Reycling of the Insulin-sensitive Glucose Transporter GLUT4

ACCESS OF SURFACE INTERNALIZED GLUT4 MOLECULES TO THE PERINUCLEAR STORAGE COMPARTMENT IS MEDIATED BY THE Phe5-Gln6-Gln7-Ile8 MOTIF

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The insulin-sensitive glucose transporter GLUT4 is translocated to the plasma membrane in response to insulin and recycled back to the intracellular store(s) after removal of the hormone. We have used clonal 3T3-L1 fibroblasts and adipocyte-like cells stably expressing wild-type GLUT4 to characterize (a) the intracellular compartment where the bulk of GLUT4 is intracellularly stored and (b) the mechanisms involved in the recycling of endocytosed GLUT4 to the store compartment. Surface internalized GLUT4 is targeted to a large, flat, fenestrated saccular structure resistant to brefeldin A that localized to the vicinity of the Golgi complex is sealed to endocytosed transferrin (GLUT4 storage compartment). Recycling of endocytosed GLUT4 was studied by comparing the cellular distributions of antibody/green fluorescent protein tagged GLUT4 and GLUT4(Ser5), a mutant with the Phe5-Gln6-Gln7-Ile8 inactivated by the substitution of Ser for Phe. Ablation of the Phe5-Gln6-Gln7-Ile8 motif inhibits the recycling of endocytosed GLUT4 to the GLUT4 store compartment and results in its transport to late endosomes/lysosomes where it is rapidly degraded.

Plasma euglycemia is maintained by the effect of insulin on muscle and to a lesser extent on adipose tissue. In these tissues insulin stimulates glucose transport, the rate-limiting event in glucose disposal (1–3). The function of GLUT4, the only glucose transporter sensitive to insulin, is essential to maintain the insulin-regulated plasma euglycemia, which is controlled through the regulation of the GLUT4 trafficking. Insulin stimulates glucose transport by promoting the translocation of GLUT4 (4, 5) from the intracellular tubulovesicular structures where it is stored (6–8) to the plasma membrane (5, 7, 9, 10). After withdrawal of the hormone, GLUT4 is removed from the plasma membrane and recycled to the intracellular stores, and the steady-state previous to insulin stimulation is re-established (11, 12).

The molecular mechanisms involved in GLUT4 trafficking are poorly understood. The 12 transmembrane domain protein displays, in its amino and carboxy-tyrosinoclastic tails, Phe5-Gln6-Gln7-Ile8-based (13) and Leu489-Leu490-based motifs (14) that when inactivated provoke its cellular redistribution. Both motifs have been involved in GLUT4 endocytosis, intracellular retention, and targeting in transfected 3T3-L1 fibroblasts, COS-7 and Chinese hamster ovary cells (15–20), myoblasts (21), and adipocytes (18, 22, 23). Whether the dileucine motif mediates the trafficking of GLUT4 in adipocytes is, however, the subject of much debate (18, 22, 23). In addition, the mechanisms that mediate the intracellular retention and recycling of GLUT4 to the intracellular store compartments remain essentially unknown.

Here we have investigated the motifs involved in the recycling of endocytosed GLUT4 to the GSC and study the organization and localization of this. For this purpose, we have stably expressed HA epitope-tagged wild-type GLUT4 and a GLUT4(Ser5), a mutant with the Phe5-Gln6-Gln7-Ile8 motif ablated, and studied the intracellular distribution of endocytosed molecules tagged with anti-HA antibodies and the GLUT4-specific reagent Bio-LC-ATB-BMPA. The results of these studies show that the Phe5-Gln6-Gln7-Ile8 motif is critical for access of endocytosed GLUT4 to the GSC where the bulk of it is stored. The GSC consists of a flat saccular structure resistant to BFA organized as a reticulum at one of the nuclear poles.

EXPERIMENTAL PROCEDURES

Cell Culture—3T3-L1 fibroblasts were cultured on plastic dishes or glass coverslips in DMEM supplemented with 10% fetal calf serum, 4.5 mM glutamine, 50 μg/ml streptomycin, 100 IU/ml penicillin, nonessential amino acids (complete medium) in a 37 °C humidified CO2 incubator.

Differentiation of 3T3-L1 Fibroblasts into Adipocyte-like Cells—Clonal 3T3-L1 fibroblasts kept confluent for 3 days were incubated for 3–7 days in IDBX medium (1.6 × 10−4 μM insulin, 10 μg/ml biotin, 2.5 μM dexamethasone, and 500 μM 1-isobutyl 3-methylxanthine) and then with 10−8 M insulin in complete medium for different time periods as required. The fibroblasts so treated developed large lipid-droplets in their cytoplasm and will be referred henceforth as adipocyte-like (ADL) cells. ADL cells were fixed with 4% paraformaldehyde and stained with 1 μM Nile blue prepared in phosphate-buffered saline to monitor the development of lipid droplets in the cytoplasm. ADL cells fixed with 4% paraformaldehyde were permeabilized for 3 min with cold (−20 °C) methanol and immunostained with specific antibodies as required for their study by immunofluorescence (IMF) microscopy.

The abbreviations used are: GSC, GLUT4 store compartment; HA, hemagglutinin; BFA, brefeldin A; DMEM, Dulbecco’s modified Eagle’s medium; ADL, adipocyte-like; IMF, immunofluorescence; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; pAb, polyclonal antibody; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; Tfn, transferrin; HDM, high density microsomes; LDM, low density microsomes; TGN, trans-Golgi network; Bio-LC-ATB-BMPA, 4-(1-azi-2,2,2-trifluoro-ethyl)-benzoyl-1-3-bis (d-mannosyl4-cycloxy)-2-napoylamine.
Antibodies—The mouse monoclonal (mAb16B12) and the rabbit polyclonal (Y11) anti-HA antibodies were from BabCO (Berkeley, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The rabbit polyclonal anti-exofacial loop antibody (pAbEL) was raised against a mixture of two peptides (NH2-CRQGPGGPDSIPQGTLTTLWA-COOH and NH2-CNAPQKVIEQSYNLWC-COOH) that specifically bind to GLUT4 (26) to residue 44 to residue 83 on the first large exofacial loop of GLUT4. The rabbit pAb828 was raised against the peptide representing the COOH- tail of GLUT4. These four antibodies were used to study the distribution of native and tagged GLUT4 molecules by IMF microscopy, electron microscopy, and Western analysis. Their use was conditioned by the protocols employed and the origin of the antibodies used as specific markers (see below). Occasionally the experiments were repeated with two different anti-GLUT4 antibodies to evaluate their effects on the system under study. pAb828 was covalently bound to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) and used to study the fraction of pAbEL bound to the GLUT4 and GLUT4(Ser5) molecules recovered with purified LDM fractions. pAbEL was conjugated to FITC to compare the distribution of surface internalized tagged GLUT4 or GLUT4(Ser5) with Texas red-Tfn and Texas red-dextran beads. The mouse anti-His mAb, rabbit anti-Rab5b, and anti-Rab4 pAb were from Santa Cruz Biotechnology (Santa Cruz, CA). The development of the rabbit pAb35C8 against LIMPII, a membrane protein marker of late endosome/lysosome, has been reported (25). The rabbit pAb against the Golgi sialoglycoprotein MG160 was a gift of Dr. Gonatas (26). The rabbit pAbIS11B against the trans-Golgi network marker GM1, was developed in the laboratory, and its properties have been reported (27).

