The v-SNARE Vti1a Regulates Insulin-stimulated Glucose Transport and Acrp30 Secretion in 3T3-L1 Adipocytes*

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Regulated exocytosis in adipocytes mediates key functions, exemplified by insulin-stimulated secretion of peptides such as adiponectin and recycling of intracellular membranes containing GLUT4 glucose transporters to the cell surface. Using a proteomics approach, the v-SNARE Vti1a (vps10p tail interacting 1a) was identified by mass spectrometry in purified GLUT4-containing membranes. Insulin treatment of 3T3-L1 adipocytes decreased the amounts of both Vti1a and GLUT4 in these membranes, confirming that Vti1a is a component of insulin-sensitive GLUT4-containing vesicles. In the basal state, endogenous Vti1a colocalizes exclusively with perinuclear GLUT4. Although Vti1a has previously been reported to be a v-SNARE localized in the trans-Golgi network, treatment with brefeldin A failed to significantly modify Vti1a or GLUT4 localization while completely dispersing Golgi and trans-Golgi network marker proteins. Furthermore, depletion of Vti1a protein in cultured adipocytes through small interfering RNA-based gene silencing significantly inhibited both adiponectin secretion and insulin-stimulated deoxyglucose uptake. Taken together, these results suggest that the v-SNARE Vti1a may regulate a step common to both GLUT4 and Acrp30 trafficking in 3T3-L1 adipocytes.

Blood glucose homeostasis is maintained by insulin in part through glucose disposal into muscle and fat tissues. This is achieved by means of a redistribution of the facilitative glucose transporter GLUT4 from an internal sequestration compartment to the cell surface where it facilitates glucose uptake into these cells. In the absence of insulin GLUT4 recycles very slowly from internal membranes to the plasma membrane, whereas insulin markedly increases the exocytic rate of these GLUT4-containing vesicles. This results in a net increase of GLUT4 at the plasma membrane. This regulated trafficking pathway for GLUT4 appears to be distinct from that of many other membrane proteins such as the transferrin receptor or the GLUT1 glucose transporter protein. Insulin stimulation of adipocytes increases the abundance of these latter proteins at the cell surface by ~2-fold, whereas the amount of GLUT4 is increased between 10–20-fold (1, 2). Other insulin-regulated exocytic pathways likely operate in adipocytes as well, such as a secretory mechanism for peptides like Acrp30/adiponectin. Like transferrin receptor recycling to the cell surface, Acrp30 secretion is only modestly stimulated by insulin (3). This large difference in insulin sensitivity indicates that the molecular mechanisms of GLUT4 sequestration and trafficking have unique regulatory elements. To understand insulin action on glucose transport it will be necessary to elucidate the nature of GLUT4 sequestration in intracellular membranes, the transit of GLUT4-containing intracellular membranes toward the cell surface, and the fusion of these membranes with the plasma membrane.

Although the trafficking of the transferrin receptor and GLUT4 differ in their response to insulin stimulation, it appears that ~50% of the intracellular GLUT4 in cultured adipocytes resides in the general endosomal compartment that also contains the transferrin receptor (2, 4, 5). The nature of the membrane compartment or compartments containing the remaining insulin-sensitive GLUT4 is not fully established and has been somewhat controversial. This membrane compartment has been referred to as the “specialized” pool or GLUT4-sensitive vesicle compartment (4). Several recent reports have indicated that this membrane compartment may include the trans-Golgi network (TGN)3 or a specialized compartment derived from it. The evidence supporting this hypothesis is that proteins that are known to traffic between the TGN and endosomes such as the cation-dependent and -independent mannose 6-phosphate receptor (6, 7) and the adaptor-related protein complex-1 (8) are found in GLUT4-containing vesicles. Recently, two well known t-SNAREs syntaxin-6 and syntaxin-16 that reside in the TGN region were found to be enriched in GLUT4-containing vesicles (9, 10). A C-terminal acidic motif of GLUT4 was responsible for targeting the GLUT4-containing vesicle to the TGN region (9, 10). There is also data derived from electron microscopy indicating GLUT4 is heavily concentrated in the TGN (8, 11). On the other hand, treatment of 3T3-L1 adipocytes with brefeldin A (BFA) which blocks adaptor-related protein complex-1 association with the TGN and disrupts TGN/endosomal sorting had no effect on insulin-stimulated GLUT4-containing vesicle trafficking to the cell surface (7). BFA treatment did seem to mildly prolong the half-time of reversal of insulin-stimulated glucose transport, however (8).

