUT4Δ₅ to late endosomes, but the localization of GLUT4Δ₅Ser⁴₈⁹ in vesicles required further confirmation. To further characterize the transport step(s) mediated by the dileucine motif in the targeting of GLUT4Δ₅ to vesicles, we introduced the Ser⁴₈⁹ mutation in the GLUT4Δ₅Phe⁵⁰² mutant, which is retained in the cycloheximide resistant GSC, and studied its effect on the cellular distribution of this. The study performed in COS-7 cells transfected for 18 hours in the absence of cycloheximide showed that the inactivation of the dileucine signal provoked the extensive accumulation of the protein in the plasma membrane (Fig. 6C). In addition, GLUT4Δ₅ Ser⁴₈⁹ Phe⁵⁰² was also found in the perinuclear area (Fig. 6C). Incubation of these cells for the three last hours of the transfection with 0.1 mM cycloheximide resulted in complete removal of GLUT4Δ₅Ser⁴₈⁹Phe⁵⁰² from both the perinuclear area and the plasma membrane as well as in its concentration in vesicles often localized to the cell periphery (Fig. 6D, arrowheads). This dramatic effect was in contrast with the retention of GLUT4Δ₅Phe⁵⁰² in the perinuclear area of cells treated with...
GLUT4 lacking the last five residues, GLUT4ΔS, is targeted to late endosomes, to characterize some of the structural motifs that regulate the targeting, retention and exit of GLUT4 from the GSC.

The distribution studies performed in stably transfected 3T3-L1 fibroblasts and transiently transfected COS-7 cells, show that these cells, as well as 3T3-L1 adipocytes, store the intracellular GLUT4 in the GSC juxtaposed to the Golgi complex as well as in a number of vesicles scattered throughout the cytoplasm. This result agrees with studies in other cell lines and confirms the universality of the intracellular distribution of GLUT4 (Haney et al., 1991).

In 3T3-L1 fibroblasts and COS-7 cells the GSC can be described as a structure adjacent to and shaped as the Golgi complex which is enriched in VAMP2, and is able to retain GLUT4 but not GLUT4ΔS.

The retention of GLUT4ΔS in the Golgi (i.e. TGN) of transiently transfected COS-7 cells incubated at 20°C, proves that, like GLUT4, GLUT4ΔS it transported through the secretory pathway to the TGN. This observation excludes the possibility that, as described for other secretory membrane proteins, removal of the carboxyl-terminal acidic DEXD cluster (i.e. Pro502AspGluAsnAsp509) might inhibit its exit from the ER (Bannykh et al., 1998). This result also discards the suggestion that GLUT4ΔS is targeted from the ER to late endosomes as previously shown for proteins retained in the ER of hyperstimulated thyrotrophs (Noda and Farquhar, 1992).

Moreover, the redistribution of the GLUT4ΔS molecules retained in the TGN to vesicles after shifting the cell temperature from 20°C to 37°C as well as the exclusion of GLUT4ΔS from the plasma membrane, demonstrate that GLUT4ΔS is targeted from the TGN to vesicles (i.e. GLUT4-positive vesicles/late endosomes).

The exclusion of GLUT4ΔS from the GSC is in dramatic contrast to the accumulation of GLUT4 in this organelle. The contrast between its exclusion and the retention of GLUT4ΔS5Ala502 and the GLUT4ΔS5Phe502 mutants in the GSC, strongly suggests that GLUT4ΔS is released from the GSC by a mechanism involving Tyr502. Tyr502 is a potential substrate for protein tyrosine-kinases and phosphatases as indicated by the Lys, Glu, Glu and Pro residues at positions –7, –3, –1 and +3, respectively (Songyang and Cantley, 1995). The possibility that the phosphorylation of Tyr502 may control the exit of GLUT4ΔS from the GSC is supported by the observation that genistein causes the retention of GLUT4ΔS within the GSC. The prospect that the effect of genistein could be caused by the facilitation of the return of GLUT4ΔS from the vesicles that host this, appears to be discarded by the observation that GLUT4ΔS was localized exclusively to vesicles after 2 hours incubation with cycloheximide and further incubation for 2 hours more with genistein and cycloheximide. Attempts to demonstrate that Tyr502 is phosphorylated in vivo, however, failed. This failure suggests that phosphorylated Tyr502 could be rapidly dephosphorylated or, alternatively, that Tyr502 is not phosphorylated at all. In this case genistein might inhibit the release of GLUT4ΔS from the GSC by acting on the phosphorylation of a molecule involved in its release. With regard to the possibility that Tyr502 may not be phosphorylated, it is interesting that a Tyr residue which can not be replaced by Phe has been recently shown to be critical
for the COPI-mediated recycling of membrane proteins from the Golgi to the ER (Cosson et al., 1998).

It is important to note that Tyr\(^{502}\) becomes critical for the release of GLUT4\(^{Δ5}\) from the GSC when the carboxy-terminal Pro\(^{505}\)AspGluAsnAsp\(^{509}\) sequence is removed from the GLUT4 molecule, but that it remains inactive in the intact molecule or after the en-block substitution of the Pro\(^{505}\)AspGluAsnAsp\(^{509}\) by five alanines. This interdependence suggests that the activity of Tyr\(^{502}\) might be regulated by the acidic cluster. The significance of this observation in vivo remains to be determined. The possibility that the interaction between Tyr\(^{502}\) and the adjacent acidic cluster may regulate the insulin-induced release of GLUT 4 from the GSC in adipocytes and myocytes, is attractive. Examination of this has been hampered by the failure to differentiate in vitro clonal 3T3-L1 fibroblasts stably expressing GLUT4\(^{Δ5}\). The importance of identifying a new target for the COPI-mediated recycling of membrane proteins at the TGN (for a review see Sandoval and Bakke, 1994). The observation that GLUT4\(^{Δ5}\) is transported to the TGN and the contrast between the targeting of GLUT4\(^{Δ5}\) to late endosomes/GLUT4-positive vesicles and the accumulation of GLUT4\(^{Δ5Phe^{502}}\) in the GSC, provided us with the opportunity to examine the possible role of the dileucine motif in the targeting of these GLUT4 mutants, and by extension of GLUT4, from the TGN to the GSC. This study, which included the comparison of the cellular distributions of GLUT4\(^{Δ5Phe^{502}}\) and GLUT4 with the corresponding mutants with the dileucine motif inactivated, clearly showed that the dileucine motif is involved in their targeting from the TGN to the GSC. It will be interesting to study if this sorting involves the AP1 clathrin adaptor as suggested the recent observation that the dileucine motif competes for binding to the TGN-based adaptor AP-1 (Rapoport et al., 1998).

Finally, the importance of the COOH-terminal residues in the trafficking and distribution of the glucose transporters is emphasized by the results reported here as well as by the recent observation that a new protein called GLUT1CBP links GLUT1 but not GLUT4 to the cytoskeleton through its interaction with the COOH-terminal four amino acids (Bunn et al., 1999).

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