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Sortilin Is Essential and Sufficient for the Formation of Glut4 Storage Vesicles in 3T3-L1 Adipocytes

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Summary

Impaired translocation of the glucose transporter isoform 4 (Glut4) to the plasma membrane in fat and skeletal muscle cells may represent a primary defect in the development of type 2 diabetes mellitus. Glut4 is localized in specialized storage vesicles (GSVs), the biological nature and biogenesis of which are not known. Here, we report that GSVs are formed in differentiating 3T3-L1 adipocytes upon induction of sortilin on day 2 of differentiation. Forced expression of Glut4 prior to induction of sortilin leads to rapid degradation of the transporter, whereas overexpression of sortilin increases formation of GSVs and stimulates insulin-regulated glucose uptake. Knockdown of sortilin decreases both formation of GSVs and insulin-regulated glucose uptake. Finally, we have reconstituted functional GSVs in undifferentiated cells by double transfection of Glut4 and sortilin. Thus, sortilin is not only essential, but also sufficient for biogenesis of GSVs and acquisition of insulin responsiveness in adipose cells.

Introduction

The regulation of blood glucose levels in mammals is achieved by insulin-dependent translocation of the fat- and muscle-specific glucose transporter, Glut4, to the plasma membrane. The inability of insulin to stimulate translocation of Glut4, i.e., insulin resistance, may represent a primary defect in the development of type 2 diabetes mellitus (Kahn, 1998; Mora and Pessin, 2002; Mueckler, 2001; Saltiel, 2001; Saltiel and Kahn, 2001; Shepherd and Kahn, 1999), which is considered one of the major threats to human health in the modern world (Zimmet et al., 2001). The molecular nature of insulin resistance is not yet known. It may lie in either a defective insulin signaling pathway or in the cell biology of Glut4 recycling or both.

In basal adipocytes and skeletal myocytes, ~75% of the total Glut4 pool is accumulated in small (less than 100 nm in diameter) 60–80 S membrane vesicles (Glut4 storage vesicles, or GSVs) with the rest of the transporter being present in large (120–500 nm), rapidly sedimenting intracellular membranes that are likely to represent endosomes and, possibly, TGN structures (Kandror et al., 1995; Kupriyanova et al., 2002; Malide et al., 2000; Ploug et al., 1998; Ramm et al., 2000; Shewan et al., 2003; Slot et al., 1991; Smith et al., 1991). Upon insulin administration, the amount of GSVs markedly decreases while the amount of Glut4 in the plasma membrane simultaneously and proportionally increases (Bryant et al., 2002). Insulin regulation of Glut4 content in endosomes and other rapidly sedimenting intracellular membranes has not been systematically studied and is largely unknown. Available morphological evidence suggests that insulin does not cause any major changes in the presence of Glut4 in large "vacuoles" and "tubules" (Ramm et al., 2000). This, however, may be explained by the rapid replenishing of the endosomal pool of Glut4 with internalized transporter. In any case, 60–80 S GSVs are likely to represent the major insulin-responsive Glut4-containing compartment in both fat and skeletal muscle cells.

In addition to Glut4 itself, several proteins have been found in the GSVs in adipose cells. These proteins are: insulin-regulated aminopeptidase, receptors for transferrin and IGFII/mannose 6-phosphate, a sorting receptor sortilin, SCAMPs, VAMP2 and/or VAMP3, and one or more rab proteins (Grusovin and Macaulay, 2003; Kandror and Pilch, 1996; Lin et al., 1997; Morris et al., 1998).

The origin and the molecular mechanism of GSV formation are still not known. In order to address this problem, we chose a developmental approach and examined the intracellular localization of ectopically expressed Glut4 in differentiating 3T3-L1 cells. These cells can differentiate in vitro into adipocytes over the course of 7–8 days (Green and Kehinde, 1976). During differentiation, 3T3-L1 cells activate the expression of endogenous Glut4 and dramatically increase insulin-stimulated glucose uptake (Garcia de Herreros and Birnbaum, 1989).

We stably expressed myc7-epitope-labeled Glut4 in undifferentiated 3T3-L1 cells and found that it is localized mainly in rapidly sedimenting endosomes with only a small fraction of the transporter present in the GSVs. In addition, in undifferentiated 3T3-L1 cells, exogenous myc7-Glut4 is highly unstable and is rapidly degraded through the lysosomal pathway with a half-life of about 2 hr. In other words, in undifferentiated cells, a major part of ectopically expressed myc7-Glut4 is targeted to the degradation pathway with only a small fraction of the transporter being recycled to the plasma membrane in an insulin-sensitive fashion.

Between day 2 and 3 of differentiation, the major pool of myc7-Glut4 is redistributed from rapidly sedimenting endosomes to GSVs. Formation of this vesicular compartment leads to stabilization of the transporter and to a dramatic increase in its insulin responsiveness.

Using an in vitro vesicle reconstitution assay, we also found that formation of GSVs in differentiating cells was limited by the expression of integral membrane protein(s) and not cytoplasmic proteins. Since induction of sortilin takes place on day 2 of differentiation immediately prior to the massive redistribution of Glut4 from heavy endosomes to the GSVs, we hypothesized that the putative membrane protein responsible for the formation of these vesicles is sortilin. To this end, we found that sortilin may interact with Glut4 via its lumenal domain. Expression of sortilin in 3T3-L1 adipocytes increases the formation of GSVs and stimulates insulin-

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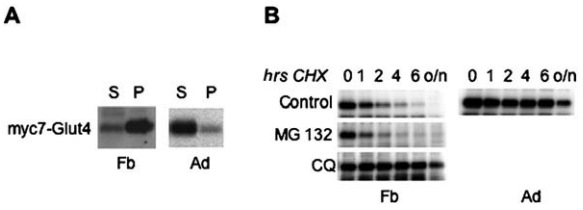

Figure 1. Subcellular Distribution and Stability of myc7-Glut4 in 3T3-L1 Cells

(A) Undifferentiated (Fb) and differentiated (Ad) G cells were homogenized and centrifuged at 16,000 × g for 20 min. myc7-Glut4 was analyzed in the supernatant (S) and the pellet (P) by Western blotting with the anti-myc antibody.

(B) Stability of myc7-Glut4 was measured in undifferentiated (Fb) and differentiated (Ad) G cells as described in Experimental Procedures. Total cell extract (100 μg per lane) was analyzed by Western blotting with the anti-myc antibody.

regulated glucose uptake, whereas partial knockdown of sortilin using an RNAi approach has the opposite effect. Finally, we stably expressed sortilin-myc/His together with myc7-Glut4 in 3T3-L1 preadipocytes that do not express these proteins endogenously. In these cells, sortilin-myc/His stimulates formation of GSVs and increases plasma membrane translocation of myc7-Glut4 as well as insulin-stimulated glucose uptake. We conclude, therefore, that sortilin is essential and sufficient for acquisition of insulin-regulated glucose transport in adipocytes and that it fulfils its biological role by stimulating formation of GSVs on donor membranes.

