

Syndet, an Adipocyte Target SNARE Involved in the Insulin-induced Translocation of GLUT4 to the Cell Surface*

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In adipocytes, insulin stimulates the translocation of the glucose transporter, GLUT4, from an intracellular storage compartment to the cell surface. Substantial evidence exists to suggest that in the basal state GLUT4 resides in discrete storage vesicles. A direct interaction of GLUT4 storage vesicles with the plasma membrane has been implicated because the v-SNARE, vesicle-associated membrane protein-2 (VAMP2), appears to be a specific component of these vesicles. In the present study we sought to identify the cognate target SNAREs for VAMP2 in mouse 3T3-L1 adipocytes. Membrane fractions were isolated from adipocytes and probed by far Western blotting with the cytosolic portion of VAMP2 fused to glutathione S-transferase. Two plasma membrane-enriched proteins, p25 and p35, were specifically labeled with this probe. By using a combination of immunoblotting, detergent extraction, and anion exchange chromatography, we identified p35 as Syntaxin-4 and p25 as the recently identified murine SNAP-25 homologue, Syndet (mSNAP-23). By using surface plasmon resonance we show that VAMP2, Syntaxin-4, and Syndet form a ternary SDS-resistant SNARE complex. Microinjection of anti-Syndet antibodies into 3T3-L1 adipocytes, or incubation of permeabilized adipocytes with a synthetic peptide comprising the C-terminal 24 amino acids of Syndet, inhibited insulin-stimulated GLUT4 translocation to the cell surface by ~40%. GLUT1 trafficking remained unaffected by the presence of the peptide. Our data suggest that Syntaxin-4 and Syndet are important cell-surface target SNAREs within adipocytes that regulate docking and fusion of GLUT4-containing vesicles with the plasma membrane in response to insulin.

In order to transiently modulate the uptake of nutrients and other factors that are required to sustain changes in cellular metabolism, eucaryotic cells have adopted mechanisms whereby proteins can be translocated from intracellular storage vesicles to the cell surface in response to extrinsic stimuli. The integral membrane protein, GLUT4,¹ one of six mammalian facilitative

glucose transporters, is a paragon example of this group of molecules. Expressed predominantly in skeletal muscle, cardiac muscle, and adipocytes, GLUT4 is almost completely sequestered intracellularly under resting or fasting conditions (1). However, in response to muscle contraction (2, 3), or acute insulin elevation in both muscle and fat (1), GLUT4 is rapidly translocated to the cell surface in an ATP-dependent manner (4). The regulated movement of GLUT4 in response to insulin is fundamental to the maintenance of glucose homeostasis because defects in this process have been implicated in the development of non-insulin-dependent diabetes mellitus (5, 6).

Immunoelectron microscopy (7) and cell surface labeling techniques (8) reveal that GLUT4 continually recycles between the cell surface and its intracellular storage site. Under fasting conditions, the majority of GLUT4 is found within intracellular structures comprised of tubulo-vesicular elements that are clustered in the *trans*-Golgi network, near sorting endosomes, or in the cytoplasm, often just beneath the plasma membrane (7). Upon stimulation with insulin there is a rapid translocation of GLUT4 to the cell surface, occurring with a *t*_{1/2} of ~3 min (9).

The precise nature of the tubulo-vesicular intracellular GLUT4 compartment is currently under intensive investigation. Two models have been formulated to describe the trafficking and biogenesis of GLUT4 and its storage compartment, and both have been reviewed in detail (10, 11). Each model predicts different modes of GLUT4 trafficking, as well as distinct loci of insulin action. The first suggests that under basal conditions, GLUT4 is sequestered within a topologically continuous subdomain of the endosomal system. This model assumes that the trafficking of GLUT4 through the general recycling pathway is regulated by its interaction with other proteins that constitute retention factors. Insulin and/or contraction are predicted to disrupt the interaction between GLUT4 and these retention factors, in turn enabling GLUT4 to re-enter the constitutive recycling pathway and gain access to the cell surface. No specialized vesicular fusion machinery is required to accompany GLUT4 in this model since, presumably, this function would be fulfilled by the constitutive machinery utilized by the endosomal system.

The second model suggests that GLUT4 is sorted and pack-

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¹ The abbreviations used are: GLUT4, insulin-regulated glucose

transporter; β -OG, β -octylglucoside; GST, glutathione S-transferase; GSVs, GLUT4 storage vesicles; PAGE, polyacrylamide gel electrophoresis; SNAP-25, synaptosomal associated protein of 25 kDa; SNAP-23, SNAP-25-like protein of 23 kDa; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor; SPR, surface plasmon resonance; SSVs, small synaptic vesicles; t-SNARE, target-SNAP receptor; VAMP2, vesicle-associated membrane protein 2; v-SNARE, vesicle-SNAP receptor; FCS, fetal calf serum; PBS, phosphate-buffered saline; PM, plasma membranes; HDM, high density microsomes; LDM, low density microsomes; PVDF, polyvinylidene fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

