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The C-terminus of GLUT4 targets the transporter to the perinuclear compartment but not to the insulin-responsive vesicles

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Abstract

Postprandial blood glucose clearance is mediated by GLUT4 (glucose transporter 4) which is translocated from an intracellular storage pool to the plasma membrane in response to insulin. The nature of the intracellular storage pool of GLUT4 is not well understood. Immunofluorescence staining shows that, under basal conditions, the major population of GLUT4 resides in the perinuclear compartment. At the same time, biochemical fractionation reveals that GLUT4 is localized in IRVs (insulin-responsive vesicles). The relationship between the perinuclear GLUT4 compartment and the IRVs is not known. In the present study, we have exchanged the C-termini of GLUT4 and cellugyrin, another vesicular protein that is not localized in the IRVs and has no insulin response. We have found that GLUT4 with the cellugyrin C-terminus loses its specific perinuclear localization, whereas cellugyrin with the GLUT4 C-terminus acquires perinuclear localization and becomes co-localized with GLUT4. This, however, is not sufficient for the effective entry of the latter chimaera into the IRVs as only a small fraction of cellugyrin with the GLUT4 C-terminus is targeted to the IRVs and is translocated to the plasma membrane in response to insulin stimulation. We suggest that the perinuclear GLUT4 storage compartment comprises the IRVs and the donor membranes from which the IRVs originate. The C-terminus of GLUT4 is required for protein targeting to the perinuclear donor membranes, but not to the IRVs.

Keywords

adipocyte; cellugyrin; glucose transporter 4 (GLUT4); protein chimaera; syntaxin 6; vesicle

INTRODUCTION

Insulin stimulates glucose uptake in fat and skeletal muscle cells by redistributing GLUT4 (glucose transporter 4) from its insulin-responsive intracellular storage compartment(s) to

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the plasma membrane. Although the nature of the insulin-responsive compartment(s) is still under debate, biochemical studies suggest that it mainly represents small 60S–80S membrane vesicles, or IRVs (insulin-responsive vesicles) that, in adipose cells, accumulate 60–75 %of the total pool of GLUT4 [1,2]. The rest of the transporter is localized in heavy, rapidly sedimenting membranes that may include the plasma membrane, endosomes [3] and TGN (*trans*-Golgi network) [4].

Immunofluorescence staining demonstrates that the major intracellular pool of GLUT4 is localized in the perinuclear membranes [5,6], which are likely to represent a combination of the IRVs, recycling endosomes and TGN [7]. The nature of signals that target GLUT4 to each of these individual compartments is not yet clear.

Available evidence indicates that the C-terminus of GLUT4 plays an important role in the acquisition of insulin responsiveness of the transporter [4–6,8–19], whereas the N-terminus may be more involved in its endocytosis [5,6,11,19,20]. Previous studies, however, did not address GLUT4 targeting to small IRVs and used the functional criterion (i.e. insulin-dependent translocation to the cell surface) as the major, or the only, proof of GLUT4 localization in the insulin-responsive compartment. Considering that a protein can arrive at the plasma membrane via several different trafficking pathways with varying degrees of insulin responsiveness, unequivocal interpretation of previous results is complicated. In addition, many of these results were obtained using a ‘loss-of-function’ approach that cannot differentiate between specific and non-specific inactivation of the reporter proteins.

In the present study, we decided to use both ‘loss-of-function’ and ‘gain-of-function’ approaches in order to examine whether or not the C-terminus of GLUT4 is sufficient for the acquisition of insulin responsiveness and targeting into the IRVs. Our plan was to exchange the C-termini tails of GLUT4 and cellugyrin which, we believe, represents an appropriate reporter molecule for such studies. GLUT4 and cellugyrin are naturally co-expressed in adipocytes at high levels and share the same general design of the molecule with both proteins having several transmembrane domains (Figure 1). The overall intracellular localization of GLUT4 and cellugyrin in 3T3-L1 adipocytes, as revealed by immunofluorescence staining, shows very little overlap (see below). In addition, major pools of GLUT4 and cellugyrin are localized in different populations of intracellular vesicles that can be readily separated by gradient centrifugation ([21] and the present study). Unlike IRVs that compartmentalize most of the intracellular GLUT4, cellugyrin-containing vesicles are completely resistant to insulin stimulation and cellugyrin is not translocated to the plasma membrane in response to insulin ([21,22] and the present study). Last, but not least, the C-terminus of cellugyrin does not carry any targeting information [23], which is essential for the interpretation of results. We have found that the C-terminus of GLUT4 is required for protein localization in the perinuclear compartment, but is not sufficient for the efficient targeting to the IRVs.

MATERIALS AND METHODS

Materials

Aprotinin, leupeptin, pepstatin A, PMSF, Triton X-100 and sucrose were obtained from American Bioanalytical. G418 (Geneticin), calf bovine serum, fetal bovine serum and DMEM (Dulbecco's modified Eagle's medium) were purchased from Invitrogen. Alexa Fluor® 488 donkey anti-mouse IgG and the SlowFade-Light Antifade kit were obtained from Molecular Probes (Carlsbad, CA, U.S.A.). Cy3 (indocarbocyanine)-conjugated donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories. All other reagents were from Sigma.

Antibodies

We used a monoclonal anti-GLUT4 antibody 1F8 [24], a monoclonal antibody against syntaxin 6 (BD Biosciences), a monoclonal antibody against EGFP (enhanced green fluorescent protein, sc-9996; Santa Cruz Biotechnology), a monoclonal anti-Myc antibody 9B11 and a polyclonal anti-Myc antibody (both from Cell Signaling Technology), a polyclonal antibody against cellugyrin [2], and Alexa Fluor® 488-conjugated, as well as Cy3-conjugated, donkey anti-mouse and anti-rabbit IgG (ImmunoResearch Laboratories).

Plasmids

The cellugyrin–GLUT4 chimaera [CG (cellugyrin with the C-terminus of GLUT4) in Figure 1] containing cellugyrin Met^{1}–Tyr^{177} and GLUT4 Val^{468}–Asp^{509} was obtained by three consecutive PCR reactions. The cellugyrin part of the molecule was obtained with the following primers: Ceg-Glut4 N (5′-GCGGATCCTTGGGTTCCATTTACAG-3′) and Ceg-Glut4 M2 (5′-CTCGAGTTTCAGGTACGTAGGCCAGGGAGG-3′). The GLUT4 part of the molecule was obtained with the following primers: Ceg-Glut4 M1 (5′-CCTCCCTGGCCTACGTACCTGA-AACTCGAG-3′) and Ceg-Glut4 C (5′-GCGCGGCCGCCTATT-AGT-3′). The two PCR products were mixed together and used as a new template. The third PCR reaction was carried out with the primers Ceg-Glut4 N and Ceg-Glut4 C. The final PCR product was subcloned into the retroviral vector pLNCX2 using BamHI and NotI restriction sites.

