Identification of SNAP receptors in rat adipose cell membrane fractions and in SNARE complexes co-immunoprecipitated with epitope-tagged *N*-ethylmaleimide-sensitive fusion protein

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The vesicle-associated membrane proteins [VAMPs; vesicle SNAP receptors (v-SNAREs)] present on GLUT4-enriched vesicles prepared from rat adipose cells [Cain, Trimble and Lienhard (1992) J. Biol. Chem. **267**, 11681–11684] have been identified as synaptobrevin 2 (VAMP 2) and cellubrevin (VAMP 3) by using isoform-specific antisera. Additional antisera identify syntaxins 2 and 4 as the predominant target membrane SNAP receptors (t-SNAREs) in the plasma membranes (PM), with syntaxin 3 at one-twentieth the level. Syntaxins 2 and 4 are enriched 5–10-fold in PM compared with low-density microsomes (LDM). Insulin treatment results in an 11-fold increase in immunodetectable GLUT4 in PM and smaller (approx. 2-fold) increases in VAMP 2 and VAMP 3, whereas the subcellular distributions of the syntaxins are not altered by insulin treatment. To determine which of the SNAP receptors (SNAREs) in PM might participate in SNARE complexes with proteins from GLUT4 vesicles, complexes were immunoprecipitated with anti-*myc* antibody from solubilized membranes after the addition of *myc*-epitope-tagged *N*-ethylmaleimide-sensitive fusion protein (NSF) and recombinant α-soluble NSF attachment protein (α-SNAP). These complexes contain VAMPs 2 and 3 and syntaxin 4, but not syntaxins 2 or 3. Complex formation requires ATP and is disrupted by ATP hydrolysis. When all membrane fractions are prepared from basal cells, few or no VAMPs and no syntaxin 4 are immunoprecipitated in SNARE complexes obtained from LDM alone (or from immunoisolated GLUT4 vesicles). The content of syntaxin 4 depends on the presence of PM, and participation of VAMPs 2 and 3 is enhanced 4–6-fold by the addition of solubilized GLUT4 vesicles to PM. The latter increase is greater than can be explained by the 2-fold higher levels of VAMPs added to the reaction mixture. When all membrane fractions are prepared from insulin-stimulated cells, SNARE complexes formed from PM alone contain similar levels of syntaxin 4 but 5–6-fold higher levels of VAMPs 2 and 3 compared with PM alone from basal cells. Addition of GLUT4 vesicle proteins to PM from insulin-treated cells results in a further 2-fold increase in VAMP 2 recovered in SNARE complexes. Therefore the VAMPs in PM of insulin-treated but not basal cells, and in GLUT4-vesicles from cells in either condition, are in a form that readily forms a SNARE complex with PM t-SNAREs and NSF. Insulin seems to activate PM and/or GLUT4 vesicles so as to increase the efficiency of SNARE complex formation.

INTRODUCTION

One of the primary actions of insulin is its stimulation of glucose transport into adipose and muscle cells. In 1980, Suzuki and Kono [1] and Cushman and Wardzala [2] reported that glucose transporters (now known to be GLUT4) in non-stimulated adipose cells are sequestered in a large intracellular pool and can be recruited to the plasma membrane in response to insulin (reviewed in [3]). Recent studies on the subcellular trafficking [4,5] and immunolocalization [6,7] of GLUT4 reveal that, in the insulin-stimulated state, GLUT4 undergoes continuous cycling through multiple intracellular compartments. Kinetic studies have indicated that at least two intracellular compartments are involved in intracellular sequestration of GLUT4. One of these compartments seems to be early endosomes involved in GLUT4 internalization [6] whereas the other seems to act as a GLUT4 reservoir from which rapid exocytosis to the plasma membrane can occur [4,5]. In addition, studies of the time courses of arrival of GLUT4 at the cell surface and its participation in glucose transport have led to the suggestion that plasma membranes (PM) might contain a subpopulation of occluded GLUT4 that might comprise an intermediate in the processing of transport-competent GLUT4 [4,8–10]. If part of the plasma membrane content of GLUT4 is in vesicles that are docked but not fully fused with the plasma membrane, these might comprise the occluded, transport-inactive GLUT4.

The recruitment of an intrinsic membrane protein such as GLUT4 to the plasma membrane from an intracellular pool of vesicles is conceptually similar to neurosecretion and other secretory processes. To explore the possibility that similar mechanisms are involved, we have looked in rat adipose cells for the presence of several protein families that are known to be

Abbreviations used: DTT, dithiothreitol; HDM, high-density microsomes; LDM, low-density microsomes; PM, plasma membranes; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; v-SNARE, vesicle SNARE; t-SNARE, target membrane SNARE; VAMP, vesicle-associated membrane protein.

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involved in the docking and/or fusion of membrane vesicles in neurons and other cells. In particular we have examined the possibility that docking and fusion might involve the interaction of SNAP receptor (SNARE) proteins (v-SNAREs on the GLUT4 vesicles and t-SNAREs in the plasma membrane) and that these proteins can form a complex with the *N*-ethylmaleimide-sensitive fusion protein (NSF) [11,12]. Although previous studies have established the presence of v-SNAREs on GLUT4 vesicles [13,14], an NSF-dependent complex in the association of GLUT4 vesicles with PM would not necessarily be expected, because in some cells exposure of membrane proteins to the cell surface can occur without the involvement of NSF and α-soluble NSF attachment protein (α-SNAP) [15].

