The luminal Vps10p domain of sortilin plays the predominant role in targeting to insulin-responsive Glut4-containing vesicles.

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In fat and skeletal muscle cells, insulin-responsive vesicles, or IRVs, deliver glucose transporter Glut4 and several associated proteins to the plasma membrane in response to hormonal stimulation. Although the protein composition of the IRVs is well studied, the mechanism of their formation is unknown. It is believed, however, that the cytoplasmic tails of the IRV component proteins carry targeting information to this compartment. In order to test this hypothesis, we have studied targeting of sortilin, one of the major IRV constituents. We have found that the reporter protein consisting of the cytoplasmic tail of sortilin and EGFP is co-localized with ectopically expressed Glut4 in the perinuclear compartment of undifferentiated 3T3-L1 cells that do not form insulin-responsive vesicles. Upon cell differentiation, this reporter protein does not enter the IRVs; moreover, it loses its perinuclear localization, and becomes randomly distributed throughout the whole intracellular space. On the contrary, the tagged luminal Vps10p domain of sortilin demonstrates partial co-localization with Glut4 in both undifferentiated and differentiated cells and is targeted to the IRVs upon cell differentiation. Using chemical cross-linking and yeast two-hybrid system, we show that sortilin interacts with Glut4 and IRAP in the vesicular lumen. Our results suggest that luminal interactions between component proteins play an important role in the process of IRV biogenesis.

Insulin increases glucose uptake in fat and skeletal muscle tissues by translocating glucose transporter isoform 4, Glut4, from an intracellular storage pool to the plasma membrane. Under the basal conditions, Glut4 is present in multiple subcellular compartments that include sorting and recycling endosomes and trans-Golgi network (1,2). However, the major pool of the transporter is localized in a distinct population of 60-80 S vesicles (IRVs for insulin-responsive vesicles, also known as GSVs for Glut4-storage vesicles) that are translocated to the plasma membrane in response to insulin stimulation (3-9). The protein composition of these vesicles is limited. In addition to Glut4, their major integral membrane proteins include insulin-regulated amino peptidase, IRAP (10-12) and the putative sorting receptor sortilin (7,13,14). Unlike Glut4 that has 12 transmembrane domains, both
sortilin and IRAP are correspondingly type I and type II single pass transmembrane proteins. It is likely that each IRV compartmentalizes several copies of Glut4, IRAP (15) and, probably, sortilin.

The mechanism of IRV biogenesis is not known. Available data suggest that IRVs may be formed from syntaxin 6-positive donor membranes of the TGN/endosomal origin localized in the perinuclear region of the cell (7,16,17). Monomeric GGA adaptors (16,18) and GGA-binding protein sortilin (7) play important roles in vesicle biogenesis. Under basal conditions, IRVs may exist in a dynamic equilibrium with the donor membranes which is maintained by multiple cycles of budding and fusion (19). It is not clear, however, what signal sequences in the IRV component proteins target them to the perinuclear donor membranes and to insulin-responsive vesicles.

In order to approach this problem, we prepared reporter molecules that comprise the luminal domain and the cytoplasmic tail of sortilin and stably expressed these proteins (mSorN and EGFP-SORTILINtail correspondingly, see Fig. 1) in 3T3-L1 cells. In agreement with our previous results (7), we found that EGFP-SORTILINtail was co-localized with ectopically expressed Glut4 in undifferentiated pre-adipocytes. However, upon cell differentiation, EGFP-SORTILINtail does not acquire insulin responsiveness and loses its co-localization with Glut4. At the same time, the luminal domain of sortilin alone is partially co-localized with ectopically expressed Glut4 in undifferentiated cells and is targeted to the IRVs upon cell differentiation. These results suggest that protein-protein interactions in the vesicular lumen may play an essential role in protein sorting and recruitment into the IRVs.

**EXPERIMENTAL PROCEDURES**

**Antibodies** - In the present study, we used the monoclonal anti-V5 antibody from Invitrogen (Carlsbad, CA), the monoclonal anti-myc-tag antibody and the polyclonal anti-myc-tag antibody from Cell Signaling Technology (Danvers, MA), the monoclonal anti-syntaxin 6 and anti-sortilin (neurotensin receptor 3) antibodies from BD Biosciences Pharmingen (San Diego, CA), the monoclonal anti-FLAG antibody from Sigma-Aldrich (St. Louis, MO), the monoclonal anti-Glut4 antibody 1F8 (20), a rabbit polyclonal antibody against cellubryin (Ac-CQNVTETQYQPPPY-OH) that has been raised and affinity-purified by BioSource International (Camarillo, CA), a rabbit polyclonal antibody against IRAP (a kind gift from Dr. Paul Pilch, Boston University School of Medicine).