A cellugyrin–GLUT4 chimaera with EGFP at the N-terminus (EGFP–CG in Figure 1) contained cellugyrin Met^{1}–Phe^{187} and GLUT4 Arg^{472}–Asp^{509} was prepared as follows. The first PCR reaction for the cellugyrin part of the molecule was performed with the following primers: CeN-Bgl-Start (5′-CGGAAGATCTC-GATGCCCTTGAGGGTCGGC-3′) and CeGl-M3–5 (5′-GTCA-AACGTCCGGCCTCGGAAGGCATCCACTCCAGC-3′). The second PCR reaction for the GLUT4 part of the molecule was performed with the following primers: CeGl-M5-3 (5′-GCTGGA-GTGGATGCCTTCCGAGGCCGGACGTTTGAC-3′) and GluC-Sal-Stop (5′-GACGCGTCGACTTAGTCATTCTCATCTGG-3′). The two PCR products were mixed together and used as a new template. The third PCR reaction was carried out with the primers CeN-Bgl-Start and GluC-Sal-Stop. The final PCR product was subcloned into the pEGFP-C2 vector using BglII and SalI restriction sites. EGFP-tagged CG was then amplified using primers: CG-Bam-GFP-Start (5′-CGGGATCCATGGTGAGC-

AAGGGC-3′) and CG-Sal-Stop and subcloned into BamHI and SalI sites of the retroviral vector pLNCX2.

In order to obtain CG–Myc (Figure 1), cellugyrin Met^{1}–Phe^{187} and GLUT4 Arg^{472}–Asp^{509} fragments were obtained by PCR reaction using GFP–CG as a template with primers: Ce-EcoRI-Start (5′-CCGGAATTCATGCCCTTGAGGGTCGGC-3′) and G4-XhoI-NoStop (5′-CGTACGCTCGAGGTCATTCTCATCTGGC-CCTAA-3′). The PCR product was subcloned into the pcDNA3. 1MycHisA vector using EcoRI and XhoI restriction sites.

A Myc_7–GLUT4–cellugyrin chimaera [Myc–GC (GLUT4 with the C-terminus of cellugyrin) in Figure 1] containing GLUT4 Met^{1}–Ile^{479} and cellugyrin Tyr^{180}–Tyr^{234} was constructed as follows. The C-terminus of cellugyrin was amplified by PCR reaction using primers: 5′-CCAGATCTACAAGGCTGGAGTG-3′ and 5′-GGCGGCCGCGTAAAACCCTCTCCT-3′, and sub-cloned into the pLNCX2 vector using BglII and NotI restriction sites in order to obtain pLNCX2-Ceg-C. A GLUT4 fragment with seven Myc tags in the first extracellular loop was excised from the pLNCX2-Myc_7-GLUT4 plasmid [25] using BglII restrictase. This fragment was subcloned into pLNCX2-Ceg-C using a BglII restriction site to obtain pLNCX2-Myc–GC.

Cell culture and retroviral infection

Murine 3T3-L1 preadipocytes were cultured, differentiated and maintained as described previously [25a]. Briefly, cells were grown in DMEM supplemented with 10 %(v/v) calf bovine serum until confluence. At 2 days later, the cells were transferred to the differentiation medium [DMEM containing 10 % (v/v) fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexa-methasone and 1.7 μM insulin]. After 48 h, the differentiation medium was replaced with the maintenance medium [DMEM supplemented with 10 %(v/v) fetal bovine serum]. The maintenance medium was changed every 48 h. The cells were used at day 8 of differentiation. For stable expression in 3T3-L1 cells, pLNCX2 empty vector or pLNCX2 carrying target genes (20 μg of each) were first added to PT67 packaging cells at 70–80 % confluency in a P100 Petri dish for 48 h. The medium was then replaced with 5.5 ml of DMEM containing 10 %(v/v) fetal bovine serum. After another 48 h, the virus-containing medium was collected, filtered through a 0.45 μm filter and added, together with 4 μg/ml polybrene, to a P100 Petri dish with 3T3-L1 preadipocytes at 20–30 % confluency for 8 h. After a 48 h recovery period, infected cells were selected in DMEM containing 10 % (v/v) calf bovine serum and 400 μg/ml G418. Clones of G418-resistant cells were combined and used for experiments. Stable transfection of 3T3-L1 cells with pBabe-Myc-Glut4 have been described previously [25].

Electroporation

3T3-L1 adipocytes grown in a 10 cm Petri dish were trypsinized, washed twice with PBS and re-suspended in 0.5 ml of PBS. Plasmid DNA (100 μg) and the suspension of adipocytes were mixed in a gene pulser cuvette with a 0.4 cm electrode gap. Electroporation was performed with a Bio-Rad Gene Pulser with 960 μF capacitance at 0.16 kV for 16–18 ms. After gene transfer, cells were transferred to 4 ml of DMEM with 10 % (v/v) calf serum for 10 min for recovery at room temperature (21 °C) and then re-plated on to cover slips.

Immunofluorescence staining

3T3-L1 adipocytes were lifted and grown overnight on cover slips coated with poly-L-lysine. Cells were fixed with 4 % (w/v) paraformaldehyde in PBS for 15 min, and permeabilized (where indicated) using PBS with 0.2 %Triton X-100. Cells were washed with PBS and incubated in blocking solution [PBS with 5 %(w/v) BSA and 5 % (v/v) donkey serum] for 1 h at room temperature. Cells were stained with primary and then with secondary antibodies and mounted on slides using the SlowFade-Light Antifade kit (Molecular Probes). Cells were examined with a Axiovert 200M fluorescence microscope and the Axiovision 3.0 program (Carl Zeiss) or with a confocal laser-scanning microscope LSM510 (Carl Zeiss). Confocal images were analysed using the image analysis program, ImageJ (National Institutes of Health).

Subcellular fractionation

Prior to harvesting, cultured 3T3-L1 adipocytes were washed three times with serum-free DMEM warmed to 37 °C and starved in the same medium for 2 h. Where indicated, cells were treated with 100 nM insulin or carrier (5 mM HCl at 1000× dilution) in DMEM for 30 min at 37 °C. Then, cells were washed three times with warm HES buffer [250 mM sucrose, 20 mM Hepes (pH 7.4), 1 mM EDTA, 1 μM aprotinin, 2 μM leupeptin, 1 μM pepstatin, 5 mM benzamidine and 1 mM PMSF]. Cells were harvested in HES buffer, homogenized by 11 strokes in a ball-bearing homogenizer (Isobiotec) with 12 μm clearance, and centrifuged at 1500 ***g*** for 5 min in order to obtain post-nuclear supernatant which was then centrifuged at 16 000 ***g*** for 20 min. IRVs were recovered in the supernatant of this centrifugation. Alternatively, plasma membrane, heavy microsomes, light microsomes and the nuclear/mitochondrial fraction were obtained by differential centrifugation as described previously [26]. Samples were re-suspended in HE buffer [20 mM Hepes (pH 7.4), 1 mM EDTA, 1 μM aprotinin, 2 μM leupeptin, 1 μM pepstatin, 5 mM benzamidine and 1 mM PMSF], and the protein concentration was determined using a BCA (bicinchoninic acid) kit (Pierce Chemical).

Sucrose-gradient centrifugation

3T3-L1 adipocytes were homogenized in a ball-bearing homogenizer, and the homogenate was centrifuged at 16 000 ***g*** for 20 min. The supernatant (400 μg) was loaded on to a 10–30 % sucrose gradient and centrifuged for 55 min in a Beckman SW-50.1 rotor at 48 000 rev./min. Each gradient was collected into 23–25 fractions starting from the bottom of the tube.