Kinetic studies on the trafficking of photolabelled GLUT4 have suggested that insulin's main effect is to increase the rate constant for externalization, or exocytosis, of the GLUT4 vesicles [4,16]. However, because exocytosis must occur through a series of intermediate steps, insulin could act at several possible sites within this limb of the recycling pathway: perhaps at the level of GLUT4 vesicle budding from a tubulo-vesicular compartment or, in a manner analogous to the regulated release of neurotransmitter vesicles, by regulating GLUT4-vesicle docking and fusion steps. These two possible mechanisms are not mutually exclusive; to simulate the effects of insulin on apparent precursor states in the plasma membrane, we have suggested that insulin might produce both a large increase in vesicle translocation to the plasma membrane and a small increase in the rate of vesicle fusion [5]. Consequently we examine here whether insulin treatment of rat adipose cells results in modulation of the interaction between v- and t-SNAREs.

EXPERIMENTAL

Materials

^{125}I-labelled Protein A and ^{125}I-labelled sheep anti-mouse immunoglobulin were from New England Nuclear. Protein G–Sepharose was from Sigma or Pharmacia. Recombinant bovine α-SNAP and NSF-*myc* were prepared as described [11]. Anti-*myc* mouse ascites fluid [17] and the recombinant protein expression vectors were gifts from Dr. T. Söllner. Monoclonal antibody to syntaxin 1A/1B (HPC-1) was from Sigma; polyclonal antisera were directed against unique (N-terminal) regions of syntaxins 2–4 [18], synaptobrevins 1 and 2 [or vesicle-associated membrane proteins (VAMPs) 1 and 2; gifts of Dr. W. Trimble] and cellubrevin (or VAMP 3; gift of Dr. P. DeCamilli) [19]. Monoclonal antibody against the α-1 subunit of the Na^+/K^+ ATPase was a gift from Dr. K. Sweadner.

Preparation of adipose cells and subcellular membrane fractions

Adipose cells were isolated by collagenase digestion from the epididymal fat pads of 180–250 g male Sprague–Dawley rats as previously described [2,20]. Homogenization and subcellular fractionation were performed as described [20], except that Na/Hepes was substituted for Tris/HCl in the homogenization buffer, EDTA was increased to 4 mM, and protease inhibitors [0.12 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin] were added.

GLUT4-containing vesicles were immunoprecipitated from the supernatant after the removal of high-density microsomes (HDM) [containing low-density microsomes (LDM) and cytosol] with affinity-purified anti-(GLUT4 C-terminal) antibody prebound to *Staphylococcus aureus* protein A-cell walls as described [13]. Elution with buffer containing 1 % (w/v) Triton X-100 at room temperature provided the 'GLUT4-vesicle-derived proteins' used in the formation of SNARE complexes with NSF (see below). Vesicles remaining in the immune supernatant were collected by centrifugation at 200000 ***g*** for 20 min at 4 °C.

Western blotting

Samples of membrane fractions or GLUT4-enriched vesicles were solubilized in SDS/PAGE sample buffer containing 1 % (w/v) SDS, 2.7 M urea and 60 mM dithiothreitol (DTT), run on 12 % polyacrylamide minigels (10–15 μg of protein per lane), transferred to nitrocellulose as described [13] and subjected to Western blotting with ^{125}I-labelled Protein A or sheep anti-mouse immunoglobulin. Blocking was done in 5 % (w/v) dried milk, whereas antisera were diluted in solutions containing 3 % (w/v) BSA. No detergent was used in these or any other steps after electrophoresis. Antisera for different proteins were used on horizontal sections of each blot, so that multiple different proteins were quantified on each lane of gel. Results are presented either as phosphorimager (Molecular Dynamics) units/mg of protein, normalized to that for PM from basal cells within each experiment (see Figures 3b, 3c and 3d), or as percentages of the recovered total found by summing the total amount found in all fractions (as in Table 1). Protein was measured by bicinchoninic acid assay (Pharmacia) with BSA as standard.

Detection and quantification of SNARE complexes

Immunoprecipitation of SNARE complexes from adipose cell membranes was performed by the method of Söllner et al. [11], except that the membranes were not washed at high salt concentration before use. Briefly, PM obtained as described above from a fixed number (1.0–1.6 ml) of packed adipose cells were suspended at 4 °C in a buffer comprising 100 mM KCl, 20 mM TRIS/HCl, 8 mM EDTA and 2 mM DTT at pH 7.2 with the same protease inhibitors as described above for the homogenization buffer. Membranes were solubilized by the addition of Triton X-100 to a final concentration of 2 % (w/v) , incubated 20 min on ice with vortex mixing, and clarified by centrifugation at 48000 ***g*** for 10 min. The clarified solution was mixed with recombinant NSF-*myc* (1–2 μg) and recombinant α-SNAP (10–20 μg), with or without LDM or GLUT4-vesicle-derived proteins (also obtained from 1.0–1.6 ml of packed cells), in the presence of 5 mM ATP, 4 mM DTT and additional KCl/Tris/EDTA buffer in a total volume of 0.7–0.8 ml. The resulting protein complexes were immunoprecipitated at 4 °C for 90 min with 200 μg of monoclonal anti-*myc* antibody prebound to 100 μl of packed Protein G–Sepharose. Each immune complex was washed four times with an additional 1 ml of ice-cold KCl/Tris/EDTA buffer containing 1 mM ATP and 0.5 % Triton X-100 and then transferred to a small nitrocellulose filter unit (Ultrafree MC, 0.4 μm pore; Millipore). Specifically bound proteins were eluted at room temperature in small volumes (2×200 μl) of buffer in which 10 mM $MgCl_2$ was substituted for EDTA. Preliminary experiments showed that immunoprecipitation of NSF-*myc* was 70–80 % complete under these conditions and was unaffected by the source of membrane proteins. In addition, elution was essentially complete with membrane preparations from both basal and insulin-treated adipose cells, because no additional SNAREs were detected by Western blotting in subsequent elutions with additional Mg^{2+} buffer or with SDS/urea buffer. Eluted proteins were concentrated by precipitation with chloroform/methanol [21] before SDS/PAGE. The resulting Western blotting data were evaluated by phosphorimage analysis. The proteins remaining in the immune supernatant were also concentrated by chloroform/methanol precipitation and a small aliquot (4–10 %) was analysed in the same manner. The percentage of

the recovered total (immune supernate plus immune precipitate) that was in the immune precipitate was determined.

