Characterization of Munc-18c and Syntaxin-4 in 3T3-L1 Adipocytes

PUTATIVE ROLE IN INSULIN-DEPENDENT MOVEMENT OF GLUT-4*

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We have previously identified three mammalian Sec1/Munc-18 homologues in adipocytes (Tellam, J. T., McIntosh, S., and James, D. E. (1995) J. Biol. Chem. 270, 5857–5863). These proteins are thought to modulate the interaction between vesicle membrane and target membrane soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and thus regulate intracellular vesicular transport. This study aimed to further characterize these Munc-18 isoforms and to define their potential role in the trafficking of GLUT-4 in adipocytes, a process reported to involve the vesicle membrane SNARE, VAMP-2. Using an in vitro binding assay with recombinant fusion proteins, we show that Munc-18a and Munc-18b bind to syntaxin-1A, -2, and -3, while Munc-18c binds only to syntaxin-2 and -4. The specific interaction between Munc-18c and syntaxin-4 is of interest because aside from syntaxin-1A, which is not expressed in adipocytes, syntaxin-4 is the only syntaxin that binds to VAMP-2. Using a three-way binding assay, it was shown that Munc-18c inhibits the binding of syntaxin-4 to VAMP-2. The subcellular distribution of syntaxin-4 and Munc-18c was almost identical, both being enriched in the plasma membrane, and both exhibiting an insulin-dependent movement out of an intracellular membrane fraction similar to that observed for GLUT-4. Munc-18b had a similar distribution to Munc-18c and so may also be involved in vesicle transport to the cell surface, whereas Munc-18a was undetectable by immunoblotting in adipocytes. Microinjection of a syntaxin-4 antibody into 3T3-L1 adipocytes blocked the insulin-dependent recruitment of GLUT-4 to the cell surface. These data suggest that syntaxin-4/Munc-18c/VAMP-2 may play a role in the docking/fusion of intracellular GLUT-4-containing vesicles with the cell surface in adipocytes.

In adipose tissue and muscle, the glucose transporter isoform 4, GLUT-4, is translocated from an intracellular vesicular pool to the cell surface in response to insulin (1, 2), a process that plays a major role in whole body glucose homeostasis. To understand the molecular mechanisms governing this vesicular transport system, it will be necessary to identify and characterize the individual components of the trafficking machinery. The SNARE hypothesis (reviewed in Ref. 3) provides a working model for studies of vesicle targeting and fusion in adipocytes. Vesicle-associated membrane protein VAMP or synaptobrevin (v-SNAREs) present on the transport vesicle and syntaxin (t-SNAREs) on the acceptor membrane form a complex, which also includes the synaptosomal-associated protein-25 (SNAP-25), soluble N-ethylmaleimide-sensitive factor attachment protein (α-SNAP), and N-ethylmaleimide-sensitive factor (NSF). This complex may facilitate the docking and/or fusion of distinct membrane compartments, the specificity being provided by the pairing of unique v- and t-SNAREs at different loci throughout the cell. Each of the SNAREs belong to large gene families. For example, in mammalian cells seven different syntaxins (4, 5), three different synaptobrevins (VAMP-1, VAMP-2, and cellubrevin) (6–12), and three SNAP-25 homologues (13–15) have been identified. Syntaxin-1A, a t-SNARE enriched in the neuronal presynaptic membrane, binds with high affinity to VAMP-2, which is found in synaptic vesicles (16). However, syntaxin-5, which appears to facilitate membrane traffic between the endoplasmic reticulum and Golgi (4), does not interact with VAMP-2.

Several other protein families regulate the interaction between v- and t-SNAREs. One of these, the Sec1-like family, modulates the assembly of v- and t-SNAREs by competitively binding to the t-SNARE protein. Loss of SEC1 function in yeast blocks the fusion of transport vesicles with the plasma membrane (17). Several mammalian Sec1-like proteins, referred to as Munc-18, have been identified (18–24). Munc-18a is expressed at high levels in the brain and competes with VAMP-2 for binding to syntaxin-1A (25). In view of the large number of Sec1-like proteins described in other eucaryotes and their putative role in regulating different vesicle transport steps, we set out to identify Sec1-like proteins that may be involved in GLUT-4 trafficking in adipocytes. Two new Munc-18 isoforms (Munc-18b and Munc-18c) were identified (22–24), both of which were ubiquitously expressed. However, the role of these new isoforms in regulating vesicle transport has not been established and their syntaxin binding specificities have not been compared.