Antibody shift assay

The 16 000 ***g*** supernatant (800 μg) was obtained from 3T3-L1 adipocytes as described above and incubated with 2.5 μg of purified non-specific mouse IgG or anti-Myc monoclonal antibody, together with 2 μg of nanogold-conjugated goat anti-mouse Fab fragments (Nanoprobes). The mixture was rotated at 4 °C for 2 h. The whole sample was then subjected to sucrose-gradient centrifugation (as described above).

FRAP (fluorescence recovery after photobleaching) analysis

Differentiated 3T3-L1 adipocytes stably expressing EGFP–cellugyrin or EGFP–CG (Figure 1) were plated on to collagen-coated, glass-bottomed dishes (MakTek Corporation) and allowed to recover for 18–36 h. The perinuclear region was photobleached at 75 %laser power using an Argon/2 laser (100 %transmission, 20 iterations). After photobleaching, fluorescence recovery was monitored by scanning at 1.5 % laser power at 5 s intervals over a total of 220 scans. During the entire FRAP experiment, cells were maintained at 37 °C using a heated stage and a × 63 objective lens was used. The extent of recovery was quantified using the Zeiss LSM software package (version 3.2 SP2), and expressed as a fraction of the initial fluorescence intensity.

Gel electrophoresis and immunoblotting

Proteins were separated by SDS/PAGE according to the method of Laemmli [26a] and transferred to a PVDF membrane in 25 mM Tris and 192 mM glycine. Following transfer, the membrane was blocked with 10 % (w/v) non-fat dried skimmed milk in PBS for 1 h at 25 °C and probed with specific antibodies overnight. The membranes were washed three times with PBST (PBS with 0.05 % Tween 20) and incubated with HRP (horseradish peroxidase)-labelled secondary antibody for 1 h at room temperature. After three more washes, the membranes were incubated with the ECL (enhanced chemiluminescent) reagent (New England Nuclear) for 1 min and then exposed in a Kodak440 Image Station. Data analysis was performed using Kodak one-dimensional image analysis software.

Statistical analysis

A Student’s unpaired two-tailed *t* test was used to evaluate the statistical significance of the results.

RESULTS

Figure 2(A) shows the intracellular localization of endogenous cellugyrin in 3T3-L1 adipocytes stably expressing Myc–GLUT4 [25]. In these cells, cellugyrin is randomly distributed throughout the cell, while Myc–GLUT4 is localized primarily in the perinuclear region where it co-localizes with syntaxin 6 (Figure 2B) (see also [4,27]).

Then we prepared 3T3-L1 cells stably expressing Myc-tagged GC (Myc–GC in Figure 1). Unlike GLUT4, the Myc–GC chimaera was not concentrated in the syntaxin-6-positive perinuclear compartment, but instead showed a diffuse intracellular distribution (Figure 2C). This observation is consistent with the results of Shewan et al. [4] who have found that the C-terminus of GLUT4 contains sequences that target the transporter to the perinuclear syntaxin-6/16-positive compartment [4].

In order to confirm this result, we used the ‘gain-of-function’ approach and stably transfected cells with either EGFP–cellugyrin or EGFP–CG (Figure 1). Addition of EGFP to the N-terminus of cellugyrin changed somewhat the total intracellular localization of the protein, as some EGFP–cellugyrin-expressing cells demonstrated perinuclear fluorescence not detectable in other cells (Figure 3A, left-hand panel). This suggests that the addition of

EGFP to the cellugyrin molecule leads to the transient association of the chimaera with perinuclear membranes by, for example, slowing down the movement of cellugyrin through this compartment. Nonetheless, the intracellular localization of EGFP–cellugyrin is clearly different from that of EGFP–CG, which demonstrates dramatic accumulation in the perinuclear region of the cell (Figure 3A, right-hand panel) where it co-localizes with GLUT4 and syntaxin 6 (Figures 3B and 3C). The statistical analysis of the data shows that 29 ± 12 %of total intracellular EGFP–cellugyrin compared with 47 ± 9 %of EGFP–CG is localized in the perinuclear region of the cell ($P < 0.0001$).

These results demonstrate that the C-terminus of GLUT4 contains the information required for protein targeting and/or retention in the perinuclear syntaxin-6-positive compartment. In order to confirm this observation, we used FRAP. In these experiments, we analysed cells with marked perinuclear localization of both EGFP–cellugyrin and EGFP–CG. The perinuclear region of these cells was photobleached with a high-intensity laser, and fluorescence intensity of the bleached area was measured every 5 s. As shown in Figure 4, EGFP–CG re-populated the perinuclear area faster than EGFP–cellugyrin, which is consistent with the idea that the C-terminus of GLUT4 is important for the perinuclear localization of the transporter.

The next question that we asked was whether or not GC and CG chimaeras possess insulin responsiveness in the adipocyte. Since Myc–GC has Myc epitopes in the first extracellular loop, we determined the extent of extracellular exposure of the Myc epitope in non-permeabilized insulin-treated and non-treated cells using a Myc antibody. Figure 5(A) shows that Myc–GC translocates to the plasma membrane upon insulin administration. GFP–cellugyrin and GFP–CG do not contain recognizable extracellular epitopes. Therefore, in order to assess insulin responsiveness of these chimaeras, we used biochemical fractionation.

Separation of basal 3T3-L1 adipocytes into the plasma-membrane fraction and the vesicular fraction showed that major pools of chimaera proteins, as well as endogenous cellugyrin and GLUT4, were recovered in the vesicular fraction (Figure 5B). Western blot analysis of the plasma-membrane fractions purified from insulin-treated and non-treated adipocytes confirmed that neither endogenous cellugyrin nor ectopically expressed EGFP–cellugyrin were translocated to the plasma membrane in response to insulin stimulation (Figures 5C and 5D; see also [21,22]). We also found that the insulin responsiveness of Myc–GC was significantly lower than that of endogenous GLUT4 (Figures 5C and 5D). Given the dramatic changes in the intracellular localization of this chimaera (Figure 2), this result is not surprising. An unexpected finding, however, is that EGFP–CG, in spite of a significant overall co-localization with GLUT4 and syntaxin 6, demonstrates only a small 2-fold translocation, which is even less than translocation of Myc–GC.

A possible explanation of this result is that, despite its presence in the perinuclear GLUT4- and syntaxin-6-containing membranes, EGFP–CG fails to target to the insulin-responsive compartment which is represented by the IRVs [2,25,28]. In order to test this hypothesis, we have performed sucrose-gradient fractionation of adipocyte extracts. Figure 6 shows that the distribution of EGFP–CG significantly, but not completely, overlaps with IRVs marked by

endogenous GLUT4. Indeed, some EGFP–CG is present in fractions 13–15 that contain very little GLUT4. Note that both endogenous GLUT4 and ectopically expressed EGFP–CG are recognized by the same 1F8 antibody raised against the C-terminus of GLUT4. It is also seen in Figure 6 that the insulin response of GLUT4 and EGFP–CG is quite different. The amount of GLUT4 in the vesicular fraction is dramatically decreased after insulin administration due to its translocation to the plasma membrane. On the other hand, the amount of EGFP–CG in the vesicular fraction is decreased only marginally, in agreement with the results shown in Figure 5.