More recently, it has been shown that there is very little overlap between insulin stimulated GLUT4-containing vesicle trafficking pathway and the furin pathway (1). This argues against the TGN as a major reservoir for GLUT4-containing vesicles as furin accumulates in the TGN. There is also very little overlap between the GLUT4-containing vesicle compartment and TGN-38, a classical marker for the TGN (9). Recent work (12, 13) has suggested that there are two distinct populations of GLUT4 vesicles, and they are marked by the presence or absence of cellugyrin, the ubiquitously expressed homologue of synaptoctin. It was also suggested that insulin stimulation of GLUT4 trans-
location results primarily from the stimulation of translocation of small preformed vesicles in differentiated 3T3-L1 adipocytes (14).

The aim of the present studies was to gain insight into the nature and formation of the specialized GLUT4-containing vesicles in adipocytes through analyzing the protein constituents of these purified membranes. We report here the identification of a v-SNARE, vps10 tail-interacting protein 1a (Vti1a), that is a highly enriched component is insulin-sensitive GLUT4-containing membranes. Vti1a was originally identified as a novel v-SNARE in yeast and was found to be involved in trafficking between the late Golgi and prevacuolar compartments and in membrane transport to the cis-Golgi compartment (15). Recently, it was identified as a Golgi soluble N-ethylmaleimide-sensitive factor attachment protein receptor and was implicated in protein trafficking in the secretory pathway (16). Vti1a-β was also reported to be enriched in small synaptic vesicles isolated from nerve terminals and could be immunoprecipitated with the cis-Golgi t-SNARE syntaxin 5 (17) and the TGN syntaxin 6 and syntaxin 16 (18, 19). We show siRNA-mediated depletion of Vti1a protein in differentiated 3T3-L1 adipocytes inhibits both insulin-stimulated deoxyglucose uptake and secretion of the peptide Acpr30 without affecting early insulin signaling events.

EXPERIMENTAL PROCEDURES

Materials—Rabbit anti-GLUT4 was prepared as described before (20). Goat anti-GLUT4 and rabbit anti-insulin receptor β-subunit antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti-IRS-1 and mouse anti-phosphotyrosine (clone 4G10) antibodies were from Upstate Biotechnology, Inc. Mouse anti-human transferrin receptor antibodies were from Zymed Laboratories Inc.. Acrp30 antibodies were purchased from Affinity Bioreagents. Mouse monoclonal anti-Vti1a antibodies were from BD Biosciences and rabbit anti-syntaxin 16 antibodies were purchased from Synaptic Systems (Gottingen, Germany). Rabbit anti-Akt and anti-phospho (Ser473)-Akt antibodies were purchased from Cell Signaling technologies. Rabbit anti-Myo1c antibodies were prepared as described before (21).

Isolation and Fractionation of GLUT4-containing Vesicles—Differentiated 3T3-L1 adipocytes were treated with or without insulin for 30 min and were then homogenized in buffer A (50 mM Hepes, pH 7.4, 10 mM NaF, 1 mM Na3P04, 0.1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and leupeptin) and then subjected to differential centrifugation to isolate the low density microsomal subcellular fractions. The GLUT4-enriched fractions were then isolated from low density microsomal fractions utilizing the sedimentation sucrose velocity gradient centrifugation method as described previously (7, 27). Briefly, 1.5–2 mg of low density microsomal fractions in buffer B were loaded on to a 10–35% sucrose velocity gradient and centrifuged for 3.5 h at 24,000 rpm in a SW28 rotor (Beckman). Fractions containing GLUT4-containing membranes (fractions 8–18) were pooled, pelleted by ultracentrifugation at 48,000 rpm for 1.5 h, resuspended in buffer B and then loaded on an equilibrium density sucrose gradient (10–65% w/v) in buffer B and centrifuged at 35,000 rpm for 18 h in a SW 50.1 rotor (Beckman). After centrifugation fractions were collected starting from the top of the gradient and analyzed for total protein content determined by Bradford assay (Bio-Rad).

Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry Analysis—Proteins resolved by SDS-PAGE were visualized by silver staining (Bio-Rad), and the bands were excised from one single dimensional 5–15% gel. The silver-stained bands were then excised and destained, and the peptide was sequenced as described before (20).
identification of the v-SNARE Vti1a in purified GLUT4-containing membranes (Fig. 1A). GLUT4-containing membranes were purified from 3T3-L1 adipocytes (15), and an additional sucrose velocity gradient centrifugation step yielded a more pure insulin-sensitive GLUT4-enriched membrane fraction (fractions 7–16) (Fig. 1B). Insulin stimulation led to a decrease of GLUT4 in these fractions consistent with its translocation to the cell surface. Vti1a was also enriched in these fractions and interestingly, its peak coincided with that of GLUT4 in the sucrose gradient. Additionally, similar to GLUT4, insulin stimulation of intact cultured adipocytes resulted in a decrease of Vti1a in these fractions (Fig. 1B).