Results

Formation of Insulin-Sensitive GSVs in Differentiating 3T3-L1 Cells

Undifferentiated and differentiated 3T3-L1 cells stably expressing myc7-Glut4 (G cells) were homogenized and centrifuged at 16,000 × g for 20 min in order to separate small GSVs from large intracellular Glut4-containing membranes (Kupriyanova et al., 2002). Figure 1A shows that in undifferentiated cells, myc7-Glut4 is localized primarily in the 16,000 × g pellet, whereas in differentiated cells, myc7-Glut4 is found mainly in 16,000 × g supernatant where it colocalizes with endogenous Glut4 in the GSVs (see Supplemental Figure S1 available with this article online). This figure also shows that the level of expression of the reporter myc7-Glut4 molecule is at least 10 times lower than endogenous Glut4, suggesting that myc7-Glut4 is unlikely to disturb compartmentalization and trafficking of the endogenous transporter.

In preadipocytes, myc7-Glut4 is highly unstable and is rapidly degraded with a half-life of ~2 hr (Figure 1B). Notwithstanding such a short half-life, myc7-Glut4 is not degraded through an ER-associated process, but reaches an endosomal compartment (Supplemental Figure S2) from where it is likely to be targeted to lyso-

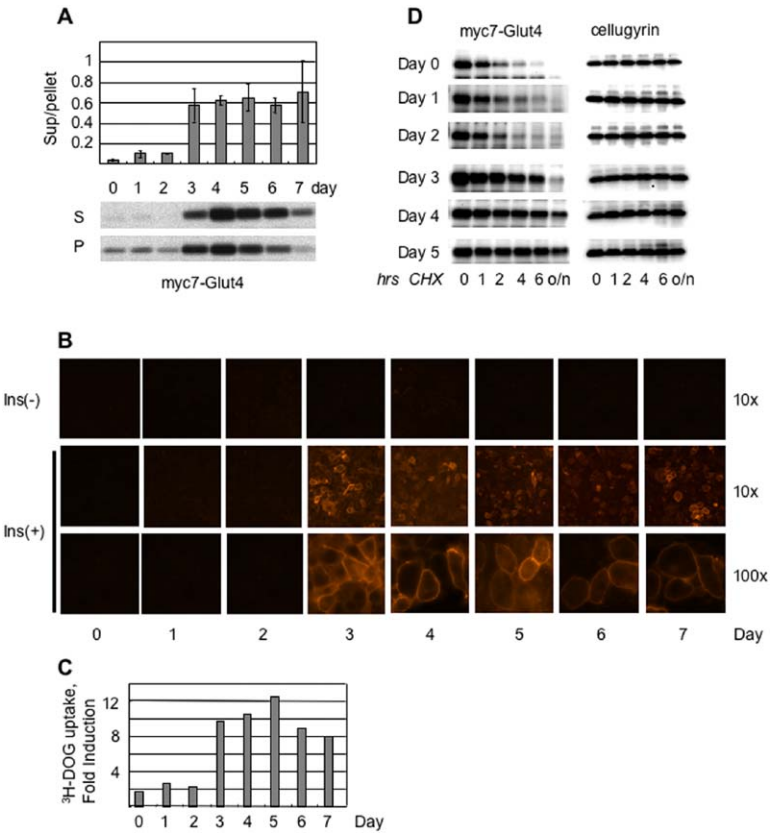

Figure 2. Formation of GSVs in Differentiation

(A) Differentiating G cells were separated into 16,000 × g supernatant (S) and pellet (P) on each day of differentiation. Membrane vesicles in the 16,000 × g supernatant were collected by centrifugation at 200,000 × g for 90 min and analyzed by Western blotting with the anti-myc antibody along with the pellet samples. The bottom panel shows results of a representative experiment (100 μg of protein per lane). The top panel shows the ratios (mean values ± SE of three independent experiments) between the total Glut4 content in 16,000 × g supernatant and pellet.

(B) Immunofluorescence microscopy of differentiating G cells treated and not treated with 100 nM insulin for 15 min. Cells were fixed and stained with anti-myc monoclonal antibody and Cy3-conjugated donkey anti-mouse secondary antibody without permeabilization.

(C) Changes in insulin-regulated ^{3}H-2-deoxyglucose (2-DOG) uptake in differentiating G cells. The fold stimulation was calculated by dividing the average of the insulin-stimulated condition by that of the basal for each time point. These data are representative of three independent experiments.

(D) Stability of myc7-Glut4 in differentiating G cells (100 μg of total protein loaded on each lane).

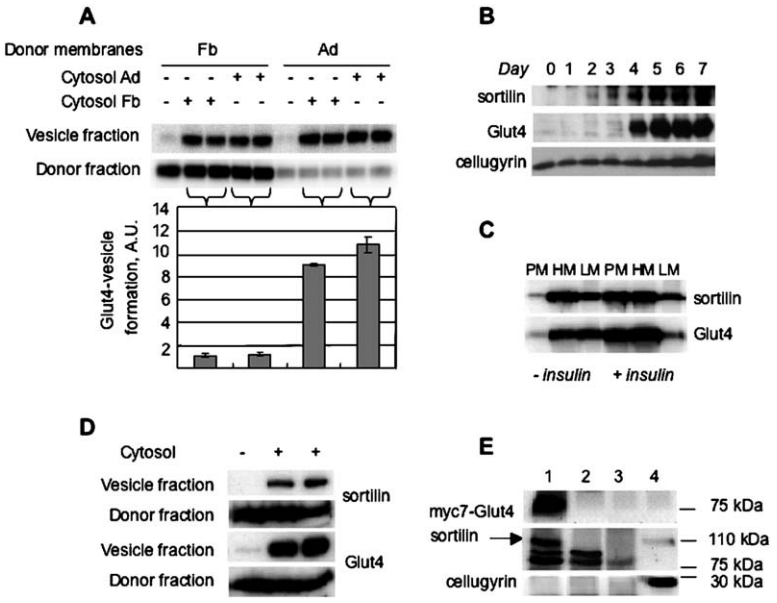

Figure 3. Induction of an Integral Membrane Protein Stimulates Formation of the GSVs in Differentiating 3T3-L1 Cells

(A) Vesicle reconstitution assay was performed in vitro in duplicate with donor membranes and cytosol isolated from differentiated and undifferentiated G cells. The lower panel shows the quantification of the Western blot stained with anti-myc antibody (values of mean and range between duplicate samples). A representative result of three independent experiments is shown.

(B) Total homogenate (100 μg per lane) of differentiating 3T3-L1 cells was analyzed by Western blotting using antibodies against sortilin, Glut4 (1F8), and cellugyrin.