In any case, co-sedimentation itself cannot be a proof of CG targeting into the IRVs. A partial overlap of EGFP–CG and GLUT4 in the sucrose gradient may indicate that the former molecule is targeted to a vesicular population with a sedimentation co-efficient similar to, but not identical with, that of the IRVs. Alternatively, EGFP–CG may be distributed among several different types of vesicular carriers with IRVs being one of them. Since the sensitivity of available antibodies and moderate levels of EGFP–CG expression in retrovirus-infected cells do not allow us to further fractionate EGFP–CG-containing vesicles, we have created additional reporter proteins, CG–Myc/His and cellugyrin–Myc/His (Figure 1). We have expressed both reporters in 3T3-L1 adipocytes by electroporation, taking advantage of the high levels of protein expression in electroporated cells and also of the specificity and the high affinity of the commercial monoclonal antibody against the Myc epitope.

Regardless of the high levels of reporter expression in electroporated cells, we found that CG–Myc/His was localized in the perinuclear region of the cell, similar to EGFP–CG (compare Figures 3A and Supplementary Figure S1 at http://www.BiochemJ.org/bj/419/bj4190105add.htm), whereas cellugyrin–Myc/His occupied the whole cell volume similar to endogenous cellugyrin. Supplementary Figure S2 (at http://www.BiochemJ.org/bj/419/bj4190105add.htm) shows that the sedimentational distribution of CG–Myc/His is the same as EGFP–CG, thereby validating the use of electroporation for further experiments. Finally, Western blot analysis of the plasma-membrane fractions purified from insulin-treated and non-treated adipocytes showed that, in agreement with Figures 5(C) and 5(D), cellugyrin–Myc/His is not translocated to the plasma membrane in response to insulin stimulation, whereas CG–Myc/His shows some tendency to such translocation, although results did not reach statistical significance (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/419/bj4190105add.htm).

The most straightforward technique to study the IRV targeting of CG–Myc/His is immunoadsorption. Unfortunately, we cannot use a well-established immunoadsorption procedure with the monoclonal 1F8 antibody raised against the C-terminus of GLUT4 as it also recognizes CG. Therefore we used a different approach. We electroporated 3T3-L1 adipocytes with cDNAs for cellugyrin–Myc/His and CG–Myc/His. Extracts of transfected adipocytes were incubated with an excess of the anti-Myc antibody (or non-specific IgG) together with nanogold-conjugated goat anti-mouse Fab fragments. During the incubation period, the heavy antibody–Fab–nanogold complex bound to the cytoplasmic Myc epitope of the target proteins, and ‘decorated’ and ‘non-decorated’ vesicles were then analysed by sucrose-gradient centrifugation (see also [23]).

Figure 7(A) shows that the complex of the anti-Myc antibody and nanogold-conjugated Fab fragments caused a dramatic shift of cellugyrin–Myc/His-containing vesicles from fractions 9–19 to the bottom of the centrifuge tube. The addition of non-specific IgG was completely without effect and did not result in any noticeable shift of vesicles (results not shown). At the same time, the position of the IRVs stays the same (fractions 7–11) in samples incubated with either non-specific IgG or with anti-Myc antibody, indicating that cellugyrin–Myc/His is not present in IRVs to any significant extent.

Next, we have applied the same technique to CG–Myc/His-expressing cells. As is the case with cellugyrin–Myc/His, the anti-Myc antibody bound to nanogold-conjugated Fab fragments caused a dramatic shift of CG-containing vesicles (Figure 7B). However, it also caused a small, but still detectable, shift of the IRVs. These results suggest that the majority of CG–Myc/His is localized in vesicles that are different from the IRVs, and only a small fraction of CG–Myc/His is present in this compartment.

DISCUSSION

During its insulin-regulated trafficking to and from the plasma membrane, GLUT4 passes through several intracellular compartments [29]. Correspondingly, the GLUT4 molecule contains multiple targeting sequences in its cytoplasmic domains. Previous studies have demonstrated that some of these sequences are localized in the C-terminus of the transporter. In particular, the C-terminus is required for the intracellular retention of GLUT4 in various cell types [5,6,9,10,12,17,30]. In addition, specific signals within the C-terminus are responsible for the localization of the transporter specifically in the perinuclear compartment [4,6,31,32]. These, or adjacent, signals also prevent GLUT4 from entering the constitutive endosomal recycling pathway [16,19,33,34]. It remains unclear, however, whether or not the perinuclear compartment is equivalent to the IRVs and if targeting to the perinuclear compartment is sufficient for effective translocation to the plasma membrane.

In the present study, we have confirmed that GLUT4 targeting to the perinuclear compartment is defined by the C-terminus of the transporter. Our experiments differ from previously published reports in that we have used both ‘loss-of-function’ and ‘gain-of-function’ approaches in order to demonstrate the targeting role of the C-terminus. Moreover, we show in the present study, for the first time, that the C-terminus of GLUT4 is not sufficient for the targeting of the transporter into the genuine insulin-responsive compartment, the IRVs. This result is consistent with the model according to which the ‘perinuclear GLUT4 storage compartment’ represents a mixture of the IRVs and donor membranes (recycling endosomes and/or TGN) that exist in a dynamic equilibrium [35,36].

We suggest that the C-terminus of GLUT4 may define its targeting to the perinuclear donor membranes. This, however, may not be sufficient for the efficient entry into the small IRVs. Additional targeting signals must facilitate the distribution of GLUT4 from the donor membranes to the IRVs. To this end, it has been shown that the entry of the newly synthesized GLUT4 into the insulin-responsive compartment requires both the N-terminus and the central loop [13]. It remains to be determined whether or not targeting of newly

synthesized GLUT4 molecules is different from the pre-synthesized pool of recycling GLUT4. Future studies should give an answer to this question.

The results of the present study show, nonetheless, that the C-terminus of GLUT4 confers some degree of insulin responsiveness to cellugyrin, which is consistent with previously published data (see, for example, [6]). In order to explain this phenomenon, we suggest that once the protein, such as CG, enters the ‘donor’ compartment, it can be captured by budding IRVs by, for example, mass-action. However, since CG, unlike GLUT4, is not actively sorted into vesicles, only a small fraction of its total population is distributed to the IRVs and acquires insulin responsiveness.

How can we explain the relatively high insulin responsiveness of GC, which is not localized in the perinuclear ‘donor’ membranes? One possibility is that GC rapidly traverses the perinuclear donor membranes with the bulk of membrane flow. However, as GC lacks the C-terminus of GLUT4, it is not retained in this compartment. Rapid transit through the perinuclear compartment may, nevertheless, provide an opportunity for GC to enter the IRVs using putative IRV targeting signals that this chimaera should still have. After multiple rounds of trafficking via the perinuclear compartment, a significant fraction of GC should re-distribute into the IRVs and thus escape constitutive endocytic recycling. Thus, according to our model, the C-terminus of GLUT4 increases the efficiency of sorting into the IRVs by retaining the protein in the IRV ‘donor’ compartment but is not absolutely required for entry into the IRVs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Cy3	indocarbocyanine
DMEM	Dulbecco’s modified Eagle’s medium
EGFP	enhanced green fluorescent protein
FRAP	fluorescence recovery after photobleaching
GLUT4	glucose transporter 4
GC	GLUT4 with the C-terminus of cellugyrin
CG	cellugyrin with the C-terminus of GLUT4
IRV	insulin-responsive vesicle