Statistical analysis

Paired *t* tests of data within each experiment were performed and two-tailed significance levels are indicated. Data are means ± S.E.M. Because of substantial variation between Western blots in the absolute radioactivity in the bands, all comparisons are made within a single Western blot. To control for possible variation between preparations of isolated adipose cells, all comparisons between basal and insulin-treated cells are within the same cell preparations, in which all parts of the experiment (preparation of cells, subcellular fractionation, isolation of SNARE complexes, electrophoresis and Western blotting) were performed together.

RESULTS

Identification of SNAP receptors in adipose cell membrane fractions

Isoform-specific antisera were used in Western blots to determine which plasma membrane and vesicle-associated SNAP receptors (syntaxins and VAMPs respectively) are expressed in white adipose cells. Figure 1 shows a blot in which replicate lanes were probed with antisera specific for syntaxins 1–4 (upper part) and VAMPs 1 and 2 (synaptobrevins 1 and 2) and VAMP 3 (cellubrevin) (lower part). The isoform-specific antisera used here exhibited no cross-reactivity. In total brain cortical membranes, shown as a positive control, bands were observed for all four syntaxins examined as well as VAMPs 1 and 2, but very little for VAMP 3 as previously reported [9]. In adipose cell PM, bands at 35 kDa corresponding to syntaxins 2, 3 and 4, but no detectable syntaxin 1A/1B, were observed. An additional, unidentified band at 30 kDa was evident in the adipose cell lane in the syntaxin 3 Western blot. Adipose cell PM also contained VAMPs 2 and 3, but not VAMP 1. LDM gave the same results (not shown). The soluble proteins NSF and α-SNAP were also present in Western blots of adipose cell PM and LDM, whereas synaptophysin and SNAP-25 were undetectable (results not shown).

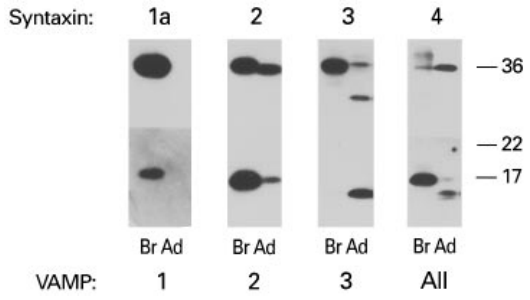

Figure 1 Identification and comparison of SNAP receptors in rat adipose cell and brain membranes by Western blotting

Unfractionated membranes from rat brain cortex (Br) or plasma membrane fractions from basal adipose cells (Ad) were subjected to SDS/PAGE [12% (w/v) acrylamide], transferred to nitrocellulose and probed with isoform-specific antisera for syntaxins 1A/1B, 2, 3 and 4 (upper bands) as well as synaptobrevins 1 and 2 (VAMPs 1 and 2) and cellubrevin (VAMP 3; lower bands) and an antiserum that recognizes a highly conserved region of the three known VAMPs (residues 51–69 of VAMP 1; All). Each lane was loaded with 10 μg of protein. The positions of prestained molecular mass markers (in kDa) are shown at the right.

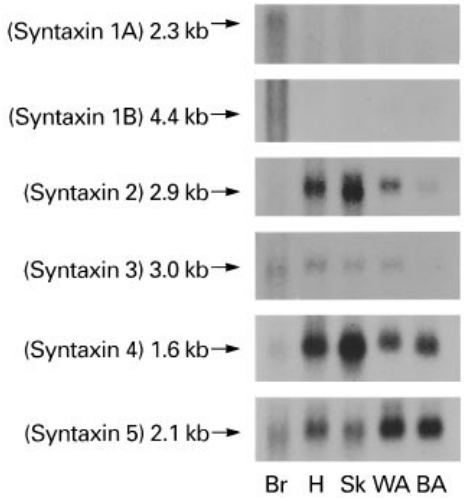

Figure 2 Northern blot analysis of syntaxin expression in rat white and brown adipose cells

Polyadenylated RNA from brain (Br, 2.5 μg), heart (H, 2.5 μg), skeletal muscle (Sk, 2.5 μg), white adipose cells (WA, 0.86 μg) and brown adipose cells (BA, 1.7 μg) were subjected to electrophoresis in 1% (w/v) agarose gels, transferred to nitrocellulose, probed with full-length cDNA probes and washed under high stringency.

Comparison of the syntaxin Western blot signals obtained by using adipose cell PM with those obtained by using known amounts of purified, bacterially expressed, recombinant syntaxins indicated 30–65 ng/mg membrane protein of syntaxins 2 and 4 (each, in three independent preparations). This is one-fiftieth to one-hundredth of the content of syntaxin 1A/1B in unfractionated brain cortical membranes. Such comparisons also indicate that adipose cell PM contain one-twentieth to one-thirtieth the amount of syntaxin 3 than of syntaxin 2 or 4. The syntaxin protein expression results were corroborated by Northern blot analysis of polyadenylated RNA shown in Figure 2, which indicates the expression of syntaxins 2, 3 and 4, but not 1A or 1B, in white adipose cells, as well as in brown adipose cells and skeletal muscle. Syntaxin 5 mRNA was also detected in all tissues examined.