GFP- and Texas red-conjugated molecules were from Cappel (Durham, NC). HRP-conjugated goat anti-mouse and F(ab')2 donkey anti-rabbit antibodies were from Amersham Pharmacia Biotech.

DNA Constructs—The cDNAs of GLUT4 were cloned into the modified pPUR vector (CLONTECH) carrying the spleen focus-forming virus (SFFV) promoter at the ApaI/EcoRI sites (20). GLUT4 was tagged by introducing the HA epitope after Thr78 into the GLUT4 cDNA carried in containing 100 mM NaCl and 2 mM EDTA. Intact and dissociated complexes protein, prepared as described above, and analyzed by Western blot analysis for 1 h at 37 °C with FITC-pAbEL, washed, incubated for 10 min with Texas red-Tfn (50 μg/ml; Molecular Probes, Eugene, OR), and then incubated for 10–50 min in plain complete medium. To study whether the endocytosed antibody-tagged GLUT4(Ser5) was down-loaded into late endosomes/lysosomes, fibroblasts stably transfected with GLUT4(Ser5) were preincubated for 1 h at 37 °C with FITC-pAbEL and then incubated for 1 h in 100 μM chloroquine and 0.4% H2O2 before their incubation with biotinylated GLUT4(Ser5) and streptavidin conjugated to Texas red-dextran beads.

Processing of the cells for IMF microscopy was as follows. The cells were quickly washed three times with phosphate-buffered saline and then fixed with 4% paraformaldehyde, permeabilized with cold methanol and stained with specific antibodies or streptavidin (25). FITC- and Texas red-conjugated antibodies were from Cappel (Durham, NC). FITC-conjugated streptavidin was prepared in the laboratory (West Grove, PA). The cellular distributions of tagged GLUT4 molecules, Tfn, and dextran beads were studied by IMF microscopy using an Axiovert 135 m inverted microscope (Zeiss) and a confocal Radiance 2000 microscope (Bio-Rad). The cells were photographed through fluorescence.

Sensitivity of GLUT4/GLUT4(Ser5)-Antibody Complexes to Low pH—To study the stability of [35S]GLUT4 and [35S]GLUT4(Ser5) antibody complexes within the pH 5.5–7.4 range, purified protein G-Sepharose complexes were incubated for 10 min or 1 h at 37 °C with either 50 mM Tris-HCl, pH 7.4, or 50 mM cacodylate buffer, pH 6.4 or 5.5, containing 100 mM NaCl and 2 mM EDTA. Intact and dissociated complexes were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and resolution by SDS-PAGE, and their levels were measured by autoradiography.

Studies on the Cellular Levels and Turnovers of GLUT4 Species—Cell membranes from 3T3-L1 fibroblasts were prepared as follows. Cells were washed twice with phosphate-buffered saline, scraped, and incubated for 30 min in 2 ml of 0.1 M Na2CO3, pH 11.3, and membrane pellets were prepared by centrifugation at 150,000 × g for 30 min. The membranes were then solubilized for 30 min with 50 mM Tris, pH 7.4, 100 mM NaCl, 1% Triton X-100 (buffer A) and, after removal of the insoluble material by centrifugation at 150,000 × g for 30 min, diluted in 1 volume of buffer A without detergent. All the manipulations were performed at 4 °C.

The cellular levels of GLUT4 and GLUT4(Ser5) mutant were measured in 30 μg of membrane protein by Western analysis using the pAb828 and the ECL technique (Amersham Pharmacia Biotech). To study the fraction of GLUT4 exposed on the surface of stably transfected cells stably expressing the transfected proteins first by IMF microscopy. The turnover of GLUT4 and GLUT4(Ser5) were studied by developing the cellular images of tagged GLUT4 molecules and, when required, washed and further incubated for different time periods in antibody-free complete medium. To bio-tynlate the molecules of GLUT4 and GLUT4(Ser5) exposed onto the cell surface, 3T3-L1 fibroblasts stably expressing the proteins were incubated with 1 mM Bio-LC-ATB-BMPA (a gift of Dr. Geoff Holman, University of Bath, Bath, UK) prepared in 30 μl of Krebs-Ringer-Hepes buffer prepared (32) and UV irradiated six times for 1 min at intervals of 5 min.

To investigate whether the GSC was a compartment distinct from the perinuclear recycling compartment traversed by Tfn, fibroblasts stably transfected with GLUT4 or GLUT4(Ser5) were preincubated for 1 h at 37 °C with FITC-pAbEL, washed, incubated for 10 min with Texas red-Tfn (50 μg/ml; Molecular Probes, Eugene, OR), and then incubated for 10–50 min in plain complete medium. To study whether the endocytosed antibody-tagged GLUT4(Ser5) was down-loaded into late endosomes/lysosomes, fibroblasts stably transfected with GLUT4(Ser5) were preincubated for 1 h at 37 °C with FITC-pAbEL and then incubated for 5 min in 100 μM chloroquine and 0.4% H2O2 before their incubation with biotinylated GLUT4(Ser5) and streptavidin conjugated to Texas red-dextran beads.

Cell membranes from 3T3-L1 fibroblasts were prepared as follows. Cells were washed twice with phosphate-buffered saline, scraped, and incubated for 30 min in 2 ml of 0.1 M Na2CO3, pH 11.3, and membrane pellets were prepared by centrifugation at 150,000 × g for 30 min. The membranes were then solubilized for 30 min with 50 mM Tris, pH 7.4, 100 mM NaCl, 1% Triton X-100 (buffer A) and, after removal of the insoluble material by centrifugation at 150,000 × g for 30 min, diluted in 1 volume of buffer A without detergent. All the manipulations were performed at 4 °C.