Staining of intact differentiated 3T3-L1 adipocytes with insulin resulted in translocation of some of the GLUT4 to the cell periphery whereas the Vti1a-containing membranes remained predominantly in the perinuclear region (data not shown). These results suggest that a significant amount of GLUT4 and Vti1a reside in the same intracellular membrane compartment in differentiated 3T3 L1 adipocytes.

The molecular composition of the intracellular GLUT4-enriched membranes has been under intense investigation over the last several years. Several investigators have postulated that at least part of the insulin-regulated GLUT4 pool resides in a trans-Golgi compartment (8), and Vti1a has also been postulated to be a TGN v-SNARE. One of the characteristic features of Golgi proteins is that they are sensitive to treatment with BFA. To test whether Vti1a resides in the TGN in differentiated 3T3-L1 adipocytes we treated these cells with BFA and analyzed the localization of Vti1a as well as several Golgi/TGN marker proteins. As shown in Fig. 3, staining of the coatamer protein β-COP
and Vti1a revealed a perinuclear Golgi localization of both proteins showing significant but not complete overlap. When these cells were treated with BFA for 1 h, β-COP was completely dispersed throughout the cell as expected. Surprisingly, there was very little change in the localization of Vti1a, which remained primarily in the perinuclear region (Fig. 3A). Similar results were obtained when cells were immunostained with two other Golgi and TGN marker proteins, p115 and γ-adaptin, respectively. As expected, both of these proteins were completely dispersed from their perinuclear compartment when these cells were treated with BFA (Fig. 3B). This effect is highlighted in a three-dimensional projection image of two cells treated with or without BFA and then stained for intracellular γ-adaptin and Vti1a (Fig. 4). This image also highlights the fact that in differentiated 3T3-L1 adipocytes, γ-adaptin, a TGN marker protein does not colocalize with Vti1a (yellow indicates overlap in two-dimensional but no colocalization in three-dimensional, white indicates colocalization in three-dimensional). The results in Figs. 3 and 4 suggest that in differentiated 3T3-L1 adipocytes Vti1a does not reside primarily in the TGN area but is rather present in a BFA-insensitive compartment.

Recent reports have indicated that almost 50% of GLUT4 in adipocytes is present in an intracellular membrane compartment that is distinct from transferrin-containing endosomal membranes (2). Our data suggests that this membrane compartment may also be enriched with the v-SNARE Vti1a. Studies have also indicated that treatment of differentiated 3T3-L1 adipocytes with BFA caused tubulation of the GLUT4-enriched membrane compartment without affecting insulin-stimulated GLUT4 translocation to the cell surface (8). To test whether Vti1a follows GLUT4 into these tubular structures, we treated differentiated 3T3-L1 adipocytes with BFA and stained these cells for endogenous Vti1a and GLUT4 proteins (Fig. 5). We could confirm that treatment with brefeldin A indeed resulted in tubulation of the GLUT4-enriched membrane compartment, as previously reported (8). Surprisingly, very little Vti1a followed GLUT4 into these tubular structures, and, instead, it remained mostly associated with perinuclear membranes that also retained a substantial amount of GLUT4.

To investigate whether Vti1a plays a role in insulin-stimulated glucose transport we attenuated the expression of this protein in differentiated 3T3-L1 adipocytes using an siRNA-based gene silencing approach. Depletion of Vti1a protein expression in these experiments varied from about 50–60%, whereas the expression of syntaxin 16 or Myo1c in these cells remained unaffected (Fig. 6A). Introduction of siRNAs specific for Vti1a in differentiated 3T3-L1 adipocytes significantly inhibited insulin-stimulated 2-deoxyglucose uptake in these cells. This inhibition was apparent at both 1 nM (~33% inhibition) and 100 nM (~45% inhibition) insulin when compared with cells treated with...
Vti1a, GLUT4, and Acrp30

Vti1a is involved in the secretion of Acrp30 in differentiated 3T3-L1 adipocytes we monitored the level of this protein secreted into the media under conditions where expression of Vti1a was attenuated by siRNA treatment (Fig. 7A). As previously reported, insulin treatment of differentiated adipocytes results in an increase in secretion of Acrp30 (3). Down-regulation of Vti1a expression led to a significant inhibition of secreted Acrp30 from these cells under serum-starved as well as insulin-stimulated conditions. However, the level of intracellular Acrp30 remained unaffected after Vti1a depletion.