Effects of insulin on subcellular distributions of SNAP receptors

The extents to which the VAMPs and syntaxins are redistributed between the intracellular membranes and the plasma membrane in response to insulin were determined. As shown in Figure 3, insulin stimulated an increase in GLUT4 in PM by an average of 11-fold at steady state in comparison with untreated cells. In contrast, VAMPs 2 and 3 showed only an approx. 2-fold redistribution from intracellular membranes to PM. These insulin-induced increases in the PM contents, expressed per mg of protein, were also evident when expressed as percentages of total recovery in PM, as shown in Table 1.

The levels of syntaxins 2 and 4 per mg of membrane protein were 3-fold and 10-fold higher in PM than in the HDM and LDM respectively (Figures 3b, 3c and 3d). Table 1 shows that, as percentages of total recovered, syntaxins 2 and 4 were largely (75–80%) confined to PM. The subcellular distributions of syntaxins 2 and 4, as well as of endogenous NSF and α-SNAP (results not shown), were not significantly affected by treatment with insulin. The subcellular distribution of the α-1 subunit of the Na^+/K^+ ATPase was somewhat less restricted than those of

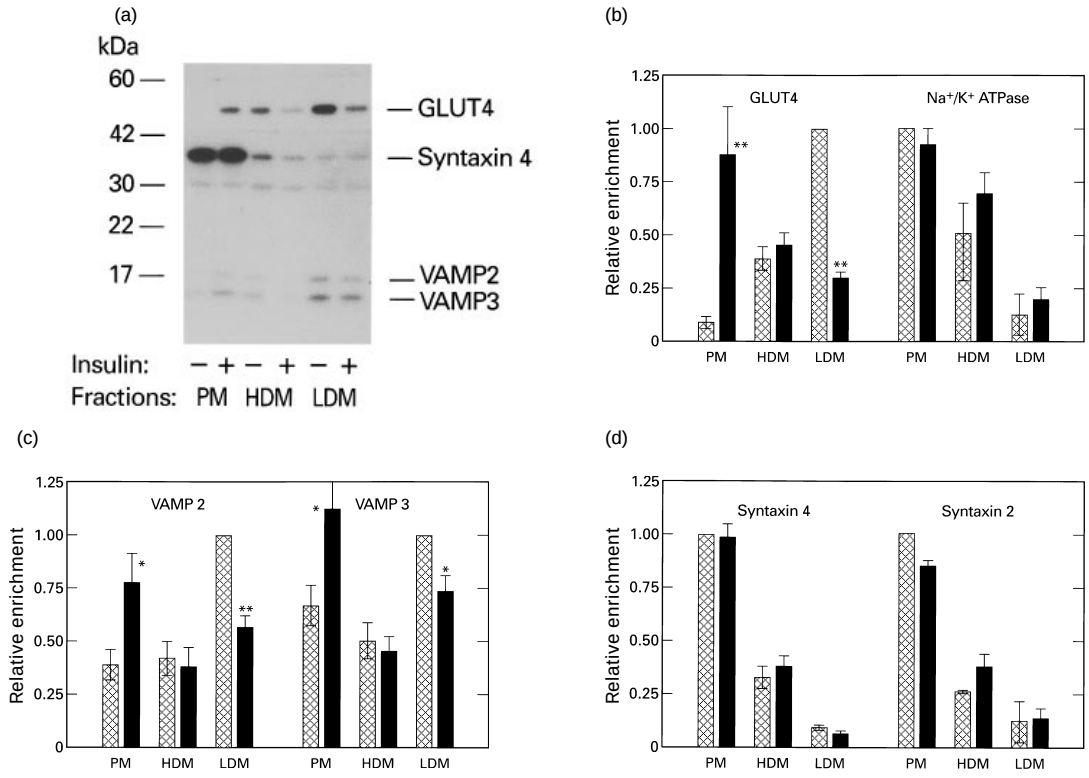

Figure 3 Effects of insulin treatment on the subcellular distributions of rat adipose cell SNAP receptors

(**a**) Membrane fractions were isolated from basal and insulin-treated cells. A single polyacrylamide gel was loaded with the subcellular membrane fractions (12 μg of protein per lane). After electrophoresis and transfer to nitrocellulose, the nitrocellulose was cut horizontally and probed with antisera against GLUT4, syntaxin 4 and VAMPs 2 and 3. The positions of prestained molecular mass markers are shown at the left. (**b**, **c**, **d**) Western blot data are expressed as phosphoimager units per mg of protein in PM, HDM and LDM obtained from basal cells (cross-hatched bars) and cells treated for 30 min with 10 nM insulin (filled bars). Values are the means $\pm$ S.E.M. for nine independent experiments, except for syntaxin 2 and Na/K ATPase: basal, three or four experiments; insulin-treated, five to seven experiments. Statistical significance was determined for pairwise comparisons within each experiment: $^{*}P < 0.05$ and $^{**}P < 0.01$, significant difference between basal and insulin-treated.

Table 1 Effects of insulin on the plasma membrane contents of SNAP receptors in rat adipose cells

Results are calculated from the experimental results shown as units/mg of protein in Figure 3 and are means $\pm$ S.E.M. Each immunoreactive protein recovered in the plasma membrane fraction is expressed as a percentage of the total recovered immunoreactivity (Western blot signal summed across all fractions; see the Materials and methods section). The totals did not differ significantly with insulin treatment. *Significant difference ($P < 0.05$) from the (basal) untreated condition.