**cDNA Constructs** - The pLenti6/V5 Directional TOPO cloning kit was purchased from Invitrogen (Carlsbad, CA). Two oligonucleotides (5'-CACCGAATTCTTTAAACTGTCGAC-3' and 5'-GTCGACAGTTTAAACGAATTCGGTG-3') were annealed and ligated in the pLenti6/V5-D-TOPO vector. This modified vector is called pLenti vector. Two more oligonucleotides (5'-GATCCGTTAACGAATTCTCGCTCGAGCCAGTGTGGTGTTAAACTCTGCAGACTAGTGTGACAGGGGCGCCG-3' and 5'-GGGCCCTGTGACACTAGTCTGCAAGTTAAACCACCACACTGGCTCGAGCGGAGAATTCTGTTAAG-3') were annealed and ligated with the BamHI and SacII cutted pLenti vector. This vector is called pLenti-m1. Full-length sortilin was released from mLNCX2-sortilin-myc/His (7) by XhoI and Pmel and ligated in the corresponding sites of the pLenti-m1 vector, creating pLenti-m1-sortilin-myc/His. The fragment (nucleotides 1816-2349) of human
sortilin was amplified with two primers: 5’-GATTTAAAGATATCCTTGAAAGGA-3’ and 5’-TTGTGAAGAAATATGTCTGTCATCACCATCACCATACTAGT-3’ (encoding the His tag) digested with EcoRV and SpeI and ligated in the corresponding sites of pLenti-m1-sortilin-myc/His. This vector (pLenti-m1-mScrN) encodes sortilin with the His/V5 tag instead of the original C terminus starting from the sixth amino acid after the transmembrane domain (Fig. 1).

EGFP was amplified from the pEGFP-C2 template using two primers 5’-ACGACGTCAGGATCCGTCGCCACCA TGGTGAGCAA-3’ and 5’-TCGAGTCTGGAATTCCTTGTACAGCT CGTCCATGCC-3’. This fragment was digested by BamHI and SalI and ligated in the corresponding sites of pLenti-m1. The resulting vector is called pLenti-m1-EGFP-C2.

EGFP fragment was amplified from the pEGFP-N3 template using two primers 5’-ACGACGACAGTCGACATCGCCACCA TGGTGAGCAAG-3’ and 5’-CTGAGTCTGCCGCGGTTACTTGTAC AGCTCGTCCATG-3’. This fragment was digested sequentially by SalI and SacII and ligated in the corresponding sites of pLenti-m1. The resulting vector is called pLenti-m1-EGFP-N3.

The C terminus of human sortilin (nucleotides 2243-2496) was amplified using two primers 5’-CAGCAGTCACTCGAGGACTACAAGGACGATGACGACAAGGATTATAAAGATGACGATGACAAGAAACAGAATTCAAAGTCAAATTCT-3’ (containing 2 FLAG tags) and 5’-TCGAGTCTGACTAGTCTATTCCAAGGGTCCATCATCT-3’. This fragment was cut with XhoI and SpeI and ligated in the corresponding sites of pLenti-m1-EGFP-C2 vector, creating pLenti-m1-EGFP-SORTILIINtail (Fig. 1).

Stable Cell Lines - G and S+ cells were stably transfected with pBabe-myc7- Glut4 and mLNCX2-sortilin-myc/His correspondingly as described previously (7). Pooled clones of G cells were infected with the lentiviral constructs described in the previous section according to the Invitrogen’s protocol. Selection of cells was performed with blasticidin (8 μg/ml) for two weeks, and pooled stable clones were used for further experiments. All cell lines were cultured, differentiated, and maintained as described previously (7).

Subcellular Fractionation of 3T3-L1 cells - Prior to harvesting, cultured 3T3-L1 adipocytes were washed three times with serum-free DMEM heated to 37ºC and starved in the same media for 3 hours. Where indicated, cells were treated with 100 nM insulin or carrier (5 mM HCl at 1000x dilution) in DMEM for 5 or 15 minutes at 37ºC. Then, cells were washed three times with warm HES buffer with protease inhibitors (250 mM sucrose, 20mM HEPES, 1 mM EDTA, pH7.4, 1 μM aprotinin, 2 μM leupeptin, 1 μM pepstatin, 5 mM benzamidine, and 1 mM PMSF). Cells were harvested in HES buffer, homogenized by 10 strokes up and down using a teflon pestle and centrifuged at 16,000xg for 20 min. Plasma membrane (PM), heavy microsomes, light microsomes (LM), and nuclear/mitochondrial fraction were obtained by differential centrifugation as described previously (21). Fractions were re-suspended in HES buffer and protein concentration was determined using a BCA kit (Pierce Chemical, Rockford, IL).

Measurements of Protein Stability - At time zero, 50 μg/ml of aqueous solution of cycloheximide or 10 μM emetine was added to plates. 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 x 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 of light microsomes (0.2 ml, 500 μg) were loaded onto a 3.8 ml linear 10-30% (wt/vol) sucrose gradient in HE buffer (20 mM HEPES, 1 mM EDTA, pH 7.4) 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.