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The abbreviations used are: v-SNARE, vesicle membrane SNARE; t-SNARE, target membrane SNARE; NSF, N-ethylmaleimide-sensitive factor; SNAPs, soluble NSF attachment proteins; SNAREs, soluble NSF attachment protein receptors; Munc-18, mammalian homologue of unc-18; VAMP, vesicle-associated membrane protein; PFR, polymerase chain reaction; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; Ig, immunoglobulin; PM, plasma membrane(s); LDM, low density microsome(s); HDM, high density microsome(s); M/N, mitochondria/nuclei.
The expression of different SNAREs in insulin-sensitive cell types and their role in GLUT-4 translocation has only recently been addressed. Adipocytes and muscle cells express VAMP-2 (10–12) and cellubrevin (10, 11). Both of these synaptotobrevin isoforms co-localize with intracellular GLUT-4 and, like GLUT-4, undergo insulin-dependent movement to the cell surface (11). Using immunoelectron microscopy on vesicles isolated from adipocytes and an endosomal ablation technique, it has been shown that the majority of cellubrevin co-localizes with approximately 40% of intracellular GLUT-4 in endosomes (26). The remaining GLUT-4, together with the majority of VAMP-2 appears to be targeted to a post-endocytic compartment, the function of which remains to be determined (26). In view of these observations, it is of considerable interest to determine the cognate t-SNAREs, particularly for VAMP-2, as this may provide some clues as to how insulin regulates the exocytosis of GLUT-4.

In the present study we provide evidence that syntaxin-4 and Munc-18c may play a role in regulating the trafficking of GLUT-4 in adipocytes. In vitro studies show that Munc-18 isoforms display unique syntaxin binding specificities. Most importantly, Munc-18c bound very strongly to syntaxin-4, one of the t-SNAREs previously identified in insulin-responsive tissues (27, 28) and shown to bind specifically to VAMP-2 (16). Using specific antisera against these proteins, we show that Munc-18c and syntaxin-4 have a similar subcellular distribution in 3T3-L1 adipocytes and both proteins exhibit insulin-dependent movement out of the intracellular membrane fraction that is highly enriched in GLUT-4. Microinjection of a syntaxin-4-specific antibody into 3T3-L1 adipocytes blocked insulin-stimulated movement of GLUT-4. These data, combined with the ability of Munc-18c to impair the interaction between syntaxin-4 and VAMP-2 in vitro, implicate these proteins in GLUT-4 trafficking in adipocytes.

**EXPERIMENTAL PROCEDURES**

**Materials**

Restriction enzymes were obtained from New England Biolabs, Inc. Radioactive nucleotides, nylon membranes (Hybond-N+), horseradish peroxidase-conjugated goat anti-rabbit IgG, and enhanced chemiluminescence (ECL) detection kits were from Amersham (Aylesbury, United Kingdom). Oligonucleotides were synthesized on an Applied Biosystems model 394 DNA synthesizer. All tissue culture reagents and Lipo- fectAMINE were from Life Technologies, Inc., with the exception of fetal calf serum, which was from Commonwealth Serum Laboratories (Australia). Polyvinylidene difluoride blotting membranes were from Millipore. BCA protein reagent was from Pierce. Glutathione-agarose beads were from Sigma and Ni2+-NTA-agarose beads were from Qiagen. Fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG, and enhanced chemiluminescence (ECL) detection kits were from Amersham (Aylesbury, United Kingdom). Oligonucleotides were synthesized on an Applied Biosystems model 394 DNA synthesizer. All tissue culture reagents and Lipo- fectAMINE were from Life Technologies, Inc., with the exception of fetal calf serum, which was from Commonwealth Serum Laboratories (Australia). Polyvinylidene difluoride blotting membranes were from Millipore. BCA protein reagent was from Pierce. Glutathione-agarose beads were from Sigma and Ni2+-NTA-agarose beads were from Qiagen. Fluorescein isothiocyanate-conjugated sheep anti-rabbit secondary antibody was purchased from The Binding Site (Birmingham, United Kingdom). Thrombin was purchased from Calbiochem. All other chemicals were of high purity commercial grade.

cDNA Cloning of 3T3-L1 Syntaxin-4

Rat syntaxin-1A, -2, -3, and -4 cDNA clones, generously provided by Dr. Richard Scheller (Stanford University, Stanford, CA), were radio-labeled with random hexamer primers (Promega Corp.) and used to screen a random-primer-labeled rat adipocyte cDNA library constructed in λ ZAP II, kindly provided by Dr. F. Fiedorek, University of North Carolina. A total of 200,000 plaques were initially screened with a 1.2-kilobase rat syntaxin-1A DNA fragment probe. Six positive clones were isolated, characterized by DNA sequencing, and shown to be identical to rat syntaxin-4. In an effort to isolate other syntaxin isoforms, a further screen was performed using a mixed cDNA probe comprising full-length cDNA inserts encompassing syntaxin-2, -3, and -4. Seven positive clones were isolated. The 13 positive clones isolated after sequential purifications were subcloned into pBluescript II SK and the inserts sequenced as described previously (22). All 13 clones were identical to mammalian syntaxin-4. Most of the clones contained the entire syntaxin-4 open reading frame, and one clone was sequenced entirely in both strands.