The cellular levels of GLUT4 and GLUT4(Ser5) mutant were measured in 30 μg of membrane protein by Western analysis using the pAb828 and the ECL technique (Amersham Pharmacia Biotech). To study the fraction of GLUT4 exposed on the surface of stably transfected 3T3-L1 fibroblasts and ADL cells, cells grown to 90% confluence on 60-mm plastic dishes were incubated for 20 min at 37 °C with 2 mM KCN prepared in Krebs-Ringer-Hepes buffer prior incubations. After washing, 10 min at 37 °C with or without 2.5 mg/ml trypsin (Sigma) (28). After stimulation with 2 mM KCN and then quickly washed twice with cold phosphate-buffered saline. Harvesting was in 2 ml of buffer 20 mM Hepes, 250 mM sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.4 (buffer B). All the subsequent manipulations were performed at 4 °C. Cells were disrupted with 30 strokes using a Potter Evehem homogenizer (0.08 mm clearance), and the resulting homogenate was centrifuged at 16,000 × g for 20 min. HDM were pelleted from the resulting supernatant by centrifugation at 30,000 × g for 90 min. LDM vesicles were resuspended in 400 μl of buffer B, loaded onto a 4.6 × 100 mM NaCl, 1 mM EDTA, and fractionated by centrifugation at 30,000 × gmax for 55 min using a SW50.1 rotor (Beckman Instruments, Palo Alto, CA). Fractions were collected from the top of the gradient. The refractive index of the collected fractions was measured to assess the linearity of the gradients. The gradient profiles of GLUT4, GLUT4(Ser5)2, Rab4, Rab5b, and endocytosed pAbEL were studied by Western analysis using specific antibodies and the ECL technique.

Phenylalanine-based Motif Involved in GLUT4 Recycling

The turnover of GLUT4 and GLUT4(Ser5) were studied in 3T3-L1 fibroblasts incubated for 30 min in methionine/cysteine-free complete medium and then labeled for 10 min or 1 h with [35S]methionine/cysteine (>1,000 Ci/mmol) (Amersham Pharmacia Biotech) before their chase for different time periods with cold complete medium (25). To study the fraction of GLUT4 exposed on the surface of stably transfected 3T3-L1 fibroblasts, the cells were preincubated for 1 h with 50 mM chloroquine and 100 μM leupeptin, and then the inhibitors were added to the labeling and chase medium. GLUT4 and GLUT4(Ser5) were immunoprecipitated using the mAb16B12 and pAbEL bound to protein G-Sepharose (25) (Amersham Pharmacia Biotech) and, after their resolution by 10% PAGE, quantitated by autoradiography.
RESULTS

To characterize the intracellular GSC and to study the role of the Phe<sup>5</sup>-Gln<sup>6</sup>-Gln<sup>7</sup>-Ile<sup>8</sup> motif in the recycling of GLUT4 from the cell surface to the GSC, we traced the intracellular distribution of endocytosed antibody/biotin tagged GLUT4 and GLUT4(Ser<sup>5</sup>) molecules by IMF microscopy, electron microscopy, and Western analysis.

**Cellular and Plasma Membrane Levels of GLUT4 and GLUT4(Ser<sup>5</sup>) in 3T3-L1 Fibroblasts and ADL Cells—**

3T3-L1 clones transfected with a modified pPUR plasmid (20) carrying the GLUT4 and GLUT4(Ser<sup>5</sup>) cDNAs (Fig. 1A) and ADL cells (D) either untransfected or stably transfected with GLUT4 or GLUT4(Ser<sup>5</sup>) were grown to 90% confluence for 72 h in complete medium on 100-mm dishes. ADL cells were further incubated for 4 h in DMEM and then for 40 min in DMEM with or without 10<sup>−7</sup> M insulin. The cells were poisoned for 20 min at 37 °C with 2 mM KCN before their incubation for 10 min at 37 °C with 2.5 mg/ml trypsin, and membranes were prepared as described under “Experimental Procedures.” The relative surface levels of the transfected proteins in 30 µg of membrane protein were quantitated by scanning of Western blots developed using pAb828 and the ECL technique. Shown in the figure is one of three separate experiments. Mean values were calculated from the three experiments. Percentages of GLUT4 and GLUT4(Ser<sup>5</sup>) in the surface of 3T3-L1 fibroblasts were 5.5 ± 1 and 22 ± 2%, respectively, whereas in ADL cells incubated without insulin they were 6.5 ± 0.5 and 38 ± 2%, and after incubation with insulin they were 71 ± 2 and 53 ± 3%. wt, wild type.

**GLUT4-Antibody Complexes are Highly Stable at Acidic pH—**

Actively endocytosed antibodies when transported through endosomes are exposed to acidic environments that...
may provoke their dissociation from bound antigens (35). The use of antibodies to study the targeting of GLUT4 from the cell surface to the GSC required, therefore, to demonstrate that HA-tagged GLUT4/antibody complexes remained stable at low pH. For this purpose, [35S]GLUT4 and [35S]GLUT4(Ser5)/antibody complexes, purified from metabolically labeled cell extracts with mAb16B12/protein G-Sepharose (Fig. 2A), were incubated for 10 min or 1 h at 37 °C at pH 7.4, 6.4, and 5.5, and the effect of pH on their stability was studied by measuring GLUT4 and GLUT4(Ser5) in the supernatants and pellets separated by low speed centrifugation. The results showed that GLUT4 (upper panel) and GLUT4(Ser5) (lower panel) remained bound to the antibody even at pH 5.5, a value lower than the pH measured in endosomes and typical of lysosomes (35). Comparable results were obtained when the study was repeated using pAbEL (not shown). From these results as well as from the results of experiments performed in vivo (see Fig. 10), we concluded that mAb16B12 and pAbEL could be used to study the recycling of GLUT4 from the cell surface to the GSC.