TGN *trans*-Golgi network

References

1. Kandror KV, Coderre L, Pushkin AV, Pilch PF. Comparison of GLUT4-containing vesicles from rat fat and muscle tissues: evidence for a unique endosomal compartment. Biochem J. 1995; 307:383–390. [PubMed: 7733873]
2. Xu Z, Kandror KV. Translocation of small preformed vesicles is responsible for the insulin activation of glucose transport in adipose cells. Evidence from the *in vitro* reconstitution assay. J Biol Chem. 2002; 277:47972–47975. [PubMed: 12393900]
3. Zeigerer A, Lampson MA, Karylowski O, Sabatini DD, Adesnik M, Ren M, McGraw TE. GLUT4 retention in adipocytes requires two intracellular insulin-regulated transport steps. Mol Biol Cell. 2002; 13:2421–2435. [PubMed: 12134080]
4. Shewan AM, van Dam EM, Martin S, Luen TB, Hong W, Bryant NJ, James DE. GLUT4 recycles via a *trans*-Golgi network (TGN) subdomain enriched in syntaxins 6 and 16 but not TGN38: involvement of an acidic targeting motif. Mol Biol Cell. 2003; 14:973–986. [PubMed: 12631717]
5. Marsh BJ, Alm RA, McIntosh SR, James DE. Molecular regulation of Glut4 targeting in 3T3-L1 adipocytes. J Cell Biol. 1995; 130:1081–1091. [PubMed: 7544796]
6. Verhey KJ, Yeh JI, Birnbaum MJ. Distinct signals in the Glut4 glucose transporter for internalization and for targeting to an insulin-responsive compartment. J Cell Biol. 1995; 130:1071–1079. [PubMed: 7657693]
7. Dugani CB, Klip A. Glucose transporter 4: cycling, compartments and controversies. EMBO Rep. 2005; 6:1137–1142. [PubMed: 16319959]
8. Asano T, Takata K, Katagiri H, Tsukuda K, Lin JL, Ishihara H, Inukai K, Hirano H, Yazaki Y, Oka Y. Domains responsible for the differential targeting of glucose transporter isoforms. J Biol Chem. 1992; 267:19636–19641. [PubMed: 1527083]
9. Corvera S, Chawla A, Chakrabarti R, Joly M, Buxton J, Czech MP. A double leucine within the Glut4 glucose transporter COOH-terminal domain functions as an endocytosis signal. J Cell Biol. 1994; 126:979–989. [PubMed: 7519625]
10. Czech MP, Chawla A, Woon CW, Buxton J, Armoni M, Wei T, Joly M, Corvera S. Exofacial epitope-tagged glucose transporter chimeras reveal COOH-terminal sequences governing cellular localization. J Cell Biol. 1993; 123:127–136. [PubMed: 8408193]
11. Garippa RJ, Judge TW, James DE, McGraw TE. The amino terminus of GLUT4 functions as an internalization motif but not an intracellular retention signal when substituted for the transferrin receptor cytoplasmic domain. J Cell Biol. 1994; 124:705–715. [PubMed: 8120093]
12. Haney PM, Levy MA, Strube MS, Mueckler M. Insulin-sensitive targeting of the Glut4 glucose transporter in L6 myoblasts is conferred by its COOH-terminal cytoplasmic tail. J Cell Biol. 1995; 129:641–658. [PubMed: 7730401]
13. Khan AH, Capilla E, Hou JC, Watson RT, Smith JR, Pessin JE. Entry of newly synthesized GLUT4 into the insulin-responsive storage compartment is dependent upon both the amino terminus and the large cytoplasmic loop. J Biol Chem. 2004; 279:37505–37511. [PubMed: 15247212]
14. Piper RC, Tai C, Kulesza P, Pang S, Warnock D, Baenziger J, Slot JW, Geuse HJ, Puri C, James DE. Glut4 NH2 terminus contains a phenylalanine-based targeting motif that regulates intracellular sequestration. J Cell Biol. 1993; 121:1221–1232. [PubMed: 8509445]
15. Piper RC, Tai C, Slot JW, Hahn CS, Rice C, Huang H, James DE. The efficient intracellular sequestration of the insulin-regulatable glucose transporter (Glut4) is conferred by the NH2 terminus. J Cell Biol. 1992; 117:729–743. [PubMed: 1577853]
16. Shewan AM, Marsh BJ, Melvin DR, Martin S, Gould GW, James DE. The cytosolic C-terminus of the glucose transporter Glut4 contains an acidic cluster endosomal targeting motif distal to the dileucine signal. Biochem J. 2000; 350:99–107. [PubMed: 10926832]

17. Verhey KJ, Hausdorff SF, Birnbaum MJ. Identification of the carboxy terminus as important for the isoform-specific subcellular targeting of glucose transporter proteins. J Cell Biol. 1993; 123:137–147. [PubMed: 7691826]

18. Song XM, Hresko RC, Mueckler M. Identification of amino acid residues within the C-terminus of the Glut4 glucose transporter that are essential for insulin-stimulated redistribution to the plasma membrane. J Biol Chem. 2008; 283:12571–12585. [PubMed: 18305115]

19. Melvin DR, Marsh BJ, Walmsley AR, James DE, Gould GW. Analysis of amino and carboxy terminal GLUT-4 targeting motifs in 3T3-L1 adipocytes using an endosomal ablation technique. Biochemistry. 1999; 38:1456–1462. [PubMed: 9931010]

20. Blot V, McGraw TE. GLUT4 is internalized by a cholesterol-dependent nystatin-sensitive mechanism inhibited by insulin. EMBO J. 2006; 25:5648–5658. [PubMed: 17139247]

21. Kupriyanova TA, Kandror V, Kandror KV. Isolation and characterization of the two major intracellular Glut4 storage compartments. J Biol Chem. 2002; 277:9133–9138. [PubMed: 11782457]

22. Kupriyanova TA, Kandror KV. Cellugyrin is a marker for a distinct population of intracellular Glut4-containing vesicles. J Biol Chem. 2000; 275:36263–36268. [PubMed: 10967091]

23. Belfort GM, Bakirtzi K, Kandror KV. Cellugyrin induces biogenesis of synaptic-like microvesicles in PC12 cells. J Biol Chem. 2005; 280:7262–7272. [PubMed: 15590695]

24. James DE, Brown R, Navarro J, Pilch PF. Insulin-regulatable tissues express a unique insulin-sensitive glucose transporter protein. Nature. 1988; 333:183–185. [PubMed: 3285221]

25. Shi J, Kandror KV. Sortilin is essential and sufficient for the formation of Glut4-storage vesicles in 3T3-L1 adipocytes. Dev Cell. 2005; 9:99–108. [PubMed: 15992544]

25a. Rubin CS, Hirsch A, Fung C, Rosen OM. Development of hormone receptors and hormonal responsiveness *in vitro* Insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3-L1 cells. J Biol Chem. 1978; 253:7570–7578. [PubMed: 81205]

26. Simpson IA, Yver DR, Hissin PJ, Wardzala LJ, Karnieli E, Salans LB, Cushman SW. Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: characterization of subcellular fractions. Biochim Biophys Acta. 1983; 763:393–407. [PubMed: 6360220]

26a. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227:680–685. [PubMed: 5432063]