**RNA Blot Analysis**

Total RNA was isolated from rat tissues, 3T3-L1 fibroblasts, and adipocytes by the guanidine isothiocyanate procedure (29). 3T3-L1 fibroblast and adipocyte poly(A)+ was obtained using the Pharmacia mRNA purification kit (Pharmacia, Uppsala, Sweden). Northern blots were probed with a 3T3-L1 syntaxin-4 DNA fragment labeled with [α-32P]dCTP by random priming. The hybridization conditions were: 50% formamide, 5 × SSPE, 5 × Denhardt’s solution, 0.1% SDS, and 100 mg/ml denatured herring sperm DNA at 42°C for 16 h. The blot was washed with 1 × SSC and 0.1% SDS at 50°C. The RNA blots were also probed with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe as an internal control.

**Cell Culture**

3T3-L1 fibroblasts were obtained from the American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium as described previously (30). Confluent cells were induced to differentiate into adipocytes (30). All experiments used adipocytes 10–14 days after initiation of differentiation. CHO cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 1% nonessential amino acids, 100 units/liter penicillin, and 100 μg/liter streptomycin at 37°C in an atmosphere of 5% CO2.

Cells were passaged at subconfluence.

**Munc-18b and Munc-18c Constructs in CHO Cells**

Recombinant Munc-18b and Munc-18c cDNA constructs were subcloned into the EcoRI site of the mammalian expression vector pMEX-neo (kindly provided by Dr. E. Santos, National Institutes of Health, Bethesda, MD) and were transiently transfected into subconfluent CHO cells using LipofectAMINE, according to the manufacturer’s instructions. Cells were harvested after 48 h, scraped in Heps buffer (20 mM, pH 7.4) containing 1 mM EDTA and 250 mM sucrose (HES), lysed using a 1-ml syringe and 27-gauge needle, and centrifuged at 208,000 × g for 30 min in a TLA-100.3 rotor (Beckman) to pellet total cellular membranes. Membrane pellets were resuspended in 1% SDS in HES buffer and subjected to SDS-PAGE and immunoblotting using antibodies specific for either Munc-18b or Munc-18c.

**Antibodies**

Recombinant glutathione S-transferase (GST)-syntaxin-4 (amino acids 5–274) was expressed in bacteria and purified by glutathione-agarose affinity chromatography (32), followed by anion exchange chromatography on a Mono Q column. The GST-syntain-4 fusion protein (M, ~59,000) was injected into rabbits with Montanide ISA-70 as adjuvant and again 4 weeks later. The rabbit received a total of 200 μg of GST-syntaxin-4 and was bled 2 weeks after the second injection. A synthetic peptide corresponding to a unique region near the NH2 terminus of syntaxin-4 (amino acids 20–38, CRKERVVLHGPTARLGSFD) that included a cysteine linker was coupled to BSA and injected into mice for production of monoclonal antibodies. An IgM antibody, which reacted specifically with recombinant syntaxin-4, was generated and purified using anti-mouse IgM (Selenus, Melbourne, Australia) coupled to a Mini-Leak™ low kit (Rem-En-Tec, Copenhagen, Denmark) support. Synthetic peptides corresponding to unique regions within each Munc-18 isoform, the carboxyl-terminal 12 amino acids of Munc-18b (VTLDQKLEGVALP) and the carboxyl-terminal 14 amino acids of Munc-18c (CMLNKSKDVKSFKDE), and to the amino-terminal 12 amino acids of cellubrevin (STGVPSGSSAATC) and all including cysteine linkers, were coupled to keyhole limpet hemocyanin according to standard procedures (33) and injected into rabbits for antibody production. Antibodies were affinity-purified using the peptide antigen conjugated to keyhole limpet hemocyanin and coupled to Sepharose beads (ImmunoPure Ag/Ab Immobilization Kit 2, Pierce).

**Subcellular Fractionation of 3T3-L1 Adipocytes**

Two separate methods were employed to determine the subcellular distribution of syntaxin-4 and Munc-18 isoforms in 3T3-L1 adipocytes.

**Differential Centrifugation—Subcellular membrane fractions were prepared from 3T3-L1 adipocytes using a differential centrifugation protocol (34). Four separate membrane fractions were obtained, and designated as plasma membranes (PM), low density microsomes (LDM), high density microsomes (HDM), and mitochondria/nuclei (MN). The protein content of all fractions was measured using the Pierce BCA reagent.**

**Plasma Membrane Lawn Assay—Plasma membrane fragments were prepared from basal and insulin-stimulated adipocytes as described previously (35, 36). Fragments were immunolabeled with an anti-
GLUT-4 polyclonal serum (43) at a dilution of 1:500, syntaxin-4 serum (1:750 dilution), or anti-Munc-18c (10 μg of protein/ml) affinity-purified polyclonal antibodies. Primary antibodies were detected with fluorescein isothiocyanate-conjugated sheep anti-rabbit secondary antibody (25 μg/ml). Coverslips were mounted on glass slides (34) and visualized with a 63× objective. Immunofluorescent images were captured using a Zeiss Axiovert fluorescence microscope (Carl Zeiss, Oberkochen, Germany). In some cases labeling of plasma membrane fragments was quantified as described previously (36).