Immunoadsorption of Glut4-containing and Cellugyrin-containing Vesicles - Protein A-purified 1F8 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. Alternatively affinity-purified polyclonal anti-cellugyrin antibody and nonspecific rabbit IgG (each - 2 μg) were coupled to 30 μl Dynabeads M-280 sheep anti-Rabbit IgG. Before usage, the beads were washed three times with ice-cold 0.1% BSA in PBS. Light microsomes (500 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 40 μl 1% Triton X-100 in PBS for 1 hour at 4°C. Then, the beads were washed three times with 1% Triton X-100 solution and the remaining proteins were eluted with 30 μl 1X non-reducing Laemmli sample buffer. An aliquot (usually ~ 100 μg) of post-absorptive supernatant was analysed by PAGE along with 100% of the eluate.

³H-2-Deoxyglucose Uptake - This assay was performed in 6-well plates. Cells were washed three times with serum-free DMEM and incubated in serum-free DMEM for 2 more hours. Cells were then washed twice with warm Krebs-Ringer-HEPES (KRH) buffer without glucose (121 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 0.33 mM CaCl₂, 12 mM HEPES, pH 7.4) and treated with either 100 nM insulin or carrier (5 mM HCl at 1000x dilution) at 37°C for 15 min. Radioactive 2-deoxyglucose (0.1 mM, 0.625 μCi/ml) was added for 4 min to adipocytes and for 15 min to confluent pre-adipocytes. 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 Cellugyrin-containing Vesicles - Cytosol and the donor membranes were isolated from undifferentiated and differentiated 3T3-L1 cells, and the assay was performed as described earlier (9). 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:

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\text{[Intensity of cellugyrin in the vesicle fraction - intensity in the “no cytosol” control lane]} \times \text{[volume of the vesicle fraction]} \quad \text{[Intensity of cellugyrin in the donor fraction]} \times \text{[volume of the donor fraction]}
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The average of duplicate reactions and the deviation is plotted on the graph.

**Immunofluorescence** - 3T3-L1 pre-adipocytes were grown in 60 mm dish. The night before the experiment, cells were reseeded onto Nunc LAB-TEK II 4-well chamber slides. 3T3-L1 adipocytes were grown and differentiated in 60 mm dish. On day 5 of differentiation, cells were lifted up by trypsin for 10 minutes at 37 ºC and reseeded to 4-well chamber slides. After growing on chamber slides for two more days, adipocytes (day 7) were used for staining. Cultured 3T3-L1 adipocytes or pre-adipocytes were washed three times with serum-free DMEM heated to 37ºC and starved in the same media for 3 hours. Then, cells were 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 PBS with 5% donkey serum and 5% bovine serum albumin, and stained with primary anti-myc monoclonal antibody or anti-syntaxin 6 monoclonal antibody and Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch). For double staining of G/mSorN cells, permeabilized cells were first stained with the mixture of anti-myc polyclonal antibody and anti-V5 monoclonal antibody followed by the mixture of Alexa 488 conjugated Donkey anti-mouse IgG (Invitrogen) and Cy3-conjugated donkey anti-rabbit 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 which were examined by fluorescence microscopy using Axiovert 200M (Carl Zeiss Inc., Thornwood, New York). Pictures were taken with the help of Axiovision 3.0 program (Carl Zeiss Inc.).

**Cross-linking and Immunoprecipitation** - Cross-linking was performed according to (22) with minor modifications. S+ cells were washed twice with PBS and once with KRP buffer (12.5 mM Heps, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO4, 1.0 mM CaCl2, 0.6 mM Na2HPO4, 0.4 mM NaH2PO4, 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 minutes 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 lyzates were cleared by centrifugation at 16,000 x 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 minutes.

**Yeast two-hybrid interactions** - The MATCHMAKER Gal4 two-hybrid system 3 (Clontech Laboratories, Mountain View, CA) was used for these experiments. The sortilin luminal Vps10p domain without signal sequence
and pro-peptide was amplified using two primers: 5'- CTAGACCTGCCATGGAG AGCGCGCCGGGCGAGGA-3' and 5'-ACGACACTCAGG GATTGACTGGAATTCTGTTTTTC-3'. The PCR product was digested by NcoI and PstI and ligated into the pGBK7 vector that encodes GAL4 DNA-binding domain. The IRAP luminal domain (IRAP LD) was amplified using two primers 5'- ACGACGTACATATGCCCTAGAT GTACCTTTACCAAAG-3' and 5'- CTAGACAGGGATCCCTACAGCCAC AGTGTCAGAG -3'. The PCR product was digested by NdeI and BamHI and ligated into the correspondingly cut pGADT7 that encodes GAL4 transcription activation domain. The first extracellular loop of Glut4 (Glut4 loop 1) was amplified using two primers 5'- CTAGACCTGGAAATTCAATGCCCTCA GAAGGTGATT-3' and 5'- ACGACGTAGGATCCCAGAGGGTGGTGAGGGTG-3'. The PCR product was digested with EcoRI and BamHI and ligated into the pGADT7 vector that encodes GAL4 transcription activation domain. Transformation was done according to the manual of YEASYMAKER yeast transformation system 2 from Clontech. Co-transformants (100 μl) were plated in parallel on SD-Leu/-Trp, SD-Leu/-Trp/-His and SD-Leu/-Trp/X-α-Gal plates. In parallel, cells transformed with pGADT7-IRAP LD (100μl) were plated on SD-Leu/-His and SD-Leu/X-α-Gal plates and cells transformed with pGBK7-Sortilin LD (100μl) were plated on SD-Trp/-His and SD-Trp/X-α-Gal plates. The number of colonies growing on the selection medium (SD-Leu/-Trp/-His) was quantified after 4 days at 30°C using MACE software from Weiss Associates (Branford, CT) and expressed as normalized mean +/- SE from three independent experiments.