(C) Differentiated 3T3-L1 adipocytes were treated or not treated with 100 nM insulin for 5 min and fractionated by differential centrifugation into the plasma membrane (PM), heavy microsomes (HM), and light microsomes (LM). Each fraction (50 μg per lane) was analyzed by Western blotting with anti-sortilin antibody (top) and 1F8 (bottom).

(D) Vesicle reconstitution assay was performed in vitro with donor membranes and cytosol isolated from differentiated 3T3-L1 adipocytes.

(E) G cells were incubated with DSP as described in Experimental Procedures, and cell lysate (1.5 mg) was immunoprecipitated with 2 μg of the anti-myc antibody (lane 1) or nonspecific IgG (lane 3) and protein G. In parallel, 2 μg of the anti-myc antibody was immobilized on protein G beads and processed as in lanes 1 and 3 using lysis buffer instead of the cell lysate (lane 2). Lane 4 shows 10 μg of the total cell lysate treated with 50 mM DTT. The arrow indicates the position of sortilin.

somes for degradation. This conclusion is based on the observation that the proteasomal inhibitor MG132 does not have any effect on myc7-Glut4 half-life, whereas the lysosomal inhibitor, chloroquine, substantially stabilizes the transporter (Figure 1B).

In order to determine at what stage of differentiation Glut4 is redistributed from heavy endosomes to GSVs, cells were fractionated by 16,000 × g centrifugation on each day of differentiation. As is seen in Figure 2A, redistribution of myc7-Glut4 from heavy membranes to GSVs takes place between day 2 and 3 of differentiation. This event coincides with a dramatic increase in insulin responsiveness of the tagged transporter (Figure 2B) as well as in fold increase of insulin-stimulated glucose uptake (Figure 2C). In addition, redistribution of myc7-Glut4 from heavy membranes to GSVs is accompanied by an increase in half-life of the transporter (Figure 2D). For comparison, stability of cellugyrin, a protein localized in a different vesicular population (Kupriyanova and Kandror, 2000; Kupriyanova et al., 2002; Xu and Kandror, 2002), does not vary during differentiation (Figure 2D). We conclude, therefore, that, in 3T3-L1 preadipocytes, ectopically expressed myc7-Glut4 is targeted primarily to the degradation pathway with only a fraction of the transporter recycling to the plasma membrane. On day 3 of differentiation, myc7-Glut4 is rerouted from the degradation to the recycling pathway and this event takes place simultaneously with the formation of GSVs. Interestingly, a complete acquisition of insulin-regulated glucose uptake in differentiation takes place on day 3, significantly prior to massive lipid accumulation, i.e., in cells that morphologically are similar to fibroblasts.

Formation of GSVs in Differentiating 3T3-L1 Cells Depends on the Induction of Membrane Protein(s)

In order to study the mechanism of formation of small insulin-responsive vesicles in differentiating cells, we used heavy membranes recovered in the 16,000 × g pellet as the donor fraction in the in vitro vesicle reconstitution assay that we recently developed (Xu and Kandror, 2002). For these experiments, we isolated donor membranes and cytosol from differentiated and undifferentiated G cells and used them in different combinations. Note that in agreement with Figure 1A, donor membranes from differentiated adipocytes have a much lower specific content of myc7-Glut4 than the analogous membrane fraction isolated from undifferentiated cells (Figure 3A). Therefore, in order to compare samples with different specific contents of Glut4, in this and in all the following figures, results of the budding reaction are expressed as the ratio between myc7-Glut4 in the vesicle fraction and in the donor fraction (Figure 3A). This is a valid approach since control experiments demonstrated that the amount of the in vitro formed vesicles was directly proportional to the amount of donor membranes regardless of their specific Glut4 content (results not shown).

When donor membranes were isolated from undifferentiated cells, formation of vesicles in vitro was low regardless of the source of cytosol. However, when donor membranes were isolated from differentiated cells, formation of vesicles increased dramatically—also regardless of the source of cytosol (Figure 3A). Stripping of donor membranes with 0.6 M KCl did not affect the results of the experiment (results not shown). Based on these results, we conclude that the induction of an in-

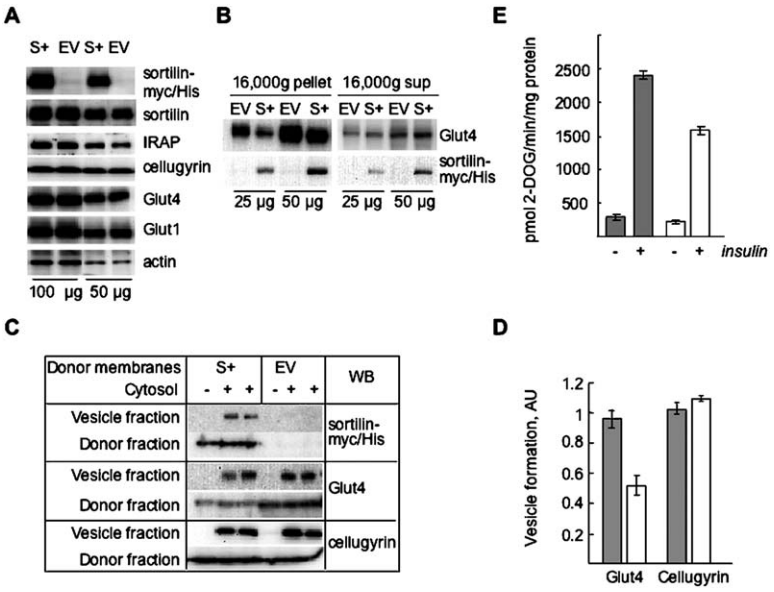

Figure 4. Expression of sortilin-myc/His Increases Formation of the GSVs and Stimulates Insulin Response in 3T3-L1 Cells

(A) Individual proteins in total homogenates of differentiated S+ and EV cells.

(B) Distribution of endogenous Glut4 between the donor fraction (16,000 × g pellet) and vesicle fraction (16,000 × g supernatant) in differentiated S+ and EV cells.

(C) Donor membranes from differentiated S+ cells and EV cells were incubated with cytosol from EV cells at 37°C for 20 min. De novo formed vesicles along with the donor fraction were analyzed by Western blotting with antibodies against cellugyrin and Glut4.

(D) Quantification of the experiment shown in (C) (values of mean and range between duplicate samples). A representative result of four independent experiments is shown. S+ cells, gray bars; EV cells, white bars.

(E) ^{3}H-2-deoxyglucose uptake in EV cells (white bars) and S+ cells (gray bars). The panel shows normalized mean values ± SE of three independent experiments, each in duplicate.

tegral membrane protein (or proteins) stimulates formation of GSVs in differentiated cells.