aged into discrete storage vesicles at some stage during transit through the endosomal recycling system. An important feature of this model is that once formed, these vesicles have the potential to dock and fuse directly with the cell surface, independently of the endocytic recycling system. Hence, one predicted loci of insulin action in this model is the machinery that mediates the docking and fusion of GLUT4 containing vesicles with the plasma membrane. Recently, elements of such insulin-regulated docking machinery were identified in adipocytes, and some of these molecules were found to be identical to those used for SSV exocytosis in neurons. These findings have provided strong support for the vesicle model of GLUT4 trafficking and also raised the intriguing possibility that GLUT4 is stored in intracellular vesicles that resemble SSVs. The discovery of vp165 (12), an aminopeptidase that co-localizes and traffics identically to GLUT4 in response to insulin, provides additional support for the storage vesicle model.

In 1993 Rothman and colleagues (13) proposed the SNARE hypothesis that suggested for all vesicular trafficking events there exists a unique vesicle-bound ligand (v-SNARE) that specifically recognizes and interacts with a unique receptor molecule (t-SNARE) found in the target membrane. The consummation of this reaction was proposed to lead to correct vesicle targeting and ultimately membrane fusion. In the case of SSVs in neurons, such functions are encoded by the v-SNARE VAMP2, which is highly enriched in SSVs, and two t-SNAREs Syntaxin-1A and SNAP-25 found at the presynaptic plasma membrane. One of the fundamental tenets of the SNARE hypothesis is that the unique interactions between different sets of v- and t-SNAREs provide the necessary specificity required to ensure that a particular vesicle will dock and fuse with a particular target membrane. Indeed it has since been shown that there are large gene families of v- and t-SNAREs in eucaryotic cells that appear to function at distinct loci throughout the cell. In the post-Golgi recycling pathway in mammalian cells, for example, at least three different v-SNAREs (VAMP1, VAMP2, and cellubrevin) and eight different t-SNAREs (Syntaxin-1, Syntaxin-2, Syntaxin-3, Syntaxin-4, Syntaxin-6, Syntaxin-7, SNAP-25, and Syndet) have been described (reviewed in Ref. 14).

The expression of neuronal VAMP homologues in adipocytes was first reported by Lienhard and colleagues (15). It was later shown that adipocytes express both VAMP2 and an additional v-SNARE, cellubrevin (16). Paradoxically, both proteins appeared to co-localize with GLUT4 in adipocytes (17). A specific role for VAMP2 in the insulin-dependent translocation of GLUT4 was implicated when it was revealed that the majority of cellubrevin localized to endosomes, as is the case in other cells (18), whereas VAMP2 was predominantly co-localized with GLUT4 in a population of vesicles segregated from recycling endosomes (17). We have proposed that these vesicles sequester GLUT4 from the constitutive recycling pathway and hence refer to them as GLUT4 storage vesicles (GSVs) (11). Consistent with studies in neurons, we have recently shown that synthetic peptides that comprise unique VAMP2 domains block GLUT4 exocytosis but not the constitutive trafficking of GLUT1 in permeabilized 3T3-L1 adipocytes (19), thus supporting a unique role for this protein in GLUT4 exocytosis.

In view of the central role of VAMP2 in insulin-regulated GLUT4 trafficking, in the present studies we have attempted to identify VAMP2-binding proteins in adipocytes. Specifically, we sought to identify the cognate t-SNAREs for VAMP2 in adipocyte plasma membranes. By employing the mouse 3T3-L1 adipocyte cell line, which exhibits a 10–40-fold increase in cell-surface GLUT4 levels following insulin stimulation (4), we have identified Syntaxin-4 and the recently described SNAP-25

homologue Syndet² (20) as the two major VAMP2-binding proteins in mouse adipocyte plasma membranes. We show, by using surface plasmon resonance, that VAMP2, Syntaxin-4, and Syndet form an SDS-resistant SNARE complex *in vitro*. Furthermore, we show that a peptide identical to the C-terminal 24 amino acids of Syndet, or anti-Syndet antibodies, inhibit insulin-stimulated GLUT4 translocation by 35–40% in cultured 3T3-L1 adipocytes. Insulin dependent trafficking of the constitutively recycling glucose transporter, GLUT1, was unaffected when challenged with the former reagent. Our work provides evidence that Syntaxin-4 and Syndet are major t-SNAREs in adipocytes that mediate GLUT4 mobilization to the plasma membrane in response to insulin and further supports the existence of discrete GSVs.