Gel Electrophoresis and Western blotting - Proteins were separated in SDS-polyacrylamide gels according to Laemmli (23), but without reducing agents, and transferred to an Immobilon-P membrane (Millipore) in 25 mM Tris, 192 mM glycine. Following transfer, the membrane was blocked with 10% non-fat milk in PBS with 0.5% Tween 20 for 1 h at 37°C. The blots were probed with specific antibodies, horseradish peroxidase-conjugated secondary antibodies (Sigma), and detected with an enhanced chemiluminescence substrate kit (PerkinElmer Life Sciences) using a Kodak Image Station 440CF (Kodak, Rochester, NY). The signals were quantified using the Kodak 1D image analysis software.

RESULTS

Previously, we have shown that full-length sortilin is faithfully targeted to the IRVs upon ectopic expression in 3T3-L1 adipocytes (7). Here, we decided to test whether or not the cytoplasmic tail of sortilin is sufficient for its targeting as it has been reported for another IRV protein, IRAP (24) and Glut4 itself (17,25-30). To this end, the cytoplasmic tail of sortilin was conjugated to EGFP and two Flag epitopes, and this molecule (EGFP-SORTILINtail) was expressed in 3T3-L1 cells stably transfected with myc7-Glut4 (G cells in (7)). We have reported earlier that an analogous molecule, mSorC (Fig. 1), co-localizes with myc7-Glut4 in undifferentiated G cells and confers some degree of insulin responsiveness to co-expressed myc7-Glut4 (7). To this end, the cytoplasmic tail of sortilin was conjugated to EGFP and two Flag epitopes, and this molecule (EGFP-SORTILINtail) was expressed in 3T3-L1 cells stably transfected with myc7-Glut4 (G cells in (7)).

We have reported earlier that an analogous molecule, mSorC (Fig. 1), co-localizes with myc7-Glut4 in undifferentiated G cells and confers some degree of insulin responsiveness to co-expressed myc7-Glut4 (7). We now confirm this result using a novel reporter molecule, EGFP-SORTILINtail (Fig. 2A). Unexpectedly, we have found that, upon cell differentiation, EGFP-SORTILINtail loses its perinuclear localization and becomes randomly distributed throughout the intracellular space (Fig. 2B, C). Naturally, in differentiated adipocytes, EGFP-
SORTILINTERNtail shows very little overlap with either myc-Glut4 or with syntaxin 6 that represents an endogenous marker of the perinuclear Glut4-containing compartment (Fig. 2B, C).

Sucrose gradient centrifugation (Fig. 3A) demonstrates that the sedimentational distribution of EGFP-SORTILINTERNtail in differentiated adipocytes is different from that of endogenous sortilin, IRAP and Glut4 but largely overlaps with cellugyrin which is localized in ubiquitous intracellular transport vesicles with random intracellular localization and no insulin response (15,31).

In order to confirm this result, we have immunoadsorbed cellugyrin-positive vesicles using the polyclonal antibody against cellugyrin. In these experiments, we sequentially elute immunoadsorbed material with 1% Triton X-100 and then – with SDS-containing Laemmli sample buffer. Triton solubilizes vesicular membrane and elutes proteins that do not bind directly to the immobilized antibody. Importantly, Triton practically does not elute antibody from the beads which makes it easier to detect various proteins in this fraction by Western blotting. On the contrary, SDS elutes immobilized antibody and vesicular proteins that directly interact with this antibody. We found that a significant fraction of EGFP-SORTILINTERNtail is co-immunoadsorbed with cellugyrin (Fig. 3B) suggesting that these two proteins are co-localized in the same vesicular population.