Antibody-tagged GLUT4 Accumulates in LDM Vesicles in a Manner Regulated by the Phe5-Gln6-Gln7-Ile8 Motif—To begin to characterize the pathway of GLUT4 recycling from the cell surface to the GSC, clonal 3T3-L1 fibroblasts either untransfected or stably expressing GLUT4 or GLUT4(Ser5) were incubated for 1 h at 37 °C with mAb16B12 (Fig. 2B) or pAbEL (data not shown), and the antibody recovered with the fractions enriched in LDM and HDM was studied by Western analysis using the ECL technique. The results showed that fibroblasts stably expressing GLUT4 preferentially accumulated the endocytosed antibody in LDM as compared with HDM (Fig. 2B). Moreover, ablation of the Phe5-Gln6-Gln7-Ile8 motif dramatically decreased the accumulation of the antibody in LDM vesicles (Fig. 2B). Comparable results were obtained when the studies were repeated in ADL cells (not shown).

Surface Internalized GLUT4 Is Targeted to the GSC—3T3-L1 fibroblasts stably expressing HA-GLUT4 immunostained with anti-GLUT4 pAb828 concentrated GLUT4 in a perinuclear structure organized as a reticulum at one of the nuclear poles, and the rest was in punctuate structures scattered throughout the cytoplasm (Fig. 3A). The perinuclear compartment will be referred henceforth as the GSC, for GLUT4 storage compartment (20).

To investigate the recycling of GLUT4 from the cell surface to the GSC, fibroblasts stably transfected with GLUT4 were incubated for periods of 15 min, 1 h, and 18 h at 37 °C with 50 μg/ml of mAb16B12 or were UV irradiated in Krebs-Ringer-Hepes buffer containing the GLUT4 affinity reagent, Bio-3C-ATB-BMPA. After treatment, the cells were washed and further incubated at 37 °C for 1 h in plain complete medium or were immediately processed for microscopy. The distribution of the tagged GLUT4 molecules was studied after cell fixation with 4% paraformaldehyde and permeabilization with methanol. mAb16B12 was stained using FITC/HRP-conjugated goat anti-mouse antibodies and Bio-3C-ATB-BMPA with FITC-streptavidin. The results with the two tags were comparable (Fig. 3, B and C). The bulk of mAb16B12 internalized by cells incubated for 1 h with the antibody and then for 1 h in plain complete medium was localized to a reticular structure localized to one of the poles of the nucleus, both organization and localization characteristic of the GSC (Fig. 3, compare A, B, and C). Comparable results were obtained when the incubation with the antibody was reduced to 15 min or extended to 18 h (data not shown). When after incubation with the antibody the cells were immediately processed, the antibody was localized, in addition to the perinuclear reticular structure, to numerous punctate structures preferentially clustered in the same perinuclear area (not shown). To further investigate the similarities between the compartment that retained the internalized antibody and the GSC, cells incubated for 1 h with mAb16B12 were double-stained for the antibody and the Golgi sialoglycoprotein MG160 (26), and their distribution was compared by IMF microscopy.
The study, performed in flat spreaded fibroblasts, showed that the compartment loaded with mAb16B12 was closely juxtaposed to the Golgi complex (Fig. 3D), again a main characteristic of the GSC (20). Moreover, when in the same experiment the distributions of the internalized antibody and the TGN marker GMP210 (27) were compared by confocal microscopy; the two proteins showed very little overlapping, thus indicating that they were localized in different cellular compartments (Fig. 4). To further characterize the compartment that retained the antibody, cells incubated for 1 h with mAb16B12 were double-stained for the endocytosed antibody and for those GLUT4 molecules that retained in the GSC were not exposed onto the cell surface. For this purpose endocytosed mAb16B12/GLUT4 complexes were cross-linked with 4% paraformaldehyde and stained with FITC goat anti-mouse pAb, whereas the GLUT4 molecules retained in the GSC and carrying antibody-free HA tags were stained with the anti-HA pAbY11 and a Texas red goat anti-rabbit pAb. The colocalization of mAb16B12 and pAbY11 to the same perinuclear reticular structure (Fig. 3E) led us to conclude that the endocytosed mAb16B12/GLUT4 complexes were targeted to the GSC.

The vacuolar compartments beyond the trans-Golgi have been shown to respond to BFA with the production of stable tubular expansions, a phenomenon in contrast with the short life expansions produced by the Golgi complex (36–38). To further characterize the GSC and to establish its localization with relation to the Golgi, fibroblasts stably expressing GLUT4 and preincubated for 1 h with mAb16B12 were further treated for 1 h with 10 mg/ml BFA and then stained for the antibody and GLUT4. In parallel, fibroblasts similarly treated were incubated for 10 min or 1 h with the drug and the Golgi stained with the anti-MG160 antibody to monitor the Golgi response to the drug. The study of the mAb16B12 distribution showed that BFA induced the production of highly stable tubular expansions from the GSC, but despite these morphological changes after 1 h of drug treatment, the GSC remained as a distinct structure in the vicinity of the nucleus (Fig. 3, F and G). This stability was in contrast with the rapid disassembly of the Golgi complex, which disappeared after 10–20 min of incubation with the drug (Fig. 3, compare F and G with H and I). This different response, while indicating differences in the flow of membranes that traverse the Golgi and GSC, strongly suggests that the GSC is organized as a distinct structure beyond the TGN. It is important that comparable results were obtained when the same experiments were repeated with cells incubated with pAbEL (Fig. 5).

**FIG. 3.** Endocytosed mAb16B12 accumulates in the perinuclear GSC of 3T3-L1 fibroblasts stably expressing GLUT4. Clonal 3T3-L1 fibroblasts stably expressing HA-tagged GLUT4 (A) were either preincubated for 1 h at 37 °C with 50 μg/ml mAb 16B12 (B and D–F) or surface biotinylated using Bio-LC-ATB-BMPA (C). After their wash and their incubation for 1 h at 37 °C in plain complete medium, the cells were either fixed or incubated for the indicated times in 10 mg/ml BFA (F–I). The distributions of GLUT4 (A, C, E, and G), endocytosed mAb16B12 (B and D–F), and the Golgi marker MG160 (D, H, and I) were studied by IMF microscopy. Cells were single (A–C, H, and I) or double-stained (D–G). GLUT4 was studied with pAbE28 (A and G), anti-HA pAbY11 (E), or FITC-conjugated streptavidin (C). Second antibodies were goat anti-mouse or anti-rabbit antibodies conjugated to FITC, Texas red, or horseradish peroxidase (B, inset). Images in D and E were simultaneously photographed through fluorescein and Texas red and processed using the Image-Pro 2.0 program. The tubular extensions of the GSC and Golgi produced in response to BFA are marked with white arrows. n, nucleus. The bars indicate: 12 μm (A), 11.4 μm (B), 7.8 μm (C), 0.8 μm (D and E), 3 μm (F–H), and 4.5 μm (I).
transported to the perinuclear area packed in the same vesicles, a result consistent with previous observations (40–42), where they were sorted and the antibody was specifically targeted to the GSC. Furthermore, the exclusion of Tf from the GSC clearly showed that the GSC and the recycling compartment were distinct structures.