	Percentage of total recovered	
Protein	Basal	Insulin
GLUT4	4.8 $\pm$ 0.8	50 $\pm$ 4*
VAMP 2	22 $\pm$ 3	43 $\pm$ 6*
VAMP 3	28 $\pm$ 4	42 $\pm$ 8*
Syntaxin 4	76 $\pm$ 3	80 $\pm$ 4
Syntaxin 2	81 $\pm$ 4	80 $\pm$ 2
Na/K ATPase	74 $\pm$ 8	64 $\pm$ 1

the syntaxins, with only 60–70 % of the total being recovered in PM (Table 1); insulin had no significant effect on this distribution. Few or no SNAREs (VAMPs 2 and 3, syntaxins 2 and 4) were detectable in the nuclear and mitochondrial fractions (results not shown).

To determine whether syntaxins and VAMPs might be preassembled in complexes on intracellular GLUT4-containing vesicles, the vesicles were immunoisolated as shown in Figure 4. The efficiency of immunoprecipitation in these experiments ($n = 3$) was 88–98 % for GLUT4; the immune precipitates also contained 70–78 % of the total VAMPs 2 and 3 in LDM. Thus most but not all of the VAMPs in the microsomal fraction were immunoprecipitable with the GLUT4. Both syntaxins 2 and 4 were largely excluded from GLUT4 vesicles (Figure 4, and results not shown). Similar results were obtained after immunoisolation of GLUT4 vesicles from LDM of insulin-treated cells.

Detection of SNAP receptors in complexes with NSF

To determine which SNAREs might participate in NSF complexes in adipose cells, experiments similar to those reported by Söllner et al. [11] were performed. Initially, PM and LDM from basal adipose cells were used. Membrane fractions were solubilized in Triton X-100 and mixed with recombinant α-SNAP and *myc*-epitope-tagged NSF in the presence of ATP and EDTA. The resulting SNARE complexes were immunoprecipitated with monoclonal anti-*myc* antibody bound to Protein G–Sepharose.

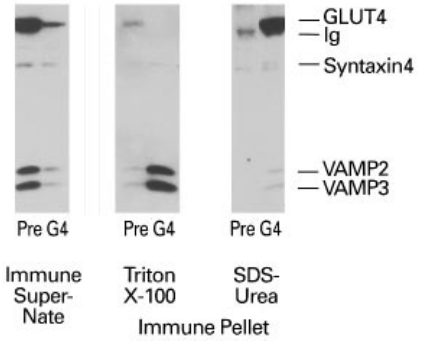

Figure 4 GLUT4-enriched vesicles contain VAMPs 2 and 3 but not syntaxin 4

Vesicles containing GLUT4 were immunoprecipitated from low-density microsomes as described in the Materials and methods section. Equivalent fractions of Triton X-100 and SDS/urea eluates of the immune precipitate and of non-precipitated vesicles (immune supernate) were loaded in each lane. A single gel transfer was cut horizontally and probed with antisera to GLUT4, syntaxin 4 and VAMPs 2 and 3 (mixed antisera). In three representative experiments, the fractional recoveries of protein in the immune pellet were 63, 88 and 98% for GLUT4; 53, 76 and 78% for VAMP 2; and 43, 70 and 73% for VAMP 3. The percentage ratios of fractional recoveries (VAMP/GLUT4) were 83±3% for VAMP 2 and 74±5% for VAMP 3. Ig, immunoglobulin.

Treatment with buffer containing $MgATP^{2-}$ at room temperature allowed hydrolysis of the bound ATP and elution of the SNAP receptors. As seen in Figure 5(a), Western blotting of the eluted proteins indicated the presence of syntaxin 4 and VAMP 3; α-SNAP and VAMP 2 were also co-immunoprecipitated, whereas syntaxins 2 and 3 were not (see below). As shown in the first lane, syntaxin 4 was co-immunoprecipitated with NSF in the presence of α-SNAP from PM alone; however, little VAMP 3 was found. A similar amount of syntaxin 4 but considerably more VAMP 3 was immunoprecipitated from a combination of LDM and PM (second lane) than from PM alone. No syntaxin 4 and only a small amount of VAMP 3 were present in complexes formed with LDM alone (third lane). Quantitative results indicate that the recovery of VAMPs in SNARE complexes from LDM alone was consistently lower (0–5%) than that from the combined membranes (6–15%; see below).

Figure 5(b) indicates that GLUT4-vesicle proteins can substitute for those present in LDM. Participation of both VAMPs 2 and 3 in complex formation was greatly enhanced by combining GLUT4-vesicle-derived membrane proteins with solubilized PM compared with PM alone (compare the second lane with the first lane). However, further inclusion of non-GLUT4-containing LDM, the LDM vesicles remaining after GLUT4 immunoprecipitation, did not further enhance the co-immunoprecipitation of VAMPs 2 and 3 (third lane). Omission of α-SNAP from the mixture resulted in failure to co-immunoprecipitate any syntaxin 4 or VAMPs 2 and 3 (fourth lane). Addition of the HDM fraction, which is relatively poor in both GLUT4 and VAMP content, had no effect (results not shown). As shown in Figure 5(c), co-immunoprecipitation of syntaxin 2 in SNARE complexes formed with either PM alone (results not shown) or PM and GLUT4 vesicles was one-tenth (less than 1% compared with 8–10%) of that of syntaxin 4 when the amount immunoprecipitated is expressed as a percentage of the total recovered (immune pellet plus immune supernatant). The syntaxin 3 signals were too weak to be evaluated quantitatively, but syntaxin 3 did not seem to be preferentially concentrated by immunoprecipitation of SNARE complexes (results not shown).