**Microinjection of 3T3-L1 Cells**

3T3-L1 adipocytes cultured on glass coverslips were incubated in Krebs-Ringer bicarbonate Hepes buffer (pH 7.4), containing 2 mM pyruvate, 0.5% BSA, and 2.5 mM glucose for 45 min prior to microinjection. All of the cells located within a corner of a 3-mm² grid (approximately 300 cells), located on the upper side of the coverslip, were microinjected with antibodies over a 45-min period using a Zeiss automated injection system coupled to an Eppendorf microinjector. The immunopurified anti-syntaxin-4 or control antibodies (an irrelevant IgG or IgM), each at 0.2 mg/ml were dissolved in a buffer containing 5 mM sodium phosphate (pH 7.2), 100 mM KCl for microinjection. Cells were transferred into fresh medium and allowed to recover for 90 min following injection, prior to incubation with insulin (100 nM) for 15 min. GLUT-4 levels in fresh medium and allowed to recover for 90 min following injection, immunofluorescence microscope under a 40× objective. Images were enhanced and relative intensities were scaled using the Bio-Rad COMOS confocal imaging software where identical gain settings were used for each experiment. Lawns from at least 24 cells were quantitated for each image, and at least five different fields for each coverslip were quantitated. Values were then averaged to obtain a single data point for each experimental condition.

**SDS-PAGE and Immunoblotting**

Subcellular membrane fractions (20 μg of protein) were subjected to SDS-PAGE using 10 or 12% polyacrylamide resolving gels (37). Proteins were electrophoretically transferred to nitrocellulose membranes and probed with GST-syntaxin-4 antiserum (1:750 dilution) or affinity-purified Munc-18c antibody (10 μg of protein/ml), affinity-purified Munc-18c antibody (10 μg of protein/ml), affinity-purified VAMP-2 antibody (10 μg of protein/ml), or cellubrevin antiserum (1:2000 dilution). Primary antibodies were detected with horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody diluted 1:10,000 (ECL kit). Autoradiograms were quantitated using a model GS-670 Imaging Densitometer (Bio-Rad).

**Purification of His₆-tagged Munc-18c**

A full-length Munc-18c cDNA construct was generated by PCR using the vector T3 primer and an oligonucleotide encompassing the XhoI restriction site and 15 nucleotides of overlapping sequence of the 5′ coding region of a 3T3-L1 Munc-18c cDNA cloned into pBluescript II K+ (22). The PCR cycling profile was 94 °C for 20 s, 55 °C for 40 s, 72 °C for 2 min, repeated for 35 cycles. The PCR product was digested with XhoI and EcoRI, and the ~2.2-kilobase fragment was subcloned into the XhoI-EcoRI sites of pSR ETA to generate the plasmid pMunc-18c-His₆. A large bacterial culture (E. coli BL21 (DE3)) was induced with isopropyl-1-thio-β-D-galactopyranoside for 5 h, and the Munc-18c-His₆ protein was purified using a Qiagen nickel resin according to the manufacturer’s instructions. The Munc-18c-His₆ protein was dialyzed against phosphate-buffered saline and stored at −80 °C.

**Purification of Recombinant Fusion Proteins**

GST fusion proteins incorporating the full cytoplasmic domains of rat syntaxin-1A, -2, -3, and -4 and rat VAMP-2 were prepared as described (16, 38). Recombinant GST-cellubrevin was produced by inserting the HindIII/EcoRI PCR fragment, incorporating the full cytoplasmic domain of a 3T3-L1 cellubrevin cDNA clone 3A (22) into the pGEX-KG vector. The oligonucleotides used to generate the cytoplasmic domain of cellubrevin (amino acid residues 1-80) were 5′-CCGGAGGTTCATGTTCACTACAGGTGTTGCTTG3′ sense primer and 5′-GGCCACGGCTTTTACTCTGGATTCTCCCACCATCC-3′ antisense primer. The cytoplasmic tails of the four syntaxin isoforms 1A, 2, 3, and 4, VAMP-2, and cellubrevin were isolated from GST using thrombin (6 units/ml) in cleavage buffer (50 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 0.1% β-mercaptoethanol, 2.5 mM calcium chloride).