**GSC Ultrastructure.—**To study the ultrastructure of the organelles involved in the recycling of GLUT4, 3T3-L1 fibroblasts stably expressing HA-tagged GLUT4 were preincubated for 1 h at 37 °C with 50 μg/ml mAb16B12 and after their wash and their incubation for 1 h at 37 °C in plain complete medium fixed, double stained for mAb16B12, with an FITC-conjugated goat anti-mouse antibody, and for the TGN protein membrane marker GMP140, with a Texas red goat anti-rabbit antibody, and their distribution was studied by confocal microscopy. The cell marked in C–F with an asterisk was not transfected with HA-tagged GLUT4. Digits in the lower left corner of each panel indicate the distance (μm) of the sections to the cell bottom. Bars, 9.7 μm.

**FIG. 4.** Endocytosed mAb16B12 accumulates in a perinuclear compartment separate from the TGN. Clonal 3T3-L1 fibroblasts stably expressing HA-tagged GLUT4 were preincubated for 1 h at 37 °C with 50 μg/ml mAb16B12 and after their wash and their incubation for 1 h at 37 °C in plain complete medium fixed, double stained for mAb16B12, with an FITC-conjugated goat anti-mouse antibody, and for the TGN protein membrane marker GMP140, with a Texas red goat anti-rabbit antibody, and their distribution was studied by confocal microscopy. The cell marked in C–F with an arrow is shown in A and B at larger magnification. The cell marked with an asterisk was not transfected with HA-tagged GLUT4. The cell marked in C–F with an arrow is shown in A and B at larger magnification. The cell marked with an asterisk was not transfected with HA-tagged GLUT4. Digits in the lower left corner of each panel indicate the distance (μm) of the sections to the cell bottom. Bars, 9.7 μm.

In dramatic contrast with the results of studies on GLUT4 in fibroblasts, treatment of ADL cells for 1 h with 10 μg/ml BFA provoked the tubulation but did not cause the disappearance of the GSC (Fig. 7C). The recycling of GLUT4 in ADL cells stably transfected with GLUT4 was studied before the beginning of the endogenous GLUT4 expression (Fig. 1B). The cells, which displayed a cytoplasm crammed with large lipid droplets and the round morphology characteristic of adipocytes (Fig. 7, D and E), were incubated at 37 °C for 1 h with mAb16B12 and the distributions of the endocytosed antibody and GLUT4 studied by IF microscopy. Again, as in fibroblasts, the bulk of the antibody was localized to the GSC (Fig. 7, F and G), thus indicating that in ADL cells the antibody-tagged GLUT4 was targeted from the cell surface to the GSC.

**Ablation of the Phε-Gln6-Gln7-Ile8 Motif Blocks the Targeting of Tagged GLUT4 to the GSC.—**To study the effect of the ablation of the Phε-Gln6-Gln7-Ile8 motif on the recycling of GLUT4 to the GSC, clonal 3T3-L1 fibroblasts stably expressing GLUT4(Ser5) were developed. Their staining with pAb828 showed that GLUT4(Ser5) was distributed between the GSC, numerous punctate cytoplasmic structures, and the plasma membrane (Fig. 8A). The steady-state distribution of GLUT4(Ser5) was therefore similar to that of GLUT4, except for its slight retention at the plasma membrane (compare Figs. 3A and 8A; see also Fig. 1B).

In dramatic contrast with the results of studies on GLUT4 recycling, when fibroblasts stably expressing GLUT4(Ser5) were incubated at 37 °C for 1 h (Fig. 8B) or 18 h (data not shown) with mAb16B12 and after their wash further incubated...
for 1 h in plain complete medium, the antibody was exclusively localized to punctuate structures scattered throughout the cytoplasm. Essentially the same results were obtained when the GLUT4(Ser5) molecules exposed on the cell surface were biotinylated using Bio-LC-ATB-BMPA and their distribution was studied after internalization using FITC-streptavidin (Fig. 8C). Furthermore, double staining of the fibroblasts for the antibody and GLUT4(Ser5) revealed that the endocytosed antibody was completely excluded from the perinuclear GSC (Fig. 8D). This exclusion of the antibody was in contrast with the detection of GLUT4(Ser5) in the GSC (Fig. 8, compare A with B and C; see “Discussion”). Altogether the results of these internalization studies strongly indicated that the ablation of the Phe5-Gln6-Gln7-Ile8 motif inhibited the targeting of endocytosed GLUT4 to the GSC.

To further characterize the vesicles that retained the antibody-tagged GLUT4(Ser5) in the GSC (Fig. 8, compare A with B and C; see “Discussion”). Altogether the results of these internalization studies strongly indicated that the ablation of the Phe5-Gln6-Gln7-Ile8 motif inhibited the targeting of endocytosed GLUT4 to the GSC.

For 1 h in plain complete medium, the antibody was exclusively localized to punctuate structures scattered throughout the cytoplasm. Essentially the same results were obtained when the GLUT4(Ser5) molecules exposed on the cell surface were biotinylated using Bio-LC-ATB-BMPA and their distribution was studied after internalization using FITC-streptavidin (Fig. 8C). Furthermore, double staining of the fibroblasts for the antibody and GLUT4(Ser5) revealed that the endocytosed antibody was completely excluded from the perinuclear GSC (Fig. 8D). This exclusion of the antibody was in contrast with the detection of GLUT4(Ser5) in the GSC (Fig. 8, compare A with B and C; see “Discussion”). Altogether the results of these internalization studies strongly indicated that the ablation of the Phe5-Gln6-Gln7-Ile8 motif inhibited the targeting of endocytosed GLUT4 to the GSC.

To further characterize the vesicles that retained the antibody-tagged GLUT4(Ser5) in the same antibody internalization experiment described above, the fibroblasts were double-stained for the endocytosed antibody and Rab5b (50) or LIMP III (51), markers of early and late endosomes/lysosomes, respectively. Their study by IMF microscopy showed no significant overlapping between the distributions of the mAb16B12 and Rab5b (Fig. 8E), whereas the antibody was often found in LIMP III-positive vesicles (Fig. 8, F–H). These results suggested that the antibody-tagged GLUT4(Ser5) was at some point deflected from the recycling pathway to the
expression of endogenous GLUT4 (Fig. 10, B and D) showed that GLUT4(Ser5) was synthesized faster and twice more rapidly degraded than GLUT4 (Fig. 10, A and B) and that the mixture of chloroquine and leupeptin slowed its degradation to a rate comparable with that of GLUT4. Altogether, the codistribution of endocytosed GLUT4(Ser5)/mAb16B12 complexes with dextran beads and the accelerated turnover of GLUT4(Ser5) as compared with GLUT4 indicated that after its internalization GLUT4(Ser5) was deflected to lysosomes.