The presence of EGFP-SORTILINTERNtail in cellugyrin-positive vesicles may help to explain differentiation-related changes in the intracellular localization of this reporter protein. Therefore, in the following experiment, we analyzed the rate of biogenesis of cellugyrin-positive vesicles in undifferentiated and differentiated cells. To this end, we have performed vesicle budding assay in vitro using the donor membranes and cytosol from undifferentiated and differentiated cells in different combinations. Results of the budding reaction were expressed as the ratio between cellugyrin in the vesicle fraction and in the donor fraction. Our results show that the formation of cellugyrin-containing vesicles from “undifferentiated” donor membranes is significantly stimulated by the cytosol obtained from differentiated cells suggesting that some unidentified cytosolic factor which stimulates the formation of cellugyrin-containing vesicles is induced during differentiation (Fig. 4). When “differentiated” donor membranes are used in this assay, formation of cellugyrin-containing vesicles was even higher regardless of the source of cytosol (Fig. 4). These results support the notion that the rate of formation of cellugyrin-containing vesicles is greatly increased with cell differentiation. This, in turn, implies that, in differentiated cells, EGFP-SORTILINTERNtail may be spread out from the perinuclear compartment to random intracellular loci by rapidly forming cellugyrin-positive vesicles.

In any case, we have to conclude that the cytoplasmic tail of sortilin does not play the major role in protein targeting in differentiated adipocytes. Therefore, we decided to explore the role of the luminal N-terminal domain of sortilin. For these experiments, we substituted the cytoplasmic tail of sortilin with two short tags, His and V5, and stably expressed this molecule (mSorN) in G cells.

In undifferentiated cells, mSorN has a broad subcellular localization and is present in both intracellular compartments and the plasma membrane (Fig. 5). This is consistent with the predominant point of view, according to which a protein, such as mSorN, with no trafficking motifs in its cytoplasmic tail follows the general membrane flow in the cell (32). Nonetheless, mSorN is clearly enriched
in the perinuclear region of the cell where it co-localizes with mycγ-Glu4 (Fig. 5). We also noticed that mSorN dramatically stabilizes mycγ-Glu4 in undifferentiated cells (Fig. 6A) and increases insulin-stimulated glucose uptake (Fig. 6B). These data suggest that mSorN does not simply co-localize with mycγ-Glu4 in the same compartment; rather, a functional connection exists between mSorN and mycγ-Glu4 in co-transfected cells. The mechanism of how mSorN affects stability and the intracellular traffic of mycγ-Glu4 is currently under investigation.

Contrary to EGFP-SORTILINtail, the intracellular localization of mSorN does not significantly change with cell differentiation. Thus, Fig. 7 shows that, in differentiated adipocytes, mSorN is also present both inside the cell and at the plasma membrane even in the absence of insulin suggesting that this protein traffics between different intracellular compartments. However, similar to the situation in undifferentiated cells, mSorN is enriched in the perinuclear region of differentiated adipocytes where it co-localizes with mycγ-Glu4.

Sucrose gradient centrifugation confirms this result and demonstrates that the sedimentational distribution of mSorN in the vesicular fraction overlaps with that of the IRVs (Fig. 8A). In addition, immunoadsorption of Glu4-vesicles with the monoclonal antibody 1F8 shows that at least 30% of mSorN in the vesicular fraction is co-localized with Glu4/IRAP-containing vesicles, that, presumably, represent IRVs (Fig. 8B).

Finally, the biochemical fractionation demonstrates that mSorN is translocated to the plasma membrane in response to insulin stimulation similar to other component proteins of the IRVs, such as Glu4, mycγ-Glu4, IRAP and sortilin (Fig. 9). At the same time, neither EGFP-SORTILINtail nor cellugyrin show insulin responsiveness in adipose cells (Fig. 9). Importantly, plasma membrane translocation of both full length sortilin and mSorN is apparent after 5 min of insulin stimulation but becomes less detectable upon longer incubations with insulin ((7) and results not shown).

Thus, the luminal domain of sortilin is required for its targeting to the IRVs and insulin-dependent translocation to the plasma membrane. In order to explain this phenomenon, we suggest that the luminal Vps10p domain of sortilin may interact with one or several IRV proteins, and that this interaction facilitates intracellular targeting of sortilin. Potential candidates for the role of sortilin-binding proteins are Glu4 and IRAP which represent the major IRV constituents. Indeed, we have previously shown that full-length sortilin may be cross-linked to mycγ-Glu4 with the help of the membrane-permeable cross-linker, DSP (7). Here, we decided to confirm this result by analyzing cross-linking of sortilin to endogenous IRAP.

Our results show that ectopically expressed full-length sortilin-myc/His may be efficiently cross-linked to endogenous IRAP in both undifferentiated and differentiated 3T3-L1 cells with the help of the membrane-permeable reagent DSP (Fig. 10). Since the protein-binding Vps10p domain of sortilin as well as the bulky catalytic domain of IRAP are localized in the vesicular lumen, we suggest that cross-linking takes place via luminal domains of these proteins. In order to test this hypothesis, we attempted to determine whether or not EGFP-SORTILINtail and mSorN may be cross-linked to mycγ-Glu4 and IRAP. Unfortunately, the results of these experiments were dirty in agreement with the broad intracellular localization of sortilin truncation constructs.