27. Perera HK, Clarke M, Morris NJ, Hong W, Chamberlain LH, Gould GW. Syntaxin 6 regulates Glut4 trafficking in 3T3-L1 adipocytes. Mol Biol Cell. 2003; 14:2946–2958. [PubMed: 12857877]

28. ElJack A, Kandror KV, Pilch PF. Formation of an insulin-responsive vesicular compartment is an early event in 3T3-L1 adipocyte differentiation. Mol Biol Cell. 1999; 10:1581–1594. [PubMed: 10233164]

29. Bryant NJ, Govers R, James DE. Regulated transport of the glucose transporter glut4. Nat Rev Mol Cell Biol. 2002; 3:267–277. [PubMed: 11994746]

30. Verhey KJ, Birnbaum MJ. A Leu-Leu sequence is essential to the C-terminal targeting signal of the Glut4 glucose transporter in fibroblasts. J Biol Chem. 1994; 269:2353–2356. [PubMed: 8300557]

31. Martinez-Arca S, Lalioti VS, Sandoval IV. Intracellular targeting and retention of the glucose transporter Glut4 by the perinuclear storage compartment involves distinct carboxyl-tail motifs. J Cell Sci. 2000; 113:1705–1715. [PubMed: 10769202]

32. Sandoval IV, Martinez-Arca S, Valdueza J, Palacios S, Holman GD. Distinct reading of different structural determinants modulates the dileucine-mediated transport steps of the lysosomal membrane protein LIMPII and the insulin-sensitive glucose transporter Glut4. J Biol Chem. 2000; 275:39874–39885. [PubMed: 10973972]

33. Araki S, Yang J, Hashiramoto M, Tamori Y, Kasuga M, Holman GD. Subcellular trafficking kinetics of GLU4 mutated at the N- and C-terminal. Biochem J. 1996; 315:153–159. [PubMed: 8670101]

34. Cope DL, Lee S, Melvin DR, Gould GW. Identification of further important residues within the Glut4 carboxy-terminal tail which regulate subcellular trafficking. FEBS Lett. 2000; 481:261–265. [PubMed: 11007975]

35. Eyster CA, Duggins QS, Gorbsky GJ, Olson AL. Microtubule network is required for insulin signaling through activation of Akt/protein kinase B: evidence that insulin stimulates vesicle docking/fusion but not intracellular mobility. J Biol Chem. 2006; 281:39719–39727. [PubMed: 17068336]

36. Karylowski O, Zeigerer A, Cohen A, McGraw TE. GLUT4 is retained by an intracellular cycle of vesicle formation and fusion with endosomes. Mol Biol Cell. 2004; 15:870–882. [PubMed: 14595108]

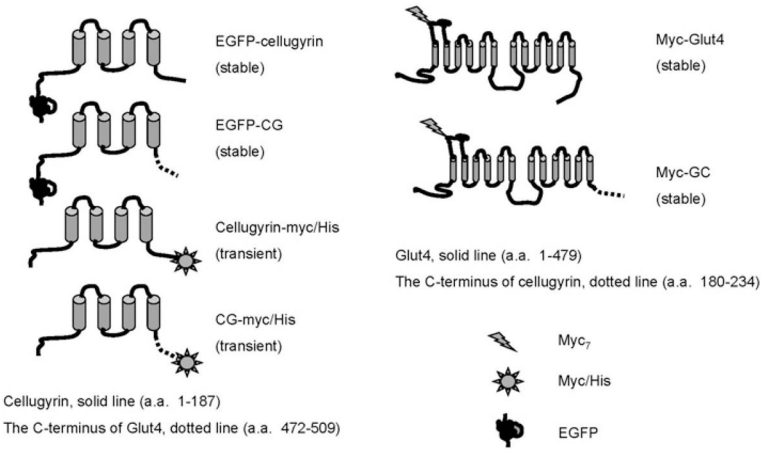

Figure 1. Schematic representation and abbreviations of the reporter molecules used in the present study

The method of protein expression is indicated in parentheses.

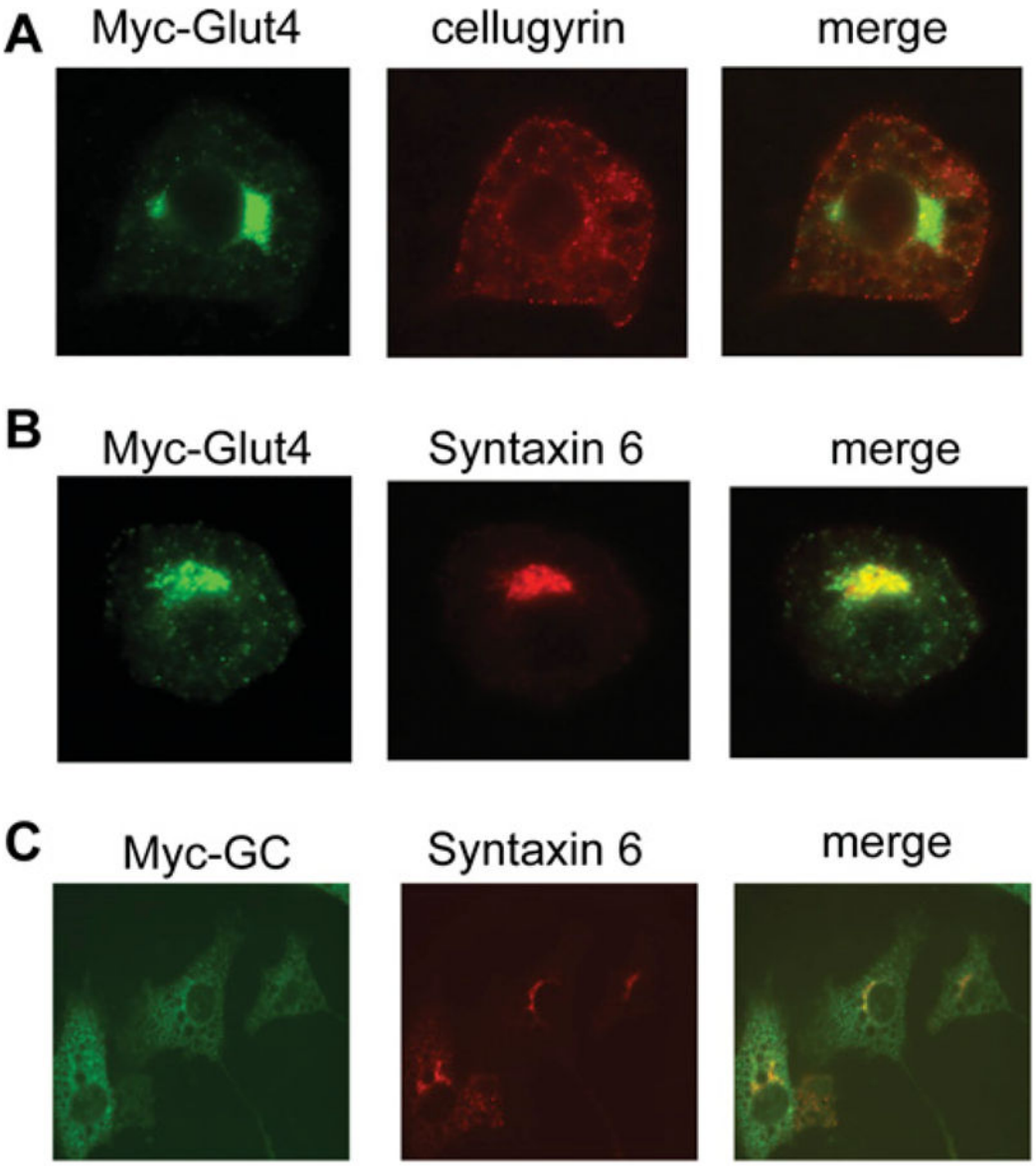

Figure 2. GLUT4 with the C-terminus of cellugyrin (GC) loses its perinuclear localization