Effects of insulin treatment on SNARE complex formation

To determine whether insulin treatment of adipose cells produces any change in the ability of the known SNAREs to form SNARE complexes, two preliminary experiments were performed to determine whether the binding capacity of the assay system, consisting of fixed amounts of recombinant NSF-*myc* and α-SNAP and of anti-*myc* antibody, was saturable. Saturation of VAMP 2 and VAMP 3 binding to the complexes was observed (results not shown), and care was taken in subsequent experiments to keep binding below the saturation level.

The effects of insulin treatment of adipose cells on the incorporation of SNAP receptors into SNARE complexes are illustrated in Figure 6 (upper panel). SNARE complexes formed with membranes from insulin-treated cells contained similar amounts of syntaxin 4, but larger amounts of VAMPs 2 and 3 than with membranes from basal cells. Quantification of several experiments is shown in Figure 6 (lower panel). For comparison purposes, the amount of each SNAP receptor eluted from the SNARE complexes formed with solubilized PM alone from a fixed number of basal cells (cross-hatched bar for each SNARE

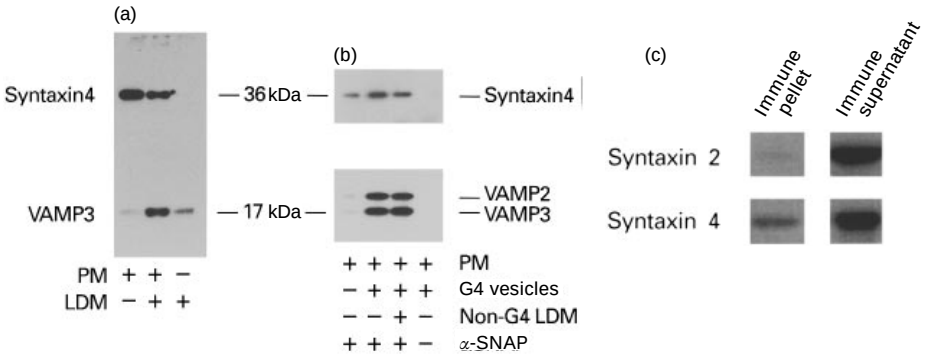

Figure 5 Incorporation of SNAP receptors from basal rat adipose cell membrane and vesicle fractions into SNARE complexes with NSF

SNARE complexes were immunoprecipitated from PM, LDM or both fractions combined (**a**); PM with or without GLUT4-enriched vesicles (**b**); PM plus GLUT4 vesicles (**c**). After elution of Mg^{2+} followed by SDS/PAGE and transfer to nitrocellulose, each blot was cut horizontally and appropriate sections were probed with antisera against syntaxin 4 and VAMP 3 (in **a**) or a mixture of antisera against VAMPs 2 and 3 (in **b**). In (**c**), duplicate Western blots of SNAP receptors eluted from a single immunoprecipitate and those remaining in the immune supernatant (non-G4 LDM) were probed with antisera to syntaxins 2 and 4.

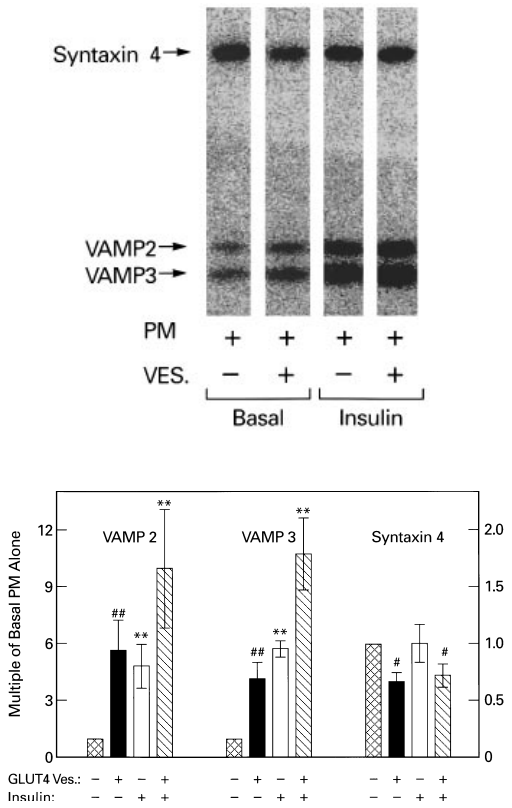

Figure 6 Treatment with insulin stimulates the incorporation of VAMPs 2 and 3 into SNARE complexes

Cells were treated with or without 10 nM insulin for 30 min, membrane fractions were prepared and the membranes were used in SNARE complex experiments under the conditions described in the legend to Figure 5. Upper panel, a representative Western blot of SNAREs eluted from complexes with NSF. PM, plasma membrane fraction; VES, GLUT4-vesicle proteins; VAMP 2, synaptobrevin 2; VAMP 3, cellubrevin. Lower panel, the combined results from six basal experiments and nine experiments with insulin-treated cells. These compare the incorporation of SNAP receptors from the plasma membrane fraction alone (cross-hatched and open bars) with PM combined with GLUT4-enriched vesicles (filled and hatched bars). In each experiment, PM from a fixed volume of packed cells were used for each sample, with or without GLUT4 vesicles from the same volume of the same batch of cells. Results are expressed as multiples (means ± S.E.M.) of results from PM of basal cells. Note the different scale (at the right) for syntaxin 4. Statistical significance was determined for pairwise comparisons within each experiment: $^{*}P < 0.05$ and $^{**}P < 0.01$, significant difference between basal and insulin-treated; # $P < 0.05$ and ## $P < 0.01$, significant difference between PM alone and combined membrane fractions.