Therefore, we decided to test the putative interaction between the luminal domains of sortilin, mycγ-Glu4 and
IRAP with the help of a yeast two-hybrid system. For that, the luminal Vps10p domain of sortilin was fused with GAL4 DNA-binding domain in the pGBK7 vector while the luminal domain of IRAP (IRAP LD) and the first extracellular loop of Glut4 (Glut4 loop 1) were fused with GAL4 transcription activation domain (AD) in the pGADT7 vector. Fig. 11A, B, C show that cells transformed with either pGADT7-IRAP LD, pGADT7-Glut4 loop 1, or pGBK7-Vps10p do not grow on the selection media (left images) and do not secret α-galactosidase when they grow on the permissive media (note lack of the blue color of colonies on the right images). By the same token, cells double transformed with pGBK7-Vps10p and empty pGADT7 vector grow on the permissive media (Fig. 11D left), do not grow on the selection media (Fig. 11D middle), and do not secret α-galactosidase on the permissive media (Fig. 11D right). However, cells double transformed with pGADT7-IRAP LD or pGADT7-Glut4 loop 1 and pGBK7-Vps10p (Fig. 11E, F) grow on the permissive media (left panels) and on the selection media (middle panels) equally well and, in addition, secret GAL4-regulated α-galactosidase (note blue colonies on the right panels). Quantification of the results obtained with the help of the yeast two hybrid system is shown in Fig. 11G.

**DISCUSSION**

Here, we analyzed the intracellular targeting of sortilin which represents one of the major component proteins of the IRVs. Our results suggest that sortilin targeting to this compartment requires both the cytoplasmic tail and the luminal domain of the protein. The role of the cytoplasmic tail is apparent in undifferentiated cells where it is sufficient for co-localization with ectopically expressed myc7-Glut4 in the perinuclear compartment that may represent a “donor” compartment for the formation of the IRVs (7). In differentiated cells, however, EGFP-SORTILINtail shows virtually no co-localization with Glut4. We explain this phenomenon by an increased capacity of differentiated cells to form small vesicles that can capture EGFP-SORTILINtail as this protein cannot interact with luminal “anchors”. It should be noted that EGFP-SORTILINtail has a GGA-binding sequence (33) that may recruit GGA adaptors and clathrin to the donor membranes and facilitate vesicle formation. Whatever the mechanism of vesicle biogenesis is, it has relatively low efficiency in undifferentiated cells. This allows the major pool of EGFP-SORTILINtail to stay in the perinuclear membranes which results in a significant co-localization between EGFP-SORTILINtail and myc7-Glut4. Upon cell differentiation, the efficiency of vesicle formation is increased, and EGFP-SORTILINtail is re-distributed to small vesicles and, likely, to their target membranes making the presence of EGFP-SORTILINtail in the perinuclear compartment virtually undetectable.

The biological nature and functions of EGFP-SORTILINtail-containing vesicles is unknown. Sucrose gradient centrifugation and immunoadsorption show that at least some of these vesicular carriers contain cellugyrin, a ubiquitous analogue of synaptogyrin, the major component of synaptic vesicles in the brain (34). In non-neuronal cells, cellugyrin represents a marker protein of ubiquitous vesicles (35) that accumulate a significant amount of recycling proteins and may thus represent transport vesicles that shuttle between different intracellular compartments (15,31).

The formation of the IRVs is also up-regulated in differentiation (7). However, the critical differentiation-related factor that controls the formation of the IRVs is not a cytosolic but, rather,
an integral membrane protein, that has recently been identified as sortilin (7). Therefore, formation of the IRVs and cellugyrin/EGFP-SORTILINtail-containing vesicles is likely to be regulated by different molecular mechanisms.

Since the cytoplasmic tail of sortilin does not seem to play a major role in protein targeting in differentiated adipocytes, we have decided to analyze the targeting role of its luminal Vps10p domain. We found that the extent of mSorN co-localization with Glut4 is less than that of full-length sortilin (7,36) indicating that the cytoplasmic tail of the protein contributes to its faithful targeting even in differentiated adipocytes. Nevertheless, mSorN is partially localized in the IRVs suggesting that the luminal Vps10p domain plays an important and, likely, predominant role in sortilin targeting in 3T3-L1 adipocytes.

According to our model, the cytoplasmic tail of sortilin may target this protein to the perinuclear IRV donor compartment and its luminal Vps10p domain may be responsible for the interaction with specific proteins within this compartment, such as Glut4 and IRAP. The heteromeric protein complex of sortilin, IRAP and Glut4 stabilized by luminal interactions may then redistribute from the perinuclear donor membranes to the IRVs via the GGA-mediated mechanism using the GGA-binding sequence in the cytoplasmic tail of sortilin (16).