(**A**) 3T3-L1 adipocytes were fixed, permeabilized and stained with the monoclonal anti-Myc antibody and polyclonal antibody against cellugyrin followed by Alexa Fluor® 488-conjugated donkey anti-mouse and Cy3-conjugated donkey anti-rabbit secondary antibodies. (**B** and **C**) Cells were stained with polyclonal anti-Myc antibody and monoclonal antibody against syntaxin 6 and then with Cy3-conjugated donkey anti-mouse and Alexa Fluor® 488-conjugated donkey anti-rabbit secondary antibodies.

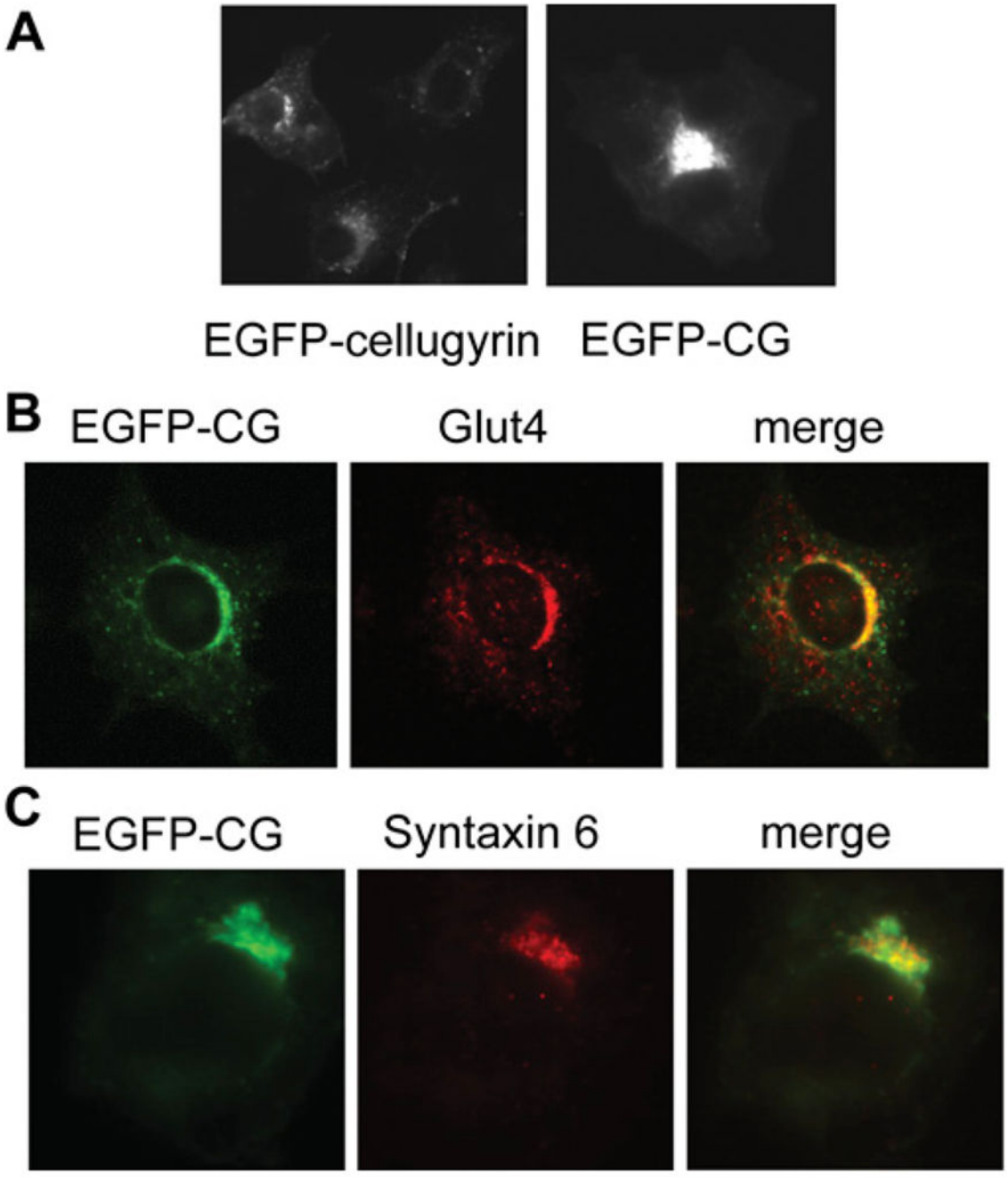

Figure 3. Cellugyrin with the C-terminus of GLUT4 (CG) co-localizes with GLUT4 and syntaxin 6 in the perinuclear compartment

(**A**) Immunofluorescence analysis of 3T3-L1 adipocytes stably expressing EGFP–cellugyrin or EGFP–CG. (**B**) 3T3-L1 adipocytes stably expressing EGFP–CG were electroporated with the cDNA for Myc–GLUT4. Cells were re-plated, grown overnight, then fixed, permeabilized and stained with monoclonal anti-Myc antibody followed by Cy3-conjugated donkey anti-mouse IgG. (**C**) 3T3-L1 adipocytes stably expressing GFP–CG were fixed, permeabilized and stained with a monoclonal antibody against syntaxin 6 followed by Cy3-conjugated donkey anti-mouse IgG.

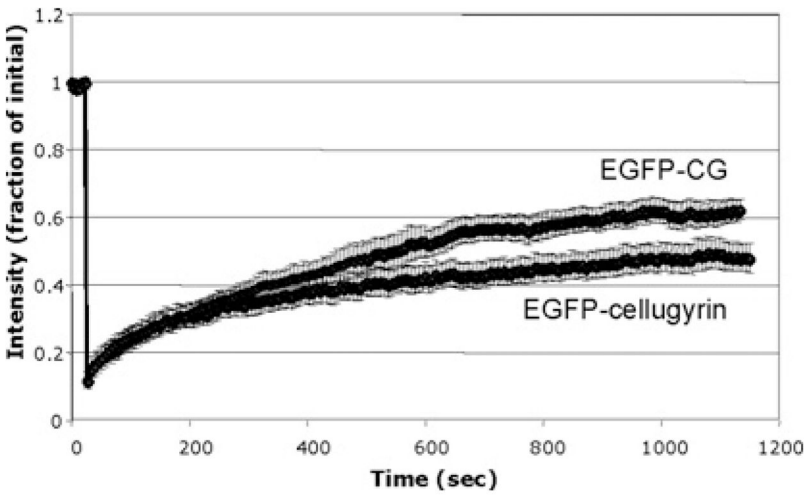

Figure 4. Perinuclear recycling kinetics of EGFP–cellugyrin and EGFP–CG

Differentiated 3T3-L1 adipocytes stably expressing EGFP–cellugyrin or EGFP–CG were imaged before and after photobleaching the perinuclear region with a high-intensity laser as described in the Materials and methods section. Cells were imaged at 5 s intervals and the relative fluorescence intensity at each interval was determined. The means ± S.E.M. from five separate FRAP experiments are shown.