protein) was set to unity. When GLUT4-vesicles were combined with PM from basal cells, 4–6-fold more VAMPs 2 and 3 were co-immunoprecipitated than with PM alone (filled bars). When PM alone from insulin-treated cells were used, VAMPs 2 and 3 incorporated into SNARE complexes were also 5–6-fold above the levels observed with PM alone from basal cells (open bars). Inclusion of GLUT4-vesicles together with PM from the same, insulin-treated cells resulted in approx. 2-fold additional increases in co-immunoprecipitated VAMPs 2 and 3 compared with PM alone from insulin-treated cells (hatched bars). After insulin treatment, the VAMPs recovered in SNARE complexes from the combined membranes increased 1.8–2.3-fold over combined membranes from basal cells. The amounts of syntaxin 4 recovered in SNARE complexes were not affected by insulin treatment of the cells with either PM alone or a combination of PM and GLUT4 vesicles, but were decreased by approx. 30% when GLUT4-vesicles were included compared with PM alone (Figure 6, lower panel).

Table 2 Effects of insulin on the contributions of SNAREs from plasma membrane and GLUT4 vesicles to SNARE complexes with NSF–*myc*

Results are presented as means ± S.E.M. for the percentage of the recovered totals (immune pellet plus immune supernatant) that were co-immunoprecipitated with NSF. For GLUT4 vesicles, the contribution of plasma membrane fraction was subtracted from data for both immune pellet and immune supernatant of samples containing the combined plasma membrane and GLUT4 vesicles. Significance testing in paired *t* tests: *, $P < 0.05$ compared with plasma membrane alone; † $P < 0.05$ for insulin effect (compared with basal in same column).

		Protein in complexes as percentage of total recovered		
SNARE	Incubation condition	Plasma membranes alone	GLUT4 vesicles + plasma membranes	GLUT4 vesicles (calculated)†
VAMP 2	Basal	3.6 ± 1.4	6.4 ± 1.3*	12.6 ± 3.3*
VAMP 2	Insulin	10.2 ± 2.5†	12.6 ± 1.8†	13.3 ± 3.4
VAMP 3	Basal	2.3 ± 0.8	6.6 ± 1.9*	10.5 ± 3.4*
VAMP 3	Insulin	6.3 ± 1.5†	8.7 ± 1.5†	8.8 ± 1.6
Syntaxin 4	Basal	9.0 ± 1.4	6.7 ± 0.8*	–
Syntaxin 4	Insulin	11.9 ± 1.2	8.8 ± 0.7*	–

Quantification of SNAP receptor participation in SNARE complexes

As shown above in Figures 3 and 4, the levels of VAMPs 2 and 3 and syntaxin 4 vary between PM and GLUT4 vesicles and between basal and insulin-treated cells. Thus the amounts of these SNAP receptors initially added to the reaction mixture from which SNARE complexes were immunoprecipitated also varied. Table 2 shows the amounts of co-immunoprecipitated VAMPs 2 and 3 and syntaxin 4 expressed as percentages of the total available SNAREs assessed by summing the amounts recovered in the immune pellet and immune supernatant. When membranes were prepared from basal cells, the percentage of available VAMP2 that participated in SNARE complex formation *in vitro* increased approx. 2-fold when GLUT4-vesicles were combined with PM compared with PM alone, whereas the percentage of VAMP 3 increased approx. 3-fold, and the percentage of syntaxin 4 decreased by approx. 25%. When membranes were prepared from insulin-treated cells, the percentages of available VAMPs 2 and 3 that participated in SNARE complex formation both increased approx. 3-fold when PM alone from insulin-treated cells were used compared with basal cells; syntaxin 4 tended to increase but the difference is not significant. In contrast with membranes from basal cells, combining GLUT4-vesicles from insulin-treated cells with PM from the same cells did not significantly increase the percentages of available VAMPs 2 and 3 participitating in SNARE complex formation (Table 2) despite increases in the amounts immunoprecipitated (Figure 6, lower panel). However, the percentage of syntaxin 4 (Table 2) decreased, as it did with membranes from basal cells. When the combination of GLUT4 vesicles and PM is compared between basal and insulin-treated cells, significant increases for both VAMPs 2 and 3 are seen in response to insulin, while syntaxin 4 is unchanged.

The apparent contribution of the GLUT4 vesicle VAMPs to SNARE complex formation with PM was estimated in the following manner. Results obtained with plasma membrane alone were subtracted from results obtained with combined PM and GLUT4-vesicles, for both the contents of SNARE complexes

and the VAMPs remaining in the supernatant after immunoprecipitation of NSF–*myc*. The calculated differences, attributable to GLUT4-vesicles, were then used to calculate the percentage incorporation of GLUT4-vesicle-derived VAMPs into SNARE complexes. With membranes from basal cells, the percentage of VAMPs 2 and 3 from GLUT4 vesicles participating in SNARE complexes was 4-fold higher than those from PM (Table 2). After insulin treatment, the percentages of VAMPs 2 and 3 contributed by GLUT4 vesicles to SNARE complexes were similar to the percentages contributed by the PM alone from the same cells.