If the cytoplasmic tail of sortilin is indeed responsible for protein targeting into the perinuclear donor membranes, how can mSorN that does not contain the cytoplasmic tail also reach this compartment? One possibility is that mSorN constantly traverses the perinuclear donor membranes that may represent either recycling endosomes (37) or TGN (17) with the bulk of membrane flow. Once there, mSorN may bind to the luminal fragments of Glut4 and IRAP. This interaction may anchor mSorN in the perinuclear compartment, so that mSorN partially escapes from the general endocytic recycling pathway that may involve ubiquitous cellugyrin-containing transport vesicles. From this standpoint, the cytoplasmic tail increases the efficiency of sortilin sorting into the perinuclear donor membranes and, eventually, into the IRVs, but is not absolutely required for these events.

An interesting problem is the role of mSorN in the recycling of myc7-Glut4 in undifferentiated pre-adipocytes. In these cells, ectopically expressed myc7-Glut4 is very unstable and is degraded via the lysosomal pathway (7). Expression of full-length sortilin (7) as well as mSorN (this paper, Fig. 6A) stabilizes myc7-Glut4 and increases insulin-stimulated glucose uptake. One possibility is that sortilin interacts with Glut4 and rescues it from the lysosomal degradation by stimulating retrograde transport from endosomes to TGN (38). Since TGN may represent the IRV donor compartment (17), this may lead to an increase in the IRV formation. This mechanism, however, is unlikely to work in case of mSorN, since it is not likely to interact with retromer. It is still possible that mSorN links Glut4 to some other retrograde cargo protein, such as CI-MPR that is known to partially follow the Glut4 pathway (39). Alternatively, both sortilin and mSorN may link Glut4 to IRAP (Figs. 10 and 11) which has been shown to undergo insulin-regulated recycling in undifferentiated 3T3-L1 cells (40). Yet another possibility is that mSorN inhibits lysosomal delivery of Glut4-degrading enzymes (41,42) and indirectly stabilizes Glut4. Regardless of the molecular mechanism, Glut4 stabilization may increase its targeting to the regulated recycling pathway and to the activation of insulin-stimulated glucose uptake (Fig. 6B). Our data suggest, for the first time, that the
luminal interactions between vesicular proteins play an important role in this process.

FOOTNOTES

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REFERENCES

LEGENDS TO FIGURES

Figure 1. Reporter proteins used in this paper. The structure of mSorc from (7) is shown for the reference.

Figure 2. EGFP-SORTILINtail co-localizes with myc7-Glut4 in the perinuclear region of 3T3-L1 pre-adipocytes, but not in differentiated adipocytes. Myc7-Glut4/EGFP-SORTILINtail-double-transfected pre-adipocytes (panel A) were stained with the anti-myc monoclonal antibody and Cy3-conjugated donkey anti-mouse secondary antibody. Bar, 10 μM. Myc7-Glut4/EGFP-SORTILINtail-double-transfected adipocytes were stained with the anti-myc monoclonal antibody (panel B) or anti-syntaxin6 monoclonal antibody (panel C) and Cy3-conjugated donkey anti-mouse secondary antibody. Bars in panels B and C, 10 μM.

Figure 3. In differentiated 3T3-L1 adipocytes, EGFP-SORTILINtail is present in cellugyrin-containing vesicles. Panel A: Light microsomes (500 μg of total protein) isolated from differentiated myc7-Glut4/EGFP-SORTILINtail-double-transfected adipocytes were fractionated in a 10-30% sucrose velocity gradient as described in Materials and Methods. The gradient fractions were blotted with the anti-IRAP polyclonal antibody, the cocktail of anti-myc monoclonal and anti-sortilin monoclonal antibodies, anti-Glut4 monoclonal antibody, anti-FLAG monoclonal antibody and anti-cellugyrin polyclonal antibody. The arrow indicates the direction of sedimentation. Panel B: Light microsomes (500 μg of total protein) of differentiated myc7-Glut4/EGFP-SORTILINtail-double-transfected adipocytes were immunoadsorbed with anti-cellugyrin- and nonspecific IgG-coupled beads. An aliquot (100 μg) of post-adsorptive supernatant along with total Triton and SDS eluates was analyzed by Western blotting with antibodies against cellugyrin (top panel) and FLAG (bottom panel). A representative result of three independent experiments is shown.

Figure 4. Formation of cellugyrin-containing vesicles is stimulated upon differentiation of 3T3-L1 cells. Vesicle reconstitution assay in vitro was performed as described previously (9) in duplicate with donor membranes (1 mg/ml) and cytosol (2 mg/ml) isolated from differentiated (Ad) and undifferentiated (Fb) 3T3-L1 cells. The lower panel shows the quantification of the Western blot stained with anti-cellugyrin antibody (values of mean and range between duplicate samples). A representative result of three independent experiments is shown.

Figure 5. mSorN partially co-localizes with myc7-Glut4 in the perinuclear region of 3T3-L1 pre-adipocytes. Myc7-Glut4/EGFP-SORTILINtail-double-transfected fibroblasts were stained with the mixture of the anti-myc polyclonal antibody and the anti-V5 monoclonal antibody followed by the mixture of Alexa 488 conjugated Donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG. Bar, 10 μM.