**In Vitro Binding Assays**

3T3-L1 Munc-18a and Munc-18b cDNA in pBluescript KG (22) were transcribed and translated in vitro with T7 RNA polymerase using a coupled reticulocyte lysate system (Promega) supplemented with [35S]methionine (ICN). Lysates from [35S]methionine-labeled Munc-18a, Munc-18b, or Munc-18c translation reactions (10 μl) were incubated for 1 h at 4 °C with GST, GST-syntaxin-1A, GST-syntaxin-2, GST-syntaxin-3, or GST-syntaxin-4 in phosphate-buffered saline containing 0.5% BSA. The concentration of the fusion proteins used in these reactions was 25 μg/ml. Glutathione-agarose beads (15 μl) were added and the mixture incubated for another 30 min at 4 °C. The beads were washed three times by brief centrifugation in phosphate-buffered saline at 4 °C. Washed beads were then incubated for 5 min with Laemmli sample buffer (20 μl) and subjected to SDS-PAGE. The gel was fixed, incubated with 1% sodium salicylate, 0.5% glycerol, dried, and subjected to autoradiography.

To assess whether Munc-18c inhibits the in vitro binding interaction between syntaxin-4 and VAMP-2, GST alone, GST-syntaxin-1A (2 μg), and GST-syntaxin-4 (2 μg) were incubated in the presence or absence of recombinant Munc-18c-His₆ (10 μg) for 1 h at 4 °C. Thrombin-cleaved recombinant-VAMP-2 (10 μg) was added to each sample and the incubation continued for another 45 min at 4 °C. Glutathione-agarose beads (15 μl) were then added to each sample and the mixture rotated at 4 °C for 30 min. The beads were washed as described above, subjected to SDS-PAGE and immunoblotting using the VAMP-2-specific antibody. This experiment was repeated to study the binding of thrombin-cleaved recombinant-cellubrevin (10 μg) to syntaxin-1A (0.5 μg) and syntaxin-4 (5 μg) in the presence or absence of recombinant Munc-18c-His₆ (10 μg).

**RESULTS**

A major goal of these studies was to identify SNAREs that may interact with the syntaxin-binding protein VAMP-2 in adipocytes, because this v-SNARE has been suggested to play a role in GLUT-4 trafficking (Refs. 26, 27, and 39). We have recently reported the identification of three Sec1-like proteins in adipocytes, Munc-18a-c (22). In an effort to determine which of these proteins might be involved in GLUT-4 trafficking, we determined the syntaxin binding specificity of these proteins because previous studies have shown that Munc-18 binds to syntaxin in vitro (18–20, 23, 24).

**Syntaxin Binding Specificity of Munc-18 Isoforms—**To determine the syntaxin binding specificity of the different Munc-18 isoforms, an in vitro binding assay was performed (Fig. 1). All three Munc-18 isoforms yielded translation products of ~65 kDa (Fig. 1). In agreement with previous studies (19, 24), there was significant binding of Munc-18a to GST-syntaxin-1A, GST-syntaxin-2, and, to a lesser extent, GST-syntaxin-3. There was no significant binding of Munc-18a to GST-syntaxin-4 or GST alone. Munc-18b also interacted with GST-syntaxin-1A, GST-syntaxin-2, and GST-syntaxin-3. The syntaxin binding specificity of Munc-18c was significantly different from Munc-18a and Munc-18b in two major ways. First, there was no significant interaction between Munc-18c and GST-syntaxin-1A or GST-syntaxin-3. Second, Munc-18c interacted strongly with GST-syntaxin-4. In addition, Munc-18c also interacted strongly with GST-syntaxin-2.

**Subcellular Localization of Munc-18 Isoforms in Adipocytes**—We were unable to detect significant expression of the Munc-18a protein in adipocytes using an isoform-specific antibody (data not shown), consistent with its predominant expression in brain (18–20). To examine the subcellular distribution of Munc-18b and 18c, we prepared antibodies specific for each isoform. The specificity of these antisera for the different Munc-18 isoforms is shown in Fig. 2A. Munc-18b and Munc-18c eDNAs were transiently transfected into CHO cells. There was no significant immunolabeling in wild type CHO cells using either the Munc-18b or the Munc-18c antibodies. The Munc-18b antibody immunolabeled a band of 65 kDa in cells transfected with the Munc-18b cDNA but not in cells transfected with Munc-18c (Fig. 2A, lanes 2 and 4). Conversely, the Munc-
18c antibody immunolabeled a band of 65 kDa in cells transfected with the Munc-18c cDNA but not in cells transfected with Munc-18b (Fig. 2A, lanes 1 and 3). These data indicate that these antibodies are specific and immunolabel proteins of the appropriate molecular size corresponding to Munc-18b and Munc-18c.

The anti-Munc-18b and Munc-18c antibodies were used to immunoblot subcellular fractions isolated from adipocytes (Fig. 2B). Four membrane fractions were isolated from adipocytes, namely PM, LDM, HDM, and M/N. The protein recovery in each fraction and the distribution of marker proteins such as GLUT-4 among these fractions were not significantly different from that described previously (34). The PM fraction is enriched in plasma membrane markers, the M/N fraction is enriched in markers for the mitochondria and nuclei, the HDM is enriched in endoplasmic reticulum markers, and the LDM in Golgi/endosomal markers (26, 34).