Since the protein composition of Glut4 vesicles is well characterized (Kandror and Pilch, 1996), we hypothesized that a candidate protein may be the putative sorting receptor, sortilin, which represents a major component of small Glut4 vesicles in adipose cells (Lin et al., 1997; Morris et al., 1998). This hypothesis is indirectly supported by the following experiment showing that induction of sortilin in 3T3-L1 cells takes place on day 2 of differentiation, i.e., immediately before the massive redistribution of myc7-Glut4 from heavy membranes to small vesicles and, also, prior to the induction of endogenous Glut4 (Figure 3B). Similar results were also obtained by Northern blot analysis (results not shown). Using subcellular fractionation according to Simpson et al. (1983), we found that in basal 3T3-L1 adipocytes, very little of sortilin and Glut4 was localized in the plasma membrane, while major pools of these proteins were present in intracellular heavy and light microsomes (Figure 3C). Insulin administration caused a significant increase in the plasma membrane content of sortilin and Glut4, indicating that both proteins were localized in the same insulin-sensitive compartment (see also Supplemental Figure S3).

In the vesicle reconstitution assay in vitro, endogenous sortilin was redistributed from the donor fraction to the vesicle fraction similar to Glut4 (Figure 3D). Finally, endogenous sortilin can be efficiently crosslinked to myc7-Glut4, suggesting that the two proteins interact either directly or indirectly. In the experiment shown in Figure 3E, we incubated differentiated G cells in the presence of the cleavable membrane-permeable crosslinking reagent DSP (Nielsen et al., 1999) and immunoprecipitated myc7-Glut4 using the monoclonal antibody against myc (lane 1) or nonspecific IgG as a control (lane 3). In parallel, we carried out a blank elution of the myc beads with no cell extract added (lane 2). We found that sortilin was crosslinked to myc7-Glut4 and was specifically immunoprecipitated with the anti-myc antibody. Since the protein binding VPS10p domain of sortilin is localized in its N-terminal lumenal domain, we suggest that sortilin interacts with Glut4 in the vesicular lumen. These results lead us to examine whether or not sortilin may play a role in the formation of GSVs.

Overexpression of Sortilin Stimulates Formation of GSVs and Potentiates the Insulin Response in 3T3-L1 Adipocytes

Sortilin-myc/His was stably expressed in 3T3-L1 adipocytes at a level of ~15% over endogenous (results not shown). We call this cell line S+ cells. Sortilin-myc/His and endogenous sortilin do not separate under our electrophoretic conditions and travel as one band. Cells were homogenized and centrifuged at 16,000 × g for 20 min. In the supernatant, sortilin-myc/His as well as endogenous sortilin was localized mainly in Glut4 vesicles as judged by immunoadsorption (Supplemental Figure S3A) and sucrose gradient centrifugation (Supplemental Figure S3B). This demonstrates the faithful targeting of sortilin-myc/His in adipocytes.

Expression of sortilin-myc/His does not change the expression levels of glucose transporters and other proteins in adipocytes (Figure 4A) but decreases the amount of Glut4 in heavy donor membranes (16,000 × g pellet) by ~40% (Figure 4B). A small (~10%) corresponding increase in Glut4 in the vesicular fraction (16,000 × g supernatant) is not easy to discern. The difference becomes apparent upon pelleting of GSVs from the 16,000 × g supernatant by high-speed centrifugation at 200,000 × g (Supplemental Figure S4).

Next, we carried out a vesicle reconstitution assay in vitro using 16,000 × g pellets from S+ and control (empty vector-transfected) adipocytes as donor membranes. As shown in Figures 4B and 4C, donor mem-

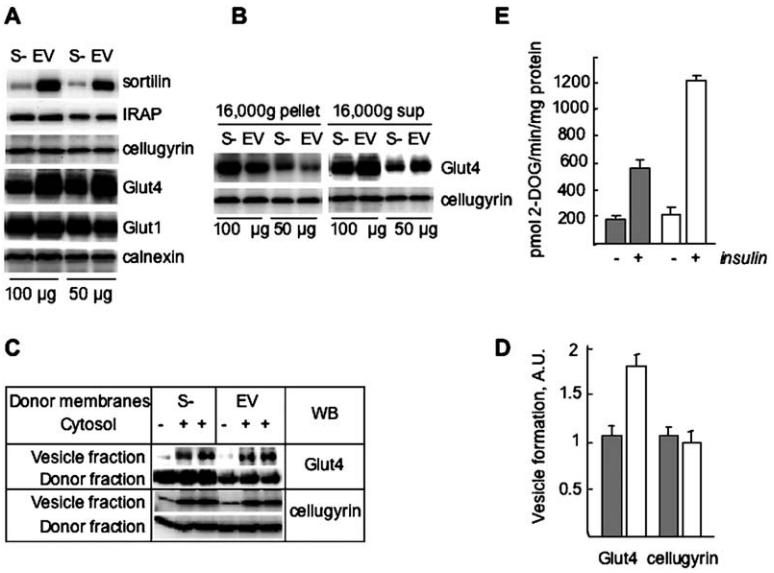

Figure 5. Sortilin Knockdown Impairs GSV Formation and Decreases Insulin-Regulated Glucose Uptake

(A) Individual protein in total homogenates of differentiated S– and EV cells.

(B) Distribution of endogenous Glut4 and cellugyrin between the donor fraction (16,000 × g pellet) and vesicle fraction (16,000 × g supernatant) in differentiated S– and EV cells.

(C) Donor membranes from differentiated S– cells and EV cells were incubated with cytosol from EV cells at 37°C for 20 min. De novo formed vesicles along with the donor fraction were analyzed by Western blotting with antibodies against cellugyrin and Glut4.

(D) Quantification of the experiment shown in (C) (values of mean and range between duplicate samples). A representative result of three independent experiments is shown. S– cells, gray bars; EV cells, white bars.

(E) ^{3}H-2-deoxyglucose uptake in EV cells (white bars) and S– cells (gray bars). The panel shows normalized mean values ± SE of three independent experiments, each in duplicate.

branes prepared from S+ cells have substantially less Glut4 than donor membranes from control cells. Nevertheless, the amount of the in vitro formed vesicles is roughly equal in both cases (Figure 4C). Since in these experiments, the yield of vesicles is directly proportional to the amount of donor membranes, these results suggest that overexpression of sortilin increases the efficiency of Glut4-vesicles formation in vitro (Figure 4D). As a control for these experiments, we studied formation of cellugyrin-containing vesicles and found that sortilin-myc/His overexpression does not have a significant impact on the biogenesis of this compartment in vitro or in the living cell (Figures 4C and 4D). An increase in GSV formation is likely to account for the fact that S+ cells have considerably higher levels of insulin-stimulated glucose transport than control cells (Figure 4E).