Figure 6. mSorN increases the half-life of myc7-Glut4 and insulin-regulated glucose uptake in 3T3-L1 pre-adipocytes. Panel A: Stability of myc7-Glut4 and mSorN in G/EGFP and G/mSorN preadipocytes. Each lane contains 100 μg of total cell homogenate. Panel B: 3H-2-deoxyglucose uptake in undifferentiated G/EGFP (white bars) and G/mSorN (grey bars) cells. The panel shows normalized mean and range values of two independent experiments, each in duplicate.
Figure 7. mSorN partially co-localizes with myc7-GLUT4 in the perinuclear region of differentiated 3T3-L1 adipocytes. Serum starved myc7-GLUT4/mSorN-double-transfected adipocytes were first stained with the mixture of the anti-myc polyclonal antibody and the anti-V5 monoclonal antibody followed by the mixture of Alexa 488 conjugated Donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG. The figure shows two representative cells with different levels of mSorN expression. Bars, 10 μM.

Figure 8. In differentiated 3T3-L1 adipocytes, EGFP-SORTILINtail is present in the IRVs. Panel A: Light microsomes (500 μg of total protein) isolated from differentiated myc7-GLUT4/mSorN-double-transfected adipocytes were fractionated in a 10-30% sucrose velocity gradient as described in Materials and Methods. The gradient fractions were blotted with the anti-IRAP polyclonal antibody, the cocktail of the anti-myc monoclonal and the anti-sortilin monoclonal antibodies, anti-GLUT4 monoclonal antibody, and anti-cellugyrin polyclonal antibody. The arrow indicates the direction of sedimentation. Panel B: Light microsomes (500 μg of total protein) of differentiated myc7-GLUT4/mSorN-double-transfected adipocytes were immunoadsorbed with 1F8- and nonspecific IgG-coupled beads. An aliquot (100 μg) of post-adsorptive supernatant along with total Triton and SDS eluates was analyzed by Western blotting with antibodies against V5 (top panel), GLUT4 (middle panel) and IRAP (bottom panel). A representative result of three independent experiments is shown.

Figure 9. mSorN, but not EGFP-SORTILINtail, demonstrate insulin-dependent translocation to the plasma membrane in differentiated 3T3-L1 adipocytes. Differentiated G/mSorN and G/EGFP-SORTILINtail adipocytes were treated or not treated with 100 nM insulin for 5 min and fractionated by differential centrifugation into the plasma membrane (PM), and light microsomal (LM) fractions which were analyzed by Western blotting (30 μg per lane). A representative result of at least three independent experiments is shown.

Figure 10. Sortilin-myc/His can be cross-linked to endogenous IRAP in 3T3-L1 cells. Undifferentiated (day 0) and differentiated (day 5) S+ cells were incubated with DSP as described in Materials and Methods, and cell lysate (1.5 mg) was immunoprecipitated with 2 μg of the anti-myc antibody or non-specific IgG and protein G. Proteins were eluted with 30 μl of Laemmli sample buffer with 50 mM DTT at 37°C for 30 minutes.

Figure 11. Luminal domains of GLUT4, IRAP and sortilin interact in yeast two-hybrid system. Panels A, B and C show negative and positive controls for single transformed cells. Panel D shows negative and positive controls for double transformed cells. Panels E and F show that double transformed cells grow equally well on the permissive media (left) and on the selection media (middle) and secret α-galactosidase (blue colonies on right images). Panel G shows normalized mean values +/- SE of 3 independent experiments.
Figure 1

Lumen

Cytoplasm

Sortilin-myc/His

mSorN

EGFP-SORTILINtail

mSorC
Figure 2

A

EGFP-SORTILIN
Myc7-Glut4
Merged

Undifferentiated pre-adipocytes

B

EGFP-SORTILIN
Syntaxin 6
Merged

Differentiated adipocytes

C

Figure 2
Figure 3

A

IRAP
sortilin
mycγ-Glut4
Glut4
EGFP-SORTILINtail
cellugyrin

P 1 3 5 7 9 11 13 15 17 19 21 23

B

α-cellugyrin

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Figure 4

Donor membranes | Fb | Ad
---|---|---
Cytosol Ad | - | - | + | +
Cytosol Fb | - | + | + | -
Vesicle fraction
Donor fraction

Vesicle formation, a.u.
Figure 5
Figure 6

A

*G cells transfected with:*  
EGFP  
mSorN  

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*hours after CHX*

B

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Figure 6
Figure 7
Figure 8
Figure 9
Figure 10
Figure 11

A. AD-IRAP LD

B. AD-Glut4 loop 1

C. DNA-BD-Vps10p

D. AD alone + DNA-BD-Vps10p

E. AD-IRAP LD + DNA-BD-Vps10p

F. AD-Glut4 loop 1 + DNA-BD-Vps10p

G. Transactivation with Vps10p, a.u.
The luminal Vps10p domain of sortilin plays the predominant role in targeting to insulin-responsive Glut4-containing vesicles
Jun Shi and Konstantin V. Kandror

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