DISCUSSION

Using a well-characterized fractionation procedure for isolation of both enriched PM, and HDM and LDM from rat adipose cells [20], we have obtained evidence that the t-SNAREs syntaxins 2 and 4 are almost exclusively localized to the plasma membrane and are excluded from GLUT4-containing intracellular vesicles. The v-SNAREs VAMPs 2 and 3 are mainly localized to intracellular membranes but are also present in substantial amounts in the PM. Apart from the high background of plasma membrane-located VAMPs, these results are consistent with the SNARE hypothesis described by Rothman and colleagues [11,12]. This hypothesis involves specific v-SNAREs docking with specific t-SNAREs, followed by binding of NSF and α-SNAP to form a 20 S complex, ATP hydrolysis by NSF, and additional events to accomplish membrane fusion [11,12]. We note that SNAP-25, a requisite component of SNARE complexes in synaptic material, is not found in adipose cell membranes, whereas a non-neuronal form of synaptotagmin has been recently reported [22]; it is likely that other SNARE proteins remain to be identified.

The increases in content of VAMPs 2 and 3 in the PM and corresponding decreases in the LDM and GLUT4 vesicles in response to insulin (Figure 3) are consistent with incorporation of VAMPs into PM as a result of GLUT4 vesicle fusion and suggest an important (though as yet ill-defined) role for these proteins in GLUT4 translocation. The small fold change of the insulin-induced increase in PM content of VAMPs might be due to involvement of the same proteins (VAMPs 2 and 3) in movements to the plasma membrane of vesicles that are constitutively recycled or that display a smaller degree of insulin stimulation [23], thus producing a high background of VAMPs 2 and 3 in the basal PM.

Distribution and redistribution results are not conclusive evidence for the role of particular SNAREs in movements of vesicles to the plasma membrane. Stronger evidence would lie in results showing direct interactions between proteins originating in vesicles with those originating in PM. The studies presented here provide that evidence, because the co-immunoprecipitations of VAMPs 2 and 3 in complexes with NSF and α-SNAP occur to a very limited extent in the presence of either basal PM or GLUT4 vesicles alone, but are greatly enhanced when the two membrane fractions are combined. In contrast, binding of PM-derived syntaxin 4 to NSF/α-SNAP can occur independently of binding of VAMPs 2 and 3 derived from GLUT4 vesicles. This behaviour of SNAREs in basal cells seems to distinguish the proteins present in the adipose cell from those in the synaptic terminal, where in the absence of a stimulus a portion of syntaxin 1, VAMP 2 and NSF are preassociated in complexes in synaptic vesicle membranes [24,25]. The ability of syntaxin 4 to participate in the SNARE complex in the near-absence of VAMPs is consistent with the ability of bacterially expressed syntaxin 1A to form a complex with α-SNAP and NSF without the participation of any v-SNARE [26].

In contrast with the enhanced co-immunoprecipitation of the v-SNAREs, VAMP 2 and VAMP 3, when PM and microsomes are combined, co-immunoprecipitation of syntaxin 4 is decreased by one-third when GLUT4 vesicle proteins are included in the reaction, compared with plasma membrane proteins alone. This is currently unexplained. It might be due to competition for binding sites on the immunoprecipitated NSF by other, unidentified t-SNAREs. The failure to detect significant co-immunoprecipitation of syntaxins 2 or 3 in the present studies is consistent with previous studies *in vitro* demonstrating that the cytoplasmic domain of VAMP 2 will form complexes with syntaxins 1A and 4 but not syntaxins 2 or 3 [27].

In synaptic vesicle docking/fusion processes, an 'active zone' has been identified at which many vesicles are closely juxtaposed to the plasma membrane and are rapidly available to respond to a secretory stimulus within milliseconds. However, the active zone at which GLUT4 vesicles can be docked might be more limited, because the response time for insulin-induced translocation of GLUT4 is comparatively lengthy ($t_{\frac{1}{2}} = 2$–3 min). The present results indicate that syntaxins 2 and 4 are present in the plasma membrane at one-fortieth to one-eightieth of the steady-state insulin-stimulated level of GLUT4 and approx. one-fiftieth to one-hundredth of the levels of syntaxin 1A and 1B in unfractionated brain cortical membranes. Such comparisons suggest that the rate of fusion of GLUT4-vesicles might be limited by low numbers of docking sites in the plasma membrane, but do not in themselves argue that the mechanisms of docking and fusion are fundamentally different from those in neurosecretion.

In addition to effects on the subcellular distributions of some of the known SNAREs, insulin seems to produce both an increase in the participation of plasma membrane-derived VAMPs 2 and 3 in SNARE complexes with NSF and an overall increase in the participation of VAMPs in complexes formed from the combination of intracellular vesicles and PM. After insulin treatment, the additional VAMPs 2 and 3 translocated to the PM seem to be available in a form that can more readily form complexes with NSF, α-SNAP and syntaxin 4. We wish to emphasize, however, that these observations involve only the behaviour *in vitro* of an identifiable subset of solubilized membrane proteins; further studies will be required to relate these results to processes occurring in the intact adipose cell, which may involve additional regulatory components.

If either vesicle- or target-membrane competence for docking and fusion is in fact insulin-regulated, then such regulation might be achieved through the removal of a molecular clamp on the formation of docking complexes. Several families of candidate molecules have been identified in the neurosecretory process that block certain interactions of SNARE proteins. These include the n-sec-1/munc-18 proteins [28,29] in the plasma membrane, and synaptotagmins [22,30], synaptophysins [11,31] and ADP-ribosylation factors [32] in vesicle membranes. Whether these or other components modulate the interactions between the SNAREs we have identified in rat adipose cells remains to be established.

We thank Dr. T. Sollner and Dr. J. Rothman for the gifts of anti-*myc* antibody and the NSF–*myc*-his$_6$ and α-SNAP-his$_6$ plasmids, Dr. P. DeCamilli for anti-cellubrevin antibody, and Dr. W. Trimble for antibodies to synaptobrevins 1 and 2.

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Received 10 June 1996; accepted 16 July 1996