**Ablation of the Phe5-Gln6-Gln7-Ile8 Motif Provokes the Redistribution of GLUT4 and Endocytosed pAbEL among LDM Vesicles**—The localization of the bulk of GLUT4 to a population of LDM vesicles that appeared to develop during differentiation of fibroblasts into adipocytes and exhibited an insulin-elicited release of GLUT4 in response to insulin (34) prompted us to compare the distribution of GLUT4 and GLUT4(Ser5) among the pool of LDM vesicles. For this purpose, LDM vesicles isolated from 3T3-L1 fibroblasts stably transfected with GLUT4 or GLUT4(Ser5) and ADL cells expressing endogenous GLUT4 were fractionated on 12–30% sucrose velocity gradients and the distribution profile of the proteins studied by Western analysis.

**Expression of GLUT4 Opens the Access of pAbEL to the GSC of Fibroblasts Stably Expressing GLUT4(Ser5)—**We next examined the possibility that in clonal 3T3-L1 fibroblasts the failure of the antibody-tagged GLUT4(Ser5) molecules to gain access to the GSC was due to a dysfunction of the transport mechanisms rather than to the ablation of the Phe5-Gln6-Gln7-Ile8 motif. For this purpose, we tested the recycling of GLUT4 in 3T3-L1 fibroblasts stably expressing GLUT4(Ser5) that were transfected for 20 h with His/HA-tagged GLUT4 and then incubated with pAbEL for 1 h at 37 °C. Their study by IMF microscopy after the staining of GLUT4 and pAbEL with a mouse anti-His mAb and a goat anti-rabbit pAb, respectively, showed that the endocytosed antibody was divided between numerous punctate structures scattered throughout the cytoplasm and the GSC (Fig. 8, I and K), whereas the bulk of the transiently expressed His/HA-tagged GLUT4 was retained in the GSC (Fig. 8, J and K). This different distribution demonstrated that endocytosed GLUT4 was targeted to the GSC in the same cells that retained the endocytosed GLUT4(Ser5) in vesicles and therefore showed that the failure of GLUT4(Ser5) to return to the GSC was provoked by the inactivation of the Phe5-Gln6-Gln7-Ile8 motif.

**Ablation of the Phe5-Gln6-Gln7-Ile8 Motif Provokes the Entrance of GLUT4(Ser5) into the Degradative Pathway and Accelerates Its Turnover**—To further investigate the accumulation of endocytosed GLUT4(Ser5) in LIMPIII positive vesicles, the download of dextran beads into the vesicles previously loaded with antibody-tagged GLUT4(Ser5) was monitored and the turnovers of GLUT4 and GLUT4(Ser5) were compared. Fibroblasts stably expressing GLUT4(Ser5) were, for that purpose, preincubated at 37 °C for 1 h with FITC-pAbEL before incubation for 10 min with Texas red conjugated dextran beads and, after washing the beads, further incubated for 50 min with plain complete medium. The distributions of the antibody and the beads were then studied by confocal microscopy after fixation of the cells in 4% paraformaldehyde. Their study revealed an extensive overlapping of their distributions (Fig. 9, A–D), thus indicating that the dextran beads were downloaded into the vesicles retaining the endocytosed GLUT4(Ser5). Because dextran beads are transported all the way down to late endosomes/lysosomes, we concluded that GLUT4(Ser5) was transported to these degradative compartments. Interestingly, when the same experiment was repeated with Tfn instead of dextran beads, the majority of the vesicles preloaded with the antibody were not stained with Texas red-Tfn (Fig. 9, E–H). This result, which agreed with the different distribution of the GLUT4(Ser5)/mAb16B12 complexes and Rab5b, strongly supported the above conclusion.

To independently assess the download of GLUT4(Ser5) into late endosomes/lysosomes, we compared its turnover with that of GLUT4. For these purpose the two proteins were metabolically labeled in vivo for 10 min or 1 h with [35S]methionine/cysteine and then chased for 0, 4, 8, 12, and 24 h with an excess of cold amino acids. The study was performed in cells incubated with and without the proteases inhibitors chloroquine (50 mM) and leupeptin (100 μM), before and during the labeling and chase periods. The radiolabeled proteins were immunoprecipitated with mAb16B12 and resolved by SDS-PAGE, and their turnovers were analyzed by autoradiography. The results showed that GLUT4(Ser5) was synthesized faster and twice more rapidly degraded than GLUT4 (Fig. 10, A and B) and that the mixture of chloroquine and leupeptine slowed its degradation to a rate comparable with that of GLUT4. Altogether, the codistribution of endocytosed GLUT4(Ser5)/pAbEL complexes with dextran beads and the accelerated turnover of GLUT4(Ser5) as compared with GLUT4 indicated that after its internalization GLUT4(Ser5) was deflected to lysosomes.