Both the Munc-18b and Munc-18c antibodies immunolabeled proteins in 3T3-L1 adipocytes that migrated to a position (65 kDa) consistent with the expected molecular mass of these proteins (see Figs. 1 and 2). Numbers on the left of the figures indicate the positions of molecular size markers.

The subcellular distribution of the 65-kDa bands corresponding to Munc-18b and Munc-18c was indistinguishable from that of the Na/K ATPase, a cell surface marker (26), and syntaxin-4 (see below). It was difficult to quantify Munc-18b levels among the different fractions due to the cross-reactivity of the antibody with BSA. However, a significant level of Munc-18c, corresponding to approximately 15% of that found in the PM fraction, was also located in the LDM fraction (Fig. 2B). This fraction primarily comprises intracellular membranes including the intracellular GLUT-4 compartment (see Fig. 5A). Interestingly, the level of Munc-18c in the LDM fraction was significantly reduced (p < 0.05) in the presence of insulin by 45 ±
is localized to the cell surface in Cos cells (4). Fractionation of subcellular fractionation studies (Fig. 2), immunolabeling of PM fragments with the Munc-18c antibody was readily detected and there was no change in the degree of labeling with insulin (Fig. 2C).

Identification of Syntaxin-4 in Adipocytes—The above studies raise the possibility that Munc-18c is involved in the insulin-dependent translocation of GLUT-4 to the cell surface because it is expressed at relatively high levels in adipocytes, it is targeted to the plasma membrane, and it undergoes a change in subcellular distribution with insulin. In addition, Munc-18c binds specifically to syntaxin-4, a t-SNARE known to interact with VAMP-2 (16). To confirm these observations, we next turned our attention to the expression of different syntaxin isoforms in adipocytes. A 3T3-L1 adipocyte cDNA library was initially screened with a radiolabeled rat syntaxin-1A probe yielding six positive clones, which all corresponded to the rat syntaxin-4 sequence. In an effort to isolate other syntaxin isoforms, a further screen was performed using a mixed cDNA probe comprising full-length cDNA inserts encompassing syntaxin-2, -3, and -4. Seven positive cDNA clones were identified following this screen, all of which corresponded to rat syntaxin-4. The murine 3T3-L1 syntaxin-4 (GenBank U76832) amino acid sequence differed from the rat sequence (4) at two positions, 35 (Ser-Gly) and 216 (Thr-Ser). When the murine 3T3-L1 syntaxin-4 (GenBank U76832) was used to probe a Northern blot, a single band was highly enriched in the plasma membrane fraction consistent with previous studies in Cos cells (4). There was a small amount of syntaxin-4 in the LDM fraction that is likely to be of intracellular origin because, like GLUT-4, in response to insulin there was a significant (p < 0.01) decrease in syntaxin-4 levels in this fraction (41 ± 3.5% mean ± S.E., n = 4) (Fig. 5A). Interestingly, the subcellular distribution of Munc-18c was almost identical to that of syntaxin-4 (Fig. 2B). To further confirm the cell surface distribution of syntaxin-4 in adipocytes, we used the plasma membrane lawn assay (36). PM fragments isolated from non-insulin-stimulated syntaxin-4 in adipocytes, the polysomal G-protein (36). PM fragments isolated from non-insulin-stimulated adipocytes showed a substantial increase in labeling (Fig. 5B). Intense syntaxin-4 labeling was observed in PM fragments isolated from both non-stimulated and insulin-treated cells. There was no significant difference in syntaxin-4 labeling of PM fragments isolated from both non-stimulated and insulin-treated cells. In combination with the immunoblotting data shown in Fig. 5A, these results indicate that syntaxin-4 is localized to the plasma membrane in 3T3-L1 adipocytes.

Syntaxin-4 and GLUT-4 Translocation in Adipocytes—The above findings raise the possibility that syntaxin-4, Munc-18c, and VAMP-2 may collectively be involved in the docking and/or fusion of GLUT-4 containing vesicles with the cell surface. In an effort to obtain direct evidence to support this hypothesis, we attempted to disrupt the function of syntaxin-4 in 3T3-L1 adipocytes. A monoclonal antibody was microinjected into adipocytes, and the PM lawn assay was used to quantify cell surface GLUT-4 levels in individual cells. In cells microinjected with buffer alone, we observed a 4–5-fold increase in surface levels of GLUT-4 following incubation with insulin. This was not significantly different from the magnesium of the effect obtained in cells that had not been microinjected (data not shown). Hence, microinjection per se does not disrupt this process, a finding that is consistent with previous studies (40). The syntaxin-4 antibody blocked the insulin-dependent increase in GLUT-4 at the cell surface.