Partial Knockdown of Sortilin Decreases Formation of GSVs and Insulin-Stimulated Glucose Transport

In the following set of experiments, we knocked down sortilin expression with the help of shRNA to 10%–20% of the basal level (Figure 5A). Cells stably expressing the shRNA interference construct are referred to as S– adipocytes. Knockdown of sortilin leads to a 35% decrease in total Glut4 levels, although expression of all other proteins tested did not change (Figure 5A). Despite a decrease in total Glut4, its content in heavy donor membranes actually increased by ~50% (Figure 5B). Note that the distribution of cellugyrin between the vesicle and the donor membrane fractions did not change. This suggests that knockdown of sortilin specifically compromised the formation of GSVs. Indeed, our in vitro budding assay confirmed that formation of Glut4 vesicles from sortilin-depleted donor membranes was decreased, whereas formation of cellugyrin-containing vesicles was not affected (Figures 5C and 5D). Furthermore, aberrant biogenesis of GSVs in S– adipocytes leads to a marked decrease in insulin-stimulated glucose uptake (Figure 5E).

Sortilin-myc/His Stimulates Formation of GSVs and Retargets myc7-Glut4 from the Degradation to the Recycling Pathway in Double-Transfected Preadipocytes

In order to determine whether or not sortilin alone can retarget Glut4 from the degradation to the regulated recycling pathway in undifferentiated cells, we prepared double-transfected 3T3-L1 preadipocytes that stably express both myc7-Glut4 and sortilin-myc/His (GS cells). As shown in Figure 6A, myc7-Glut4 and sortilin-myc/His demonstrated a high degree of colocalization by immunofluorescence staining. Figure 6B shows that sortilin-myc/His has a stabilizing effect on myc7-Glut4, which leads to a moderate increase in the total myc7-Glut4 while expression levels of other proteins do not change (Figure 6C). It is noteworthy that in all isolated clones of GS cells, the level of myc7-Glut4 directly correlates with the level of sortilin-myc/His expression (Supplemental Figure S5).

Expression of sortilin-myc/His dramatically increases the amount of myc7-Glut4 in the GSV fraction of double-transfected cells (Figure 6D), an effect that cannot be explained solely by upregulation of myc7-Glut4 (compare Figures 6C and 6D). Also, sortilin-myc/His targets myc7-Glut4 to the regulated recycling pathway, as illustrated by staining of nonpermeabilized G and GS preadipocytes with the anti-myc antibody (Figure 6E). We also measured basal and insulin-stimulated glucose uptake in G and GS preadipocytes (Figure 6F) and found that coexpression of sortilin-myc/His and myc7-Glut4 significantly increases insulin-stimulated glucose uptake. Importantly, expression of sortilin-myc/His alone does not change glucose uptake in preadipocytes (although it does so in differentiated adipocytes that express Glut4 endogenously—see Figure 4E), strongly suggesting that the stimulating effect of sortilin on glucose transport in GS preadipocytes is mediated by Glut4 and not by Glut1.

It thus appears that expression of sortilin and Glut4

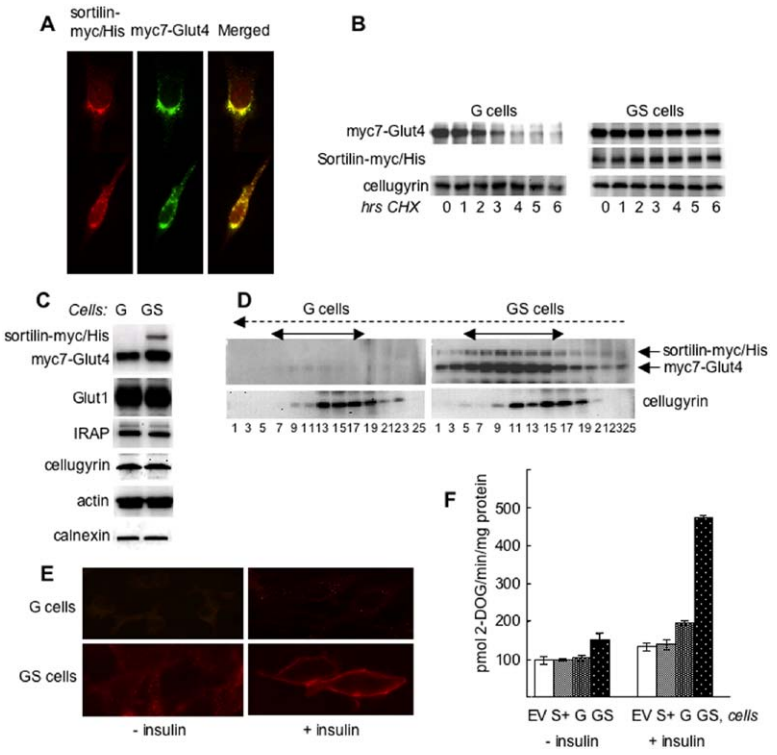

Figure 6. Double Transfection of myc7-Glut4 and sortilin-myc/His Creates Functional GSVs in 3T3-L1 Preadipocytes

(A) Colocalization of myc7-Glut4 and sortilin-myc/His in undifferentiated GS cells. The cells were stained with the polyclonal antibody against Glut4 and the monoclonal antibody against the His epitope followed by Alexa 488-conjugated donkey anti-mouse IgG (Molecular Probes) and Cy3-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch).

(B) Stability of myc7-Glut4 in G and GS cells. Each lane contains 100 μg of total homogenate.

(C) Individual protein in total homogenates of undifferentiated G and GS cells. Each lane contains 100 μg of protein.

(D) Light microsomes (800 μg of total protein) isolated from undifferentiated G and GS cells were fractionated in a sucrose velocity gradient. Top panels were blotted with the anti-myc antibody, bottom panels with anti-cellugyrin antibody. The position of GSVs is indicated by double end arrows. The dotted arrow indicates the direction of sedimentation.

(E) Undifferentiated G and GS cells were serum starved and treated or not treated with 100 nM insulin for 15 min. Cells were fixed and stained with anti-myc monoclonal antibody and Cy3 donkey anti-mouse secondary antibody without permeabilization. 100× magnification fields are shown.

(F) ^{3}H-2-deoxyglucose uptake in undifferentiated EV, S+, G, and GS cells. The panel shows normalized mean and range values of two independent experiments, each in duplicate.

is sufficient for the formation of the insulin-responsive vesicular compartment and reconstitution of insulin-regulated glucose uptake in 3T3-L1 preadipocytes. We observed an analogous effect also in Swiss and NIH 3T3 fibroblasts that do not naturally express Glut4 and sortilin (Supplemental Figure S6). Although these cells possess the insulin-signaling pathway, they are not considered as genuine "insulin-sensitive" cells. Our preliminary results suggest that the presence of the insulin-responsive compartment rather than the insulin signal transduction pathway defines the difference between insulin-sensitive and -nonsensitive cells.