In an attempt to examine whether the effect of Vti1a knockdown on Acrp30 secretion was specific or a result of Vti1a regulating constitutive secretion in adipocytes, total proteins secreted into the medium from scrambled control or Vti1a depleted cells were resolved by SDS-PAGE and visualized by silver staining. As depicted in Fig. 7B, no major differences were observed in total proteins secreted in Vti1a knockdown cells, when compared with controls. Taken together, these data suggest that Vti1a may function in the trafficking of secretory vesicles carrying Acrp30 to the cell surface but does not affect the biogenesis of these vesicles or Acrp30.

The data presented here reports the identification of the v-SNARE Vti1a in GLUT4-enriched membranes from differentiated 3T3-L1 adipocytes. Immunofluorescence analysis indicates that a significant portion of GLUT4 resides in the same intracellular membrane compartment as Vti1a. We show that down-regulation of Vti1a protein expression inhibits insulin stimulated 2-deoxyglucose uptake indicating that Vti1a plays a key role in the insulin-stimulated trafficking of GLUT4-enriched vesicles. Additionally, Vti1a also plays a key role in the secretory pathway leading to secretion of Acrp30 in differentiated 3T3-L1 adipocytes.

Vti1a was originally discovered in a two-hybrid screen designed to identify novel yeast proteins involved in the trafficking of the carboxypeptidase Y sorting receptor Vps10p (15). It was reported to be involved in two distinct membrane trafficking steps, from the Golgi to the prevacuolar compartment by its interaction with Pep12p and to the cis-Golgi compartment through its interaction with Sed5p. Additionally, in higher eukaryotes Vti1a has been shown to interact with syntaxin 5, syntaxin 6, and syntaxin 16 and has been implicated in trafficking of secreted proteins (16, 18). In the absence of insulin, GLUT4-enriched vesicles are retained in an intracellular compartment, and insulin appears to trigger the release of these vesicles from this compartment. Significant portions of these intracellular GLUT4-enriched vesicles overlap with the localization of Vti1a, which has been shown before and confirmed by us to bind to the TGN t-SNARE syntaxin 16 (data not shown). However, as has been previously suggested this compartment does not resemble the classical Golgi or the trans-Golgi compartment.

FIGURE 6. Knockdown of Vti1a inhibits insulin-stimulated 2-deoxyglucose uptake in differentiated 3T3-L1 adipocytes. A, protein levels of Vti1a, syntaxin 16, and the unrelated protein Myo1c after siRNA-induced Vti1a knockdown. B, fold increase over scrambled siRNA control in 2-deoxyglucose uptake of cells treated with 1 or 100 nM insulin after Vti1a knockdown. C, depicted are the tyrosine phosphorylation levels in the insulin receptor β-subunit (pIR), and IRS-1 (pIRS-1), and (Ser-473)-phosphorylation level of Akt in scrambled control or Vti1a-depleted cells, treated without or with different doses of insulin (0, 0, 1, 10, 100 nM) for 30 min. Insulin receptor, IRS-1, and Akt total protein levels are also shown. D, Total GLUT4 levels in cells transfected with either scrambled or Vti1a-directed siRNA. 35 and 70 μg of total cell extract were used for this experiment.

FIGURE 7. Knockdown of Vti1a inhibits Acrp30 secretion in differentiated 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were transfected with siRNA against Vti1a and the level of secreted and intracellular Acrp30 was measured by Western blot of the medium and the total cell extract, respectively (A). Total proteins secreted in the medium were resolved by SDS-PAGE and silver-stained (B).
Two lines of evidence strongly suggest the hypothesis that Vti1a resides outside these Golgi compartments. First, there is very little overlap between Vti1a and γ-adaptin, a classical trans-Golgi marker protein. Secondly, treatment of differentiated 3T3-L1 adipocytes with BFA dramatically disperses the Golgi membranes, as is evident from the total dispersion of γ-adaptin, p115, and β-COP, two known Golgi proteins distributed throughout the stacks. However, under the same conditions the subcellular distribution of Vti1a remains largely unaffected.

BFA treatment also causes tubulation of the GLUT4-enriched membranes but very little Vti1a follows GLUT4 into these tubular structures. Rather, Vti1a colocalizes with the remaining perinuclear GLUT4-enriched membrane compartment, which is distinct from trans-Golgi membranes. Another novel observation presented here is that Vti1a is likely regulating a common and early step in the trafficking of GLUT4 and Acrp30, previously thought to involve different trafficking pathways (3). Thus, Vti1a is likely regulating a common and early step in the trafficking of GLUT4 and Acrp30-containing vesicles such as the budding of these vesicles from their individual compartments. Experiments designed to rigorously test these hypotheses are currently underway.

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