**Fig. 3.** Expression of syntaxin-4 mRNA among rat tissues and 3T3-L1 cells. Total RNA from eight different rat tissues, 3T3-L1 fibroblasts, 3T3-L1 adipocytes, and poly(A) RNA from 3T3-L1 adipocytes were hybridized with an α-32P-labeled syntaxin-4 DNA probe as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described under “Experimental Procedures.” The size (kilobases) of the relevant transcripts are indicated at the left.

5.2% (mean ± S.E., n = 4) similar to that observed for GLUT-4 (Fig. 5A). To further confirm the enrichment of Munc-18c in the plasma membrane, the Munc-18c antibody was used to immunolabel plasma membrane fragments isolated from non-insulin-stimulated and insulin-treated cells. Consistent with the subcellular fractionation studies (Fig. 2B), immunolabeling of PM fragments with the Munc-18c antibody was readily detected and there was no change in the degree of labeling with insulin (Fig. 2C).

**Fig. 4.** Specificity of the syntaxin-4 antiserum. GST-syntaxin-1A (lane 1), GST-syntaxin-2 (lane 2), GST-syntaxin-3 (lane 3), and GST-syntaxin-4 (lane 4) were cleaved with thrombin and subjected to SDS-PAGE. The upper panel shows the Coomassie Blue-stained gel showing the cleaved products: the cytoplasmic tails of the four syntaxin isoforms (~32 kDa) and the GST fragment (27 kDa). The lower panel shows an immunoblot using an antibody to GST-syntaxin-4. While the cleaved GST product in each lane was labeled (due to the presence of antibodies specific for glutathione S-transferase in the serum), the antiserum was highly specific and only recognized the cytosolic domain of syntaxin-4 (lane 4).
surface by approximately 70%, whereas a control monoclonal antibody of the same isotype as the syntaxin-4 antibody had no significant effect (Fig. 6).

**Munc-18c Inhibits v-SNARE Binding to Syntaxin-4**—Based on these data and previous studies (26, 28, 39), it seems evident that the interaction between syntaxin-4 and VAMP-2 may play an important role in the docking and/or fusion of GLUT-4-containing vesicles with the cell surface in adipocytes. Munc-18c, which binds to syntaxin-4 in vitro and which is targeted to the plasma membrane in adipocytes, may also be involved in regulating GLUT-4 trafficking in adipocytes. Munc-18c, which binds to syntaxin-4 in vitro and which is targeted to the plasma membrane in adipocytes, may also be involved in regulating GLUT-4 trafficking in adipocytes. It has been suggested that Munc-18a has an inhibitory role in the synaptic vesicle docking in neurons, because it inhibits the interaction between VAMP-2 and syntaxin-1A (25). Hence, we next examined whether Munc-18c modulates the interaction between syntaxin-4 and VAMP-2 in a similar manner. GST-syntxin-1A and GST-syntxin-4 were incubated in the presence or absence of the recombinant His$_6$-Munc-18c fusion protein. The purified recombinant VAMP-2 fragment, corresponding to the cytoplasmic tail of this protein, was then added to each sample. After binding, the GST-syntxin fusion protein complex was purified on glutathione-agarose beads and immunoblotted with a VAMP-2-specific antibody. In agreement with previous studies (16), VAMP-2 specifically associated with both syntaxin-1A and syntxin-4 (Fig. 7). The binding affinity of VAMP-2 with syntaxin-1A was significantly higher than observed with syntaxin-4 (lane 1 versus lane 3) also consistent with previous studies (25). In the presence of Munc-18c, there was a 76.9 ± 6.3% (n = 3) reduction in the binding of VAMP2 to syntaxin-4 (Fig. 7). There was also a partial reduction (29%) in the binding of syntaxin-1A to VAMP2 in the presence of Munc-18c (Fig. 7).

However, this may be accounted for by a direct interaction between Munc-18c and VAMP2.2

**Cellubrevin** is another v-SNARE that is highly homologous to VAMP2 (9), interacts with syntaxin-1A and -4 (41). Cellubrevin is also expressed in adipocytes and, like VAMP2, undergoes

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2 J. Tellam and D. James, unpublished results.
an important role in regulating the docking of intracellular GLUT-4 imposed by microinjection of syntaxin-4 antibodies into adipocytes, it seems plausible that different interactions between these proteins and other adipocyte SNAREs may play an important role in regulating the docking of intracellular GLUT-4 vesicles with the cell surface.