In order to determine how important the interaction between the lumenal domains of sortilin and Glut4 is for the acquisition of insulin-stimulated glucose uptake in 3T3-L1 preadipocytes, we have replaced the lumenal N-terminal domain of sortilin with EGFP. This construct (mSorC for membrane-anchored sortilin C terminus) was expressed, along with myc7-Glut4, in 3T3-L1 cells. As controls for this experiment, we used full-length sortilin, soluble sortilin C terminus (sSorC) without the transmembrane domain, and EGFP alone (Figure 7A).

We found that mSorC demonstrated the same high degree of colocalization with myc7-Glut4 as full-length sortilin (compare Figures 6A and 7B), suggesting that the C terminus of sortilin contains sufficient information for cotargeting with Glut4. Both sSorC and EGFP showed a diffuse staining pattern typical for free soluble proteins (not shown). Interestingly, mSorC could not be crosslinked to myc7-Glut4 (not shown) and was only able to partially recapitulate the effect of full-size sortilin on insulin-stimulated glucose uptake in 3T3-L1 preadipocytes (Figure 7C). These results suggest that the interaction between lumenal domains of Glut4 and sortilin is important for the recruitment of Glut4 into the insulin-responsive vesicular compartment.

Discussion

Here we demonstrate that myc7-Glut4 ectopically expressed in 3T3-L1 preadipocytes is localized primarily in endosomes—which is consistent with previously published results (Hudson et al., 1992). A novel observation, however, is that from endosomes, myc7-Glut4 is targeted primarily to the lysosomal degradation pathway that prevails over the regulated recycling pathway. Indeed, insulin-stimulated translocation of myc7-Glut4 in preadipocytes is minute and can be detected only by the most sensitive technique of our arsenal, radioactive 2-deoxyglucose uptake (Figure 6F), while immunofluorescence staining does not provide enough sensitivity to detect a small and, likely, transient increase in the plasma membrane myc7-Glut4 (compare Figures 6E and 6F). Between day 2 and 3 of differentiation, the major pool of myc7-Glut4 is redistributed from heavy donor membranes to the GSVs (Figure 2A). This result

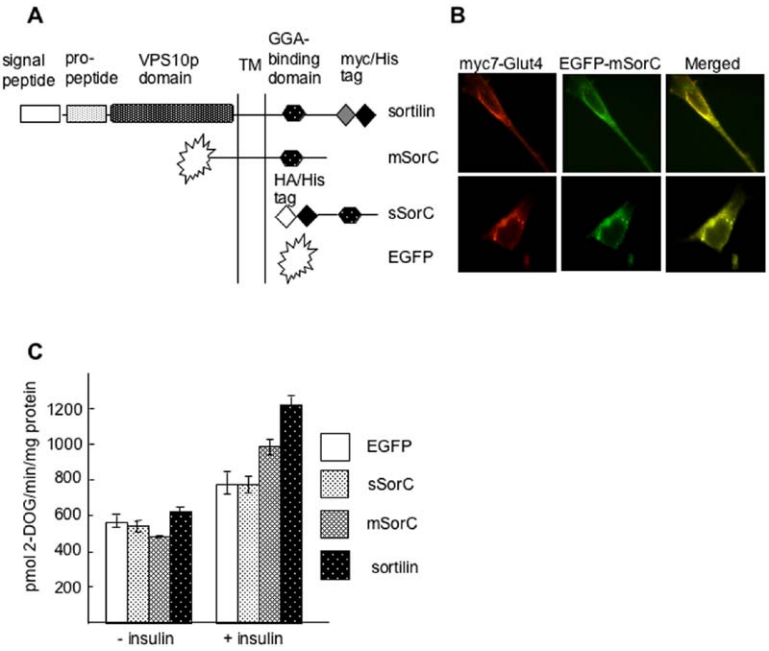

Figure 7. Sortilin without the Lumenal N-Terminal Domain Is Cotargeted with Glut4 and Partially Recapitulates the Effect of Full-Length Protein on Insulin-Regulated Glucose Uptake in 3T3-L1 Preadipocytes

(A) Constructs used in this experiment.

(B) myc7-Glut4 and mSorC double-transfected fibroblasts were stained with the anti-myc monoclonal antibody and Cy3-conjugated donkey anti-mouse secondary antibody.

(C) ^{3}H-2-deoxyglucose uptake in undifferentiated 3T3-L1 preadipocytes stably expressing myc7-Glut4 and indicated constructs. The panel shows normalized mean values ± SE of three independent experiments, each in duplicate.

is consistent with our previous report showing that the transferrin receptor and Glut1 are sequestered from the plasma membrane into a distinct insulin-responsive vesicular pool on day 3 of differentiation (ElJack et al., 1999). Sequestration of myc7-Glut4 into GSVs may lead to its stabilization (Figure 2D) and to a dramatic increase in its insulin responsiveness (Figure 2B).

These results provide further support to the model according to which GSVs represent the major insulin-responsive compartment in adipocytes. Therefore, we next addressed the mechanism of GSV biogenesis and found that it depends on the expression of sortilin. Sortilin is a homolog of the yeast vacuolar sorting receptor, Vps10p (Petersen et al., 1997). The intralumenal domain of sortilin has the ability to interact with various unrelated proteins, such as receptor-associated protein rap (Petersen et al., 1997), lipoprotein lipase (Nielsen et al., 1999), neurotensin (Mazella et al., 1998), and pro-NGF (Nykjaer et al., 2004), which may or may not represent its physiological ligands in adipocytes. In any case, published evidence is consistent with sortilin functioning as a sorting receptor in mammalian cells. In various different cells, sortilin may be involved in protein binding/sorting at the plasma membrane (Mazella et al., 1998; Nielsen et al., 1999; Nykjaer et al., 2004), in Golgi to endosome (Nielsen et al., 2001), or lysosomal protein traffic (Lefrancois et al., 2003). In adipocytes, sortilin shows a high degree of colocalization with Glut4 and represents one of the major component proteins of Glut4 vesicles (Lin et al., 1997; Morris et al., 1998; this manuscript). Moreover, it may be directly responsible for the formation of insulin-sensitive Glut4 storage vesicles in differentiating adipocytes. Its expression is induced prior to redistribution of myc7-Glut4 from donor membranes to GSVs and prior to the induction of endogenous Glut4 (Figure 3B). Sortilin can be crosslinked to Glut4 (Figure 3C), suggesting that these two proteins interact with each other. Overexpression of sortilin stimulates formation of Glut4 vesicles and increases insulin-stimulated glucose uptake (Figure 4). Partial knockdown of sortilin using the shRNA approach has the opposite phenotype (Figure 5). Most significantly, transfection of sortilin-myc/His into myc7-Glut4-expressing preadipocytes leads to the biogenesis of GSVs (Figure 6D) and targets myc7-Glut4 to the regulated plasma membrane recycling pathway (Figures 6E and 6F). All these data strongly suggest that the formation of GSVs depends primarily on sortilin expression. Our preliminary results (Supplemental Figure S6) indicate that ectopic expression of sortilin and Glut4 leads to reconstitution of insulin-stimulated glucose uptake also in Swiss and NIH 3T3 fibroblasts. These results suggest that Swiss and NIH 3T3 fibroblasts have all the necessary components of the insulin signal transduction pathway but lack the specialized insulin-responsive compartment, which may thus represent the major distinction of the insulin-sensitive cell.