**Expression of GLUT4 Opens the Access of pAbEL to the GSC**—We next examined the possibility that in clonal 3T3-L1 fibroblasts the failure of the antibody-tagged GLUT4(Ser5) molecules to gain access to the GSC was due to a dysfunction of the transport mechanisms rather than to the ablation of the Phe5-Gln6-Gln7-Ile8 motif. For this purpose, we tested the recycling of GLUT4 in 3T3-L1 fibroblasts stably expressing GLUT4(Ser5) that were transfected for 20 h with His/HA-tagged GLUT4 and then incubated with pAbEL for 1 h at 37 °C. Their study by IMF microscopy after the staining of GLUT4 and pAbEL with a mouse anti-His mAb and a goat anti-rabbit pAb, respectively, showed that the endocytosed antibody was divided between numerous punctate structures scattered throughout the cytoplasm and the GSC (Fig. 8, I and K), whereas the bulk of the transiently expressed His/HA-tagged GLUT4 was retained in the GSC (Fig. 8, J and K). This different distribution demonstrated that endocytosed GLUT4 was targeted to the GSC in the same cells that retained the endocytosed GLUT4(Ser5) in vesicles and therefore showed that the failure of GLUT4(Ser5) to return to the GSC was provoked by the inactivation of the Phe5-Gln6-Gln7-Ile8 motif.
The steady-state distribution of GLUT4(Ser5) was studied using pAb828 and a FITC-goat anti-rabbit pAb (conjugated to Texas-red). In contrast, the distributions of GLUT4(Ser5) and the antibody to the GSC in fibroblasts stably expressing GLUT4(Ser5). Clonal 3T3-L1 fibroblasts stably expressing GLUT4(Ser5) were transiently transfected of it being accumulated between fractions 6 and 11 (Fig. 11A). When the same study was repeated in 3T3-L1 fibroblasts stably expressing GLUT4(Ser5) were incubated in normal medium (A and C) or with mAb16B12 (B and D–H) as described in the legend to Fig. 3. The steady-state distribution of GLUT4(Ser5) was studied using pAb828 and a FITC-goat anti-rabbit pAb (A). The distributions of mAb16B12 and Bio-LC-ATB-BMPA tagged GLUT4 were studied with FITC-goat anti-mouse pAb (B, D, E, G, and H) and FITC-streptavidin (C), respectively. Cells were single (A–C) or double stained for mAb16B12 and GLUT4 (D), Rab5b (E), or LIMPIII (F and H) using specific pAbs and a goat anti-rabbit pAb conjugated to Texas-red. Boxed areas in F–H are shown enlarged in the inserts in the same panels. Expression of GLUT4 opens the access of pAbEL to the GSC in fibroblasts stably expressing GLUT4(Ser5). Clonal 3T3-L1 fibroblasts stably expressing GLUT4(Ser5) were transiently transfected for 20 h with HIS-tagged GLUT4 and after their incubation for 1 h with pAbEL (prepared in complete medium to ~50 μg IgG/ml) processed and double-stained for the antibody, using a FITC-goat anti-rabbit pAb (I and K) and for His-GLUT4, using a mouse anti-His mAb and a Texas red-goat anti-mouse pAb (J and K). The cells were separately (I and J) or simultaneously (K) photographed through fluorescence. The bars indicate: 12 μm (A), 5 μm (B and E), 3 μm (C and D), 4 μm (F–H), and 4.8 μm (I–K).
FIG. 9. Endocytosed dextran is downloaded into the compartment retaining GLUT4(Ser5). Cells stably transfected with GLUT4(Ser5) were pre-incubated with FITC-pAbEL for 1 h before incubation for 10 min with Texas red-dextran beads (molecular weight, 70,000; at 0.1 mg/ml) and after their wash further incubated for 50 min in medium complete medium (A–D). The same experiment was repeated substituting Texas red-Tfn for dextran beads (E–H). Note the overlapping between the endocytosed antibody and the dextran beads distributions and the segregation of the antibody and Tfn into different punctuate organelles. Digits in the lower left corner of each panel indicate the distance (μm) of the sections to the cell bottom. Bars, 9.7 μm.

Our studies show a dramatic difference between the distributions of endocytosed GLUT4 and GLUT4(Ser5). According to the results, the bulk of endocytosed tagged GLUT4 complexes is downloaded in a reticular compartment localized to one pole of the nucleus and juxtaposed to the Golgi complex, as shown by double-immunostaining of cells with antibodies against mAb16B12 and the Golgi marker MG160, whereas tagged GLUT4(Ser5) is targeted to the lysosomal degradative pathway.

With regard to the morphology, localization, and links of the GSC with the Golgi complex, the GSC can be described as an organelle constituted by flat saccular structures with multiple fenestration in their walls and arranged as a reticulum at one of the nuclear poles, all of them characteristics often associated with the Golgi cisternae (43). The previous localization of the bulk of intracellular GLUT4 in cisternae and tubulovesicular structures clustered on the trans side Golgi is consistent with these observations (7, 8, 57). The localization of endocytosed GLUT4(Ser5) complexes in a structure separated from the cis-Golgi, a TGN membrane protein that resides and stains uniformly all the TGN subcompartments (27), strongly suggests the GSC is a compartment distinct from the TGN. It is, however, very little of what we know regarding the nature, organization, and boundaries of the TGN, an organelle with a very complex organization defined as the site of sorting of the proteins that traverse the Golgi. To define the boundaries between the TGN and adjacent compartments requires the use of antibody markers that are not always available. An important difference, however, between the Golgi and the neighboring compartments is their distinct sensitivity to BFA. The relatively rapid disassembly of all the Golgi parts in response to BFA and the resistance of the GSC to the drug, also noted on skeletal myotubes (57) and a characteristic of the vacuolar compartments localized beyond the Golgi involved in the transport of proteins to the cell surface (36–38), strongly suggest that the GSC is a compartment separated from the Golgi. This conclusion is also supported by the recent demonstration that GLUT4 is targeted from the TGN to the GSC by a mechanism involving the carboxyl Leu489-Leu491-based motif (20).