Microinjection of a syntaxin-4 monoclonal antibody into adipocytes resulted in a marked inhibition of insulin-stimulated GLUT-4 translocation (Fig. 6). This provides a compelling case for the involvement of this protein in GLUT-4 trafficking in adipocytes, and so identifying other proteins that interact with syntaxin-4 in adipocytes is of potential relevance to this important physiological process. Syntaxin-4 is predominantly localized to the cell surface in adipocytes, where it may mediate the docking of intracellular vesicles containing GLUT-4 and VAMP-2. It is noteworthy that a significant, albeit smaller, pool of intracellular syntaxin-4 was also demonstrable in the intracellular membrane fraction. This is not likely due to contamination of this fraction with surface membranes because, like GLUT-4, there was a significant decrease in the amount of syntaxin-4 in this fraction following insulin treatment (Fig. 5A). Hence, we cannot exclude a role for syntaxin-4 in intracellular trafficking as well as in docking and fusion with the cell surface. During the course of these studies, similar findings concerning the role of syntaxin-4 in adipocytes were reported (27). In these studies it was shown that syntaxin-4 is expressed in 3T3-L1 adipocytes, and that it is found both in the plasma membrane and to a lesser extent in intracellular membranes. Most importantly, using a different experimental system to that reported in the present study, namely streptolysin-O-permeabilized 3T3-L1 adipocytes, it was shown that a syntaxin-4 polyclonal antibody caused a marked inhibition of insulin-stimulated glucose transport. Thus, the two studies complement each other and together provide convincing evidence that syntaxin-4 is involved in the regulation of GLUT-4 trafficking in adipocytes.

The precise locus of insulin action to trigger an increase in cell surface levels of GLUT-4 is not clear. Kinetic experiments reveal that insulin primarily effects exocytosis of GLUT-4 from the intracellular compartment with a relatively minimal change in GLUT-4 endocytosis (44). However, this could include an effect of insulin on vesicle budding, vesicle transport, or vesicle docking and fusion with the cell surface. Thus, it remains to be seen if the types of proteins under investigation in the present study serve as a focal point for modulation by insulin. This would imply that a major effect of insulin is to facilitate the docking and/or fusion of GLUT-4 vesicles with the cell surface. In the presynaptic nerve terminus, both of these parameters appear to be regulated by a change in potential difference across the cell surface. In this instance, numerous predock vesicles are observed within the active zone, implying that fusion is rate-limiting. Morphological studies in adipocytes and muscle cells (45) have failed to reveal GLUT-4 vesicles that are predock. Furthermore, the time course for accumulation of GLUT-4 at the surface is much slower than synaptic vesicle exocytosis, suggesting that vesicle fusion is...
probably not rate-limiting (46).

The modulatory role imposed by Munc-18c on the interaction between syntaxin-4 and VAMP-2 provides one avenue for insulin regulation. For instance, in non-stimulated cells Munc-18c may preferentially bind to syntaxin-4 and inhibit its ability to bind to VAMP-2 and so effectively impose a clamp on vesicle docking. Insulin may trigger a conformational change, possibly by phosphorylation or some other mechanism, in either syntaxin-4 or Munc-18c, thus changing the equilibrium in favor of VAMP-2. This model implicates syntaxin-4 and/or Munc-18c as potential targets of insulin action. In support of such a possibility, it has recently been shown that phosphorylation of Munc-18a by protein kinase C blocks its interaction with syntaxin-1A (47). This model provides a useful framework for future investigations. In particular, it will be necessary to determine if syntaxin-4 and Munc-18c interact in vivo and if such an interaction is disrupted in the presence of insulin. The localization of Munc-18c and syntaxin-4 to the plasma membrane certainly pre-empts this type of regulatory model.

The modulatory effect of Munc-18c on syntaxin-4 was not specific to VAMP-2. Syntaxin-4 also binds to cellubrevin, another v-SNARE that is expressed in adipocytes, and this interaction is also disrupted by Munc-18c (Fig. 8). Thus, the functional requirement for these unique v-SNAREs in adipocytes and other cell types is not evident from these interactions. However, it is thought that numerous proteins are involved in the formation of a docking/fusion complex, and so, until each of these interactions has been tested, the unique role for these different v-SNAREs remains unknown. We have recently shown that VAMP-2 and cellubrevin are differentially targeted in intracellular vesicles in adipocytes (26), and so it is conceivable that the unique function of these proteins is in their ability to differentially regulate the sequestration of different populations of vesicles. This is intriguing because in the presence of insulin both cellubrevin and VAMP-2 accumulate at the cell surface of adipocytes, consistent with the fact that both of these proteins and both types of vesicles that they are found in undergo parallel regulation (11, 26). Thus, this argues in favor of a model in which insulin may modulate the ability of syntaxin-4 to bind different types of vesicles, both those containing VAMP-2 and those containing cellubrevin. The inhibitory role of Munc-18c on the binding of syntaxin-4 to both VAMP-2 and cellubrevin would again suggest a key role as a regulator of this process. Hence, in this model syntaxin-4 and Munc-18c may facilitate the docking and fusion of different kinds of exocytotic vesicles, and this is consistent with their ubiquitous tissue distribution (22).

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