How can sortilin form Glut4 vesicles? It is known that the cytoplasmic tail of sortilin specifically interacts with monomeric adaptor protein, GGA2 (Nielsen et al., 2001; Shiba et al., 2002). As we have recently shown, expression of a dominant-negative GGA mutant in 3T3-L1 adipocytes inhibits the formation of small insulin-sensitive vesicles and blocks insulin-regulated glucose uptake (Li and Kandror, 2005; Watson et al., 2004). Thus, GGA adaptors are likely to represent the partners of sortilin in vesicle biogenesis. Since GGA adaptors are present in both undifferentiated and differentiated 3T3-L1 cells (Li and Kandror, 2005), we suggest that induction of sortilin is the major event that leads to the formation of insulin-responsive vesicles and acquisition of insulin-regulated glucose uptake in adipose cells. According to this model, sortilin interacts with Glut4 in the endosomal lumen via its N-terminal domain and couples

Glut4 to the preexisting GGA-dependent budding machinery via its cytoplasmic tail, thus rerouting Glut4 from the degradation to the recycling pathway. mSorC is targeted to the same donor membranes as Glut4 (Figure 7B), likely because it contains all the targeting information in its cytoplasmic tail. Furthermore, mSorC may increase budding from Glut4-containing donor membranes since it possesses the GGA binding sequence. Therefore, a fraction of Glut4 molecules may redistribute from donor membranes to newly formed vesicles. However, mSorC cannot interact with Glut4 and cannot actively recruit Glut4 into GSV. For this reason, mSorC can only partially recapitulate the effect of full-length sortilin on insulin-regulated glucose uptake.

Experimental Procedures

Antibodies

In the present study, we used monoclonal anti-myc and anti-His antibodies from Invitrogen (Carlsbad, CA), the monoclonal anti-Glut4 antibody 1F8 (James et al., 1988), a rabbit polyclonal antibody against cellugyrin (Ac-CQNVETTEGYQPPPVY-OH) that has been raised and affinity purified by BioSource International (Camarillo, CA), a rabbit polyclonal anti-Glut4 antibody (a kind gift from Dr. Samuel W. Cushman, NIDDK), a rabbit polyclonal antibody against sortilin (a kind gift from Dr. Gustav E. Lienhard, Dartmouth Medical School), and a rabbit polyclonal antibody against IRAP (a kind gift from Dr. Paul Pilch, Boston University School of Medicine).

cDNA Constructs

shRNA were designed and created as specified at http://www.cshl.edu/public/SCIENCE/hannon.html. Oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX). We screened seven shRNA constructs comprising 29 nucleotides starting from sites 267, 1034, 1253, 1553, 1945, 2344, and 2438 of the mouse sortilin open reading frame (GenBank accession number BC056343). The shRNA that starts at position 267 was the most effective and was chosen for further experiments. Another shRNA that starts at the position 2438 was less effective. Cells stably transfected with the other five shRNAs showed no difference from empty vector-transfected cells. For the preparation of the active shRNA that starts at position 267, we used the universal SP6 primer 5′-GATTTAGGTGACACTATAG-3′ and a primer 5′- AAAAAAGCGCATTGTCGGTCAACTTGGCGACGAAGCAAGCTTCCTTCATCGCCAAGCTGACCAACAATACGCGGTGTTTCGTCCTTTCCACAA-3′. The second primer contains the RNA hairpin and 21 nucleotides homologous to the human U6 RNA promoter. This PCR fragment was ligated with the pGEM-T easy vector from Promega (Madison, WI). In order to obtain pBabe-shSor 267, the EcoRI/EcoRI fragment was released from T-easy-shSor 267 and cloned into EcoRI cutted pBabe-puro vector.

Preparation of pBabe-myc7-Glut4, mLNCX$_2$-sortilin-myc/His, pLenti-m1-sortilin-myc/His, pLenti-m1-EGFP-mSorC, pLenti-m2-HA/His-sSorC, and pLenti-m1-EGFP-N3 is described in Supplemental Data.

Stable Cell Lines

G cells were stably transfected with pBabe-myc7-Glut4. S+ cells were stably transfected with mLNCX$_2$-sortilin-myc/His. S– cells were stably transfected with the shRNA interference construct pBabe-shSor 267. GS cells were stably double transfected with pBabe-myc7-Glut4 and mLNCX$_2$-sortilin-myc/His. EV cells were stably transfected with either pBabe or mLNCX$_2$ empty vectors or both and used as controls. Retrovirus production was performed as described in the Stratagene (La Jolla, CA) protocol for retrovirus production using the pVPack-GP and pVPack-VSV-G vectors. At least two individual clones of pBabe-myc7-Glut4 or mLNCX$_2$-sortilin-myc/His infected 3T3-L1 cells along with pooled populations of infected cells were analyzed. In the experiments with shRNA interference constructs, two independent pooled clones were generated and analyzed. pBabe-myc7-Glut4 and mLNCX$_2$-sortilin-myc/His double-infected 3T3-L1 cells were made by infecting the already established single clone of G cells with sortilin-mycHis-expressing retrovirus. Ten individual clones and two pooled clones were analyzed with similar results.

Alternatively, GlentiS, GmSorC, GsSorC, and GEGFP cells were created by infecting 3T3-L1 preadipocytes with retrovirus encoding pBabe-myc7-Glut4 and then by infecting the pooled clone of G cells with the following lentiviral constructs according to Invitrogen protocol. GlentiS cells stably express pLenti-m1-sortilin-myc/His, GmSorC cells express pLenti-m1-EGFP-mSorC, GsSorC cells express pLenti-m2-HA/His-sSorC, and GEGFP cells express pLenti-m1-EGFP-N3.

All cells were cultured, differentiated, and maintained as described previously (Stephens et al., 1997). Prior to homogenization, cells were starved in serum-free DMEM for 2 hr and treated with 100 nM insulin or carrier (5 mM HCl at 1000× dilution) in DMEM for 5 min at 37°C. Then, cells were washed and processed as described previously (Xu and Kandror, 2002).