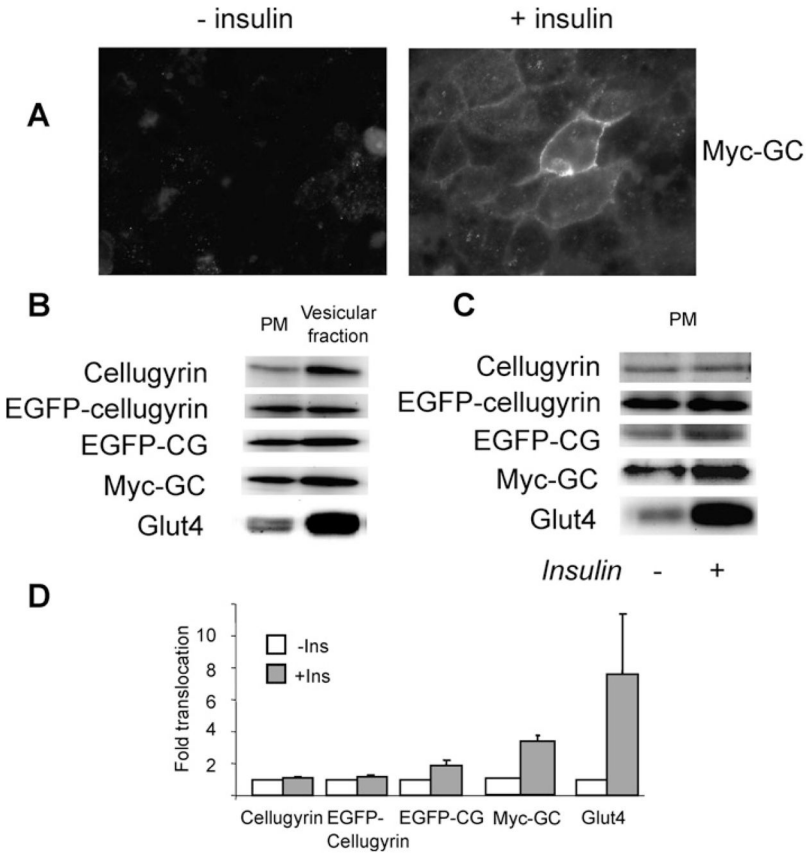

Figure 5. Insulin responsiveness of the reporter molecules

(**A**) 3T3-L1 adipocytes stably expressing Myc–GC were incubated with or without insulin for 30 min. Cells were then fixed and stained with an anti-Myc monoclonal antibody and Cy3-conjugated donkey anti-mouse secondary antibody without permeabilization. (**B**) Basal 3T3-L1 adipocytes stably expressing reporter molecules were homogenized and fractionated into the plasma-membrane fraction (PM; recovered in the 16 000 ***g*** pellet) and the vesicular fraction (16 000 ***g*** supernatant). Samples (50 μg of each) were analysed by Western blotting. (**C**) 3T3-L1 adipocytes stably expressing reporter molecules were incubated with or without insulin for 30 min, homogenized and the plasma-membrane fraction (PM) was isolated. Samples (50 μg of each) were analysed by Western blotting. (**D**) Quantification of results shown in (**C**). Values are means ± S.E.M. from three to five independent experiments is shown. − Ins, without insulin; + Ins, with insulin.

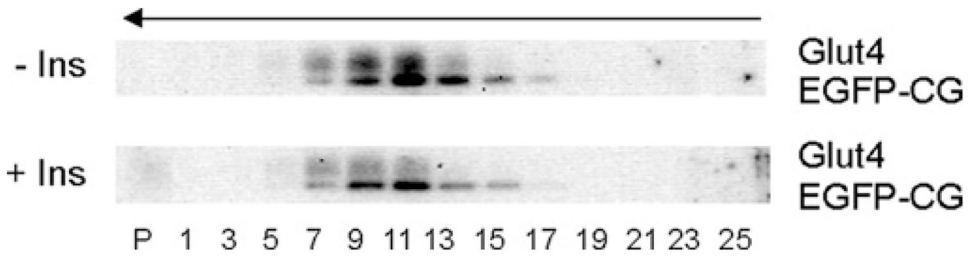

Figure 6. Cellugyrin with the C-terminus of GLUT4 is localized in small vesicles that partially overlap with the IRVs upon sucrose-gradient centrifugation

3T3-L1 adipocytes stably expressing EGFP–CG were incubated without or with insulin (− Ins and + Ins respectively) for 30 min, homogenized and centrifuged at 16 000 ***g*** for 20 min. The supernatant (400 μg) was analysed by sucrose-gradient centrifugation as described in the Materials and methods section. The arrow indicates the direction of sedimentation. Odd gradient fractions were analysed by Western blotting with the 1F8 monoclonal antibody. A representative result from three independent experiments is shown. P, pellet of the gradient.

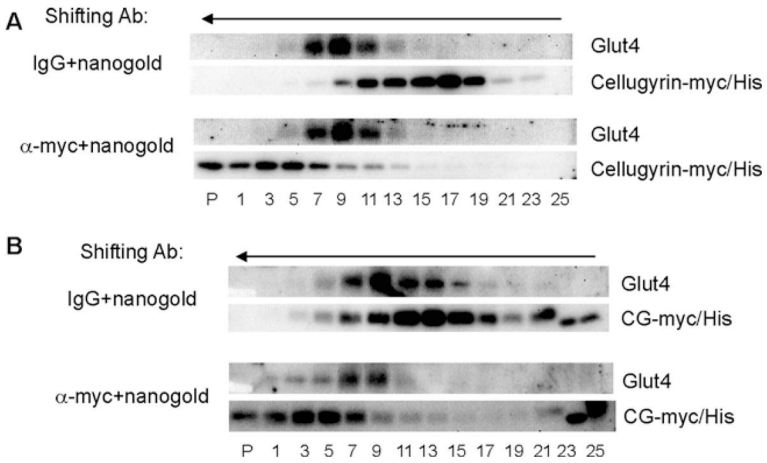

Figure 7. Cellugyrin with the C-terminus of GLUT4 (CG) is partially targeted to the IRVs

3T3-L1 adipocytes were electroporated with the cDNA for cellugyrin–Myc/His (**A**) and CG–Myc/His (**B**), homogenized and centrifuged at 16 000 ***g*** for 20 min. The 16 000 ***g*** supernatants (800 μg) were incubated with 2.5 μg of purified non-specific mouse IgG or monoclonal anti-Myc antibody together with 2 μg of nanogold-conjugated goat anti-mouse Fab fragments for 2 h at 4 °C. Samples were then fractionated in a 10–30 % sucrose gradient for 55 min in a Beckman SW-50.1 rotor at 48 000 rev./min. The arrow indicates the direction of sedimentation. Gradient fractions were analysed by Western blotting with the 1F8 monoclonal antibody and the monoclonal anti-Myc antibody. A representative result from three independent experiments is shown. P, pellet of the gradient.