In agreement with a recent study we have observed that the bulk of intracellular GLUT4 is retained within a pool of LDM vesicles with a pLo = 1.072–1.083 (34). The recovery of the endocytosed antibody with this pool of vesicles points to these as the most likely constituents of the GSC. Furthermore, our studies on 3T3-L1 fibroblasts stably transfected with GLUT4 and adipocytes expressing endogenous GLUT4, indicate that the profiles of LDM fractionated by differential centrifugation on sucrose gradients are comparable (Fig. 11, compare A and B, left panel). This result strongly suggests that the GSC already exists in 3T3-L1 fibroblasts, a result also confirmed by the IMF microscopy studies. The universality of the GSC, repeatedly demonstrated by studies that examined the distribution of transfected GLUT4 in a variety of cells (20, 21, 41, 58) is, however, not totally accepted. The recent demonstration that GLUT4 is expressed in specific sets of neurons (44–49) and within them structures with the morphology characteristic of Golgi sacculles (44) shows that GLUT4 is not exclusively expressed in insulin-responsive cells and tends to support the universality of the GSC distribution. We would like to speculate that cells that do not express GLUT4 in vivo use the GSC to regulate the store and trafficking of other protein loads. Cells expressing GLUT4 in vivo might contain in their GSC a specific set of proteins used to respond to changes in glucose homeostasis by controlling the access, retention, and exit of GLUT4. This set of proteins could be replaced in cells that do not express GLUT4 by other sets designed to regulate the store and trafficking of different protein loads. The notion of GLUT4 being stored within a single compartment is not new, but a model in which the transporter is stored in scores of vesicles ready to fuse with the plasma membrane has also met ample support. The recent demonstration that the rate-limiting step for insulin-stimulated GLUT4 translocation is the trafficking of GLUT4 vesicles and not their fusion with the plasma mem-
GLUT4(Ser5) turnover, 50 mM chloroquine and 100 μM leupeptin were included during the preincubation, labeling, and chase periods (33). The gradient profiles of GLUT4(Ser5) (D, left panel), Rab4 and Rab5b (C), GLUT4(Ser5) (D, left panel), and endocytosed pAbEL (B and D, right panels) were studied by Western analysis. GLUT4 and GLUT4(Ser5) were probed with pAb828 and a second HRP goat anti-rabbit antibody using the ECL technique. Endocytosed pAbEL was directly probed with a HRP-conjugated goat anti-rabbit antibody, whereas the endosomal markers Rab4 and Rab5b were probed with specific rabbit pAb and a HRP-conjugated goat anti-rabbit antibody. The percentages of GLUT4 (72 ± 3%) and GLUT4(Ser5) (49 ± 2.8%) accumulated in fractions 6–11 were calculated from values measured by scanning the ECL developed films from three separate experiments. LDM enriched fractions loaded with pAbBEL prepared from 3T3-L1 fibroblasts stably expressing GLUT4(Ser5) and GLUT4 were solubilized in buffer B and then incubated overnight at 4°C with Sepharose alone (−) or with anti-GLUT4 pAb and a HRP-conjugated goat anti-rabbit antibody (E).

Among the organelles jamming the perinuclear area can be counted those constituting the secretory pathway, the structures involved in the recycling of endocytosed and transcytosed proteins, late endosomes, and lysosomes. It was therefore important to examine whether the GSC has morphological, physical, and functional properties distinct from those neighboring compartments. As discussed above, the immunostaining of fibroblasts and ADL cells with specific antibodies against GLUT4 and the Golgi marker MG160 clearly shows that the two organelles, although closely juxtaposed, are distinct. Furthermore, the codistribution of endocytosed GLUT4 and Tfn in vesicles and the decrease in the number of these following the chase of two endocytosed molecules agree with previous observations describing their endocytosis and transport to the perinuclear area packed in the same vesicles (40–42). On the other hand, the sealing of the GSC to Tfn disagrees with the results of previous studies performed by conventional IMF microscopy, which described as identical the perinuclear distributions of GLUT4 and the Tfn receptor (41) and tends to agree with the segregation of GLUT4 and the Tfn receptor in skeletal myotubes (57). The emergence of the GSC as an extended reticular structure among the pleiad of vesicles loaded with Tfn and GLUT4 dramatically illustrates the distinct nature of the GSC and the recycling compartment traversed by Tfn. Furthermore, the different distributions of GLUT4 and GLUT4(Ser5) and the identification of the vesicles that retain GLUT4(Ser5) as late endosomes/lysosomes show that GSC and lysosomes are distinct organelles.

The detection of GLUT4(Ser5) in the GSC and the exclusion of the internalized antibody/GLUT4(Ser5) complexes from that opens the question of what is the source of the GLUT4(Ser5) molecules detected in the GSC. There could be at least two not mutually exclusive sources: newly synthesized molecules and molecules recycled through a pathway that excludes the
plasma membrane. With regard to the first possibility, we have noted that T3T-L1 fibroblasts stably expressing GLUT4 that were loaded for 1 h with FITC-conjugated anti-GLUT4 pAbEL and then chased for 1 h with antibody-free medium that further incubation for 1, 2, and 3 h with anti-FITC antibodies did not decrease significantly the staining of the GSC. This observation, together with the relative rapid recycling of endocytosed GLUT4 to the GSC, strongly suggest that in unstimulated cells the targeting of GLUT4 from the GSC to the plasma membrane is slow as compared with its recycling. Although the retention of internalized GLUT4(Ser5) in endosomes, as precluded to compare the speed of these two processes in cells transfected with of GLUT4(Ser5), a difference comparable with that found in the GLUT4 studies could explain the detection of GLUT4(Ser5) in the GSC because this would never completely depleted of GLUT4(Ser5) molecules. With respect to the possibility that a pool of GLUT4(Ser5) and, perhaps, also of GLUT4 molecules could be recycled to the GSC through a pathway that excludes the plasma, although it is an attractive possibility, it requires a technology that, as far as we know, is not available.

While this study was in progress Melvin et al. (24) reported that the inactivation of the Phe5-Gln6-Gln7-Ile8 motif in adipocytes results in retention of GLUT4 in an endosomal compartment that can be partly ablated in cells incubated for 3 h with Tfn-HRP before developing the peroxidase reaction. This and our observations suggest that the Phe5-Gln6-Gln7-Ile8 motif mediates a transport step in endosomes. The different distributions of endocytosed mAb16B12/GLUT4(Ser5) complexes and Tfn, the down load of dextran beads into the vesicles that retain GLUT4(Ser5), and the staining of these vesicles with an anti-LIMPIII antibody, indicate, however, that the mutant is protected by microscopy, we have observed that the levels of GLUT4(Ser5) and, perhaps, also of GLUT4 by the trypsin added to the incubation medium, therefore suggesting that GLUT4(Ser5) is significantly retained at the plasma membrane. Previous studies have involved the NH2 tail of GLUT4 and, specifically, the Phe5-Gln6-Gln7-Ile8 motif (16, 19), in the internalization of GLUT4. It should be pointed, however, that the GLUT4(Ser5) and the possibility to load the organelles that retain these two molecules with probes (i.e. HRP-conjugated antibodies) successfully used in organelle isolation (52) should help progress in their biochemical characterization. As part of this effort, it will be interesting to compare the bioemical properties of the GSC from cells expressing or not the transporter.

The different cellular distributions of GLUT4 and GLUT4(Ser5) and the possibility to load the organelles that retain these two molecules with probes (i.e. HRP-conjugated antibodies) successfully used in organelle isolation (52) should help progress in their biochemical characterization. As part of this effort, it will be interesting to compare the bioemical properties of the GSC from cells expressing or not the transporter.

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Recycling of the Insulin-sensitive Glucose Transporter GLUT4: ACCESS OF SURFACE INTERNALIZED GLUT4 MOLECULES TO THE PERINUCLEAR STORAGE COMPARTMENT IS MEDIATED BY THE Phe5-Gln6-Gln7-Ile8MOTIF

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