Measurements of Protein Stability

At time zero, 50 μg/ml of aqueous solution of cycloheximide or 10 μM emetine was added to plates along with 40 μM MG132 (Alexis Biochemicals, Carlsbad, CA) or 0.4 mM chloroquine (Sigma, St. Louis, MO) when specified. At the indicated time intervals, cells were rinsed three times with PBS and harvested in ice-cold 1% Triton X-100 in HES buffer with protease inhibitors. Cell lysates were vortexed, rotated at 4°C for an hour, and spun for 30 min at 16,000 × g in a microfuge at 4°C. The presence of the individual proteins in the supernatants was analyzed by Western blotting.

Sucrose Gradient Centrifugation

For velocity gradient centrifugation, samples (0.2 ml, 800 μg) were loaded onto a 3.8 ml linear 10%–30% (wt/vol) sucrose gradient in HE buffer and centrifuged for 65 min in a Sorvall TST60.4 rotor (Kendro Laboratory Products, Asheville, NC) at 48,000 rpm. Each gradient was separated into 20–25 fractions starting from the bottom of the tube. The protein profile was determined using a BCA kit (Pierce), and the linearity of the gradients was confirmed by measuring the refractive index of fractions. The fractions were further analyzed by gel electrophoresis and Western blotting.

Crosslinking and Immunoprecipitation

Crosslinking was performed according to Nielsen et al. (1999) with minor modifications. Differentiated G+ cells were washed twice with PBS and once with KRP buffer (12.5 mM HEPES, 120 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1.0 mM $CaCl_2$, 0.6 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 2.5 mM D-Glucose [pH 7.4]), and dithiobis(succinimidyl propionate) was added to final concentration 2 mM for 30 min at room temperature. Then, quenching buffer (50 mM Tris, 10 mM EDTA, 150 mM NaCl, 1 μM aprotinin, 2 μM leupeptin, 1 μM pepstatin, 5 mM benzamidine, and 1 mM PMSF [pH 7.4]) was added for 15 min at 4°C followed by two washes with the same buffer. The cells were lysed in 500 μl of quenching buffer with 1% Triton X-100, and cell lysates were cleared by centrifugation at 16,000 × g for 30 min. This material (1.5 mg) was incubated with the monoclonal anti-myc antibody and nonspecific mouse IgG (2 μg each) along with 30 μl of protein G beads overnight at 4°C with rotating. The beads were then washed three times with 1% Triton X-100 in quenching buffer. Elution was carried out with 30 μl of Laemmli sample buffer with 50 mM DTT at 37°C for 30 min.

Immunoadsorption of Glut4-Containing Vesicles

Protein A-purified 1F8 antibody, monoclonal anti-myc antibody, and nonspecific mouse IgG (each 2 μg) were coupled to 30 μl Dynabeads M-280 sheep anti-Mouse IgG (Dynal Biotech) according to the manufacturer's instructions. Before usage, the beads were washed three times with ice-cold 0.1%BSA in PBS. The 16,000 × g supernatants (800 μg) from 3T3-L1 adipocytes were incubated separately with each of the specific and nonspecific antibody-coupled beads overnight at 4°C with rotating. The beads were washed three times with PBS, and then eluted with 1% Triton X-100 in PBS for 1 hr at 4°C. The beads were then washed three

times with 1% Triton X-100 solution and the remaining protein was eluted with equal volume of 2× nonreducing Laemmli sample buffer. An aliquot (usually ~100 μg) of postabsorptive supernatant was analyzed by PAGE along with 100% of the eluate.

^{3}H-2-Deoxyglucose Uptake

This assay was performed in 6-well plates. Cells were washed three times with serum-free DMEM with 0.5% BSA and then serum starved for 2 hr. Then, cells were washed twice with warm Krebs-Ringer-HEPES (KRH) buffer without glucose (121 mM NaCl, 4.9 mM KCl, 1.2 mM $MgS0_4$, 0.33 mM $CaCl_2$, 12 mM HEPES [pH 7.4]) and treated with either 100 nM insulin or carrier (5 mM HCl at 1000× dilution) at 37°C for 15 min, and radioactive 2-deoxyglucose (0.1 mM, 0.625 μCi/ml) was added for 4 min for adipocytes and for 15 min for confluent preadipocytes. The assay was terminated by aspirating the radioactive media, and the cells were washed three times with 2 ml of ice-cold KRH containing 25 mM D-glucose. Each well was then extracted with 400 μl of 0.1% SDS in KRH buffer without glucose, and 300 μl aliquots were removed for determination of radioactivity by liquid scintillation counting. Under these conditions, glucose uptake was linear for at least 30 min. Measurements were made in duplicates and corrected for specific activity and nonspecific diffusion (as determined in the presence of 5 μM cytochalasin B), which was <10% of the total uptake. The protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL) and was used to normalize counts.

In Vitro Reconstitution of Glut4 Vesicles

This assay was performed as described earlier (Xu and Kandror, 2002). In some experiments, donor membranes were prewashed with 0.6 M KCl in budding buffer. The vesicle fraction along with the donor fraction were analyzed by Western blotting. The yield of small vesicles was linear when heavy membrane fraction was used at a concentration 0.2–1.6 mg/ml. In order to compare different experiments, we use arbitrary units defined as:

$$\frac{\text{[Intensity of Glut4 in the vesicle fraction – intensity in the “no cytosol” control lane] × [volume of the vesicle fraction]}}{\text{[Intensity of Glut4 in the donor fraction] × [volume of the donor fraction]}}$$

The average of duplicate reactions and the deviation is plotted on the graph.

Immunofluorescence

3T3-L1 preadipocytes were grown and differentiated on coverslips coated with poly-L-lysine and fixed with 4% paraformaldehyde in PBS for 30 min. Fixed cells were washed with PBS, permeabilized with 0.2% Triton X-100 for 5 min, blocked with 5% donkey serum in 5% bovine serum albumin, and stained with primary anti-myc monoclonal antibody and Cy3-conjugated donkey anti-mouse IgG (Jackson Immunoresearch). For double staining, permeabilized cells were first stained with the mixture of primary antibodies followed by the mixture of secondary antibodies. Nonpermeabilized cells were fixed and stained with anti-myc monoclonal antibody and Cy3-conjugated donkey anti-mouse IgG (Jackson Immunoresearch). Each incubation with antibody lasted for 60 min at room temperature and was followed by six quick washes with PBS. A SlowFade-Light Antifade kit (Molecular Probes) was used for mounting cells on slides. Slides were examined by fluorescence microscopy using Axiovert 200M (Carl Zeiss Inc., Thornwood, NY), and the pictures were taken by Axiovision 3.0 program (Carl Zeiss Inc.).

Supplemental Data

Supplemental Data include six figures and Supplemental Experimental Procedures and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/9/1/99/DC1/.

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