MATERIALS AND METHODS

General Reagents—All tissue culture reagents were purchased from BioWhittaker (Sydney, Australia), except fetal calf serum (FCS), which was purchased from Trace Biosciences (Clayton, Australia). Protein G-Sepharose, the supersignal enhanced chemiluminescence (ECL) kit, and the bicinchoninic acid (BCA) protein assay kit were purchased from Pierce. Donkey anti-rabbit IgG conjugated to horseradish peroxidase was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich (Sydney, Australia). MC21 antisera, specific for the C-terminal 12 amino acids of mouse SNAP-25, was a gift from Pietro De Camelli (Yale University). Anti-glutathione *S*-transferase (GST) antisera (catalog number 90001605) was purchased from Amersham Pharmacia Biotech (Melbourne, Australia). Antisera specific for Syntaxin-4 and the N terminus of Syndet have been described elsewhere (20, 21). Standard techniques (22) were used to generate rabbit antisera against full-length Syndet-GST (prepared as described below).

Cell Culture—3T3-L1 adipocytes were cultured as described previously (23). Briefly, 3T3-L1 fibroblasts, purchased from the American Type Culture Collection (ATCC), were grown to confluence in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum on 35-mm or 10-cm dishes (Nalgene). Cells were then transferred into 10% fetal calf serum (FCS) containing 100 ng/ml biotin, 2 μ g/ml insulin, 250 nM dexamethasone, and 500 μ M 3-isobutyl-1-methylxanthine for 72 h. Subsequently, cells were cultured in 10% FCS containing 2 μ g/ml insulin for an additional 72 h, after which they were maintained in 5% FCS for at least 48 h before being harvested as described below. Typically, adipocytes were used between 14 and 21 days post-confluence, and fresh medium was applied every 72 h. Greater than 95% of 3T3-L1 fibroblasts undergo adipogenesis using this regime.

Cell Fractionation—Subcellular fractionation of 3T3-L1 adipocytes using differential centrifugation is an established procedure that has been described in detail elsewhere (24, 25). This procedure generates four membrane-enriched fractions as follows: mitochondria/nuclei, plasma membranes (PM), and high and low density microsomes (HDM and LDM, respectively). The HDM is enriched in endoplasmic reticulum markers and fluid phase markers; the LDM contains Golgi markers, recycling endosomes, and the majority of the intracellular insulin-responsive GLUT4 storage compartment. To establish basal conditions, 3T3-L1 adipocytes were washed three times in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄) and then incubated in Dulbecco's modified Eagle's medium for 16 h. When necessary, insulin was added for the last 15 min at 4 μ g/ml final concentration.

Western and Far Western Blotting—For Western immunoblotting, membrane fractions (25 or 75 μ g) were separated by SDS-PAGE using 10% polyacrylamide resolving gels (minigel 7 \times 8 cm or large gel 13 \times 14 cm), and then electrophoretically transferred to pre-wet Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore), following the procedure of Towbin *et al.* (26). After overnight blocking in 5% skim milk powder/PBS (Blotto), at 4 °C, membranes were probed for 1 h at room temperature with primary antisera against Syntaxin-4 (1:500), the N terminus of Syndet (1:1000), full-length Syndet (1:2000), or SNAP-25 (1:200). Membranes were then washed 3 \times for 15 min in PBS, 0.1% Tween 20 and incubated for an additional 1 h with goat anti-rabbit secondary antibody, conjugated to horseradish peroxidase (1:10,000) in PBS, 0.1%

² Despite exhibiting greatest sequence similarity to the non-neuronal, SNAP-25-like protein, human SNAP-23 (29), it remains unclear whether Syndet and human SNAP-23 represent the product of the same or two closely related genes (see "Results").

Tween 20, and 20 $\mu\text{g/ml}$ bovine serum albumin. A second set of washes was performed and immunoreactive proteins identified by ECL.

Far Western immunoblotting was performed with the following modifications of the Western immunoblotting procedure described above. After electrophoretic transfer of proteins, membranes were blocked in 5% skim milk powder, 0.1% Tween 20, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5) for 1 h at 4 °C. They were then transferred into a fresh solution of the same buffer (typically 20 ml for large membranes) containing the appropriate GST fusion protein at a final concentration of 20 $\mu\text{g/ml}$ and incubated for 18 h at 4 °C with gentle mixing. Membranes were subsequently washed 3 \times for 15 min in blocking buffer, then incubated with anti-GST primary antibody (diluted 1:5000 with 20% Blotto), and treated identically thereafter as a typical Western blot.

In some cases membranes were stripped of bound antibodies and re-probed with additional antibodies as described. Following the manufacturer's instructions, membranes were immersed in stripping buffer (62.5 mM Tris-HCl (pH 6.7), 2% SDS, 100 mM β -mercaptoethanol), for 30 min at 50 °C, then washed extensively in PBS, 0.1% Tween 20 at 22 °C for 20 min (2 \times 400 ml). Membranes were subsequently re-blocked in Blotto and incubated with antibodies as described above.

Production of Recombinant Fusion Proteins—The glutathione *S*-transferase (GST) protein purification system (Amersham Pharmacia Biotech) was used to generate all recombinant proteins. Rat VAMP2₁₋₉₄/GST and Syntaxin-4₁₋₂₇₄/GST were supplied by Dr. Richard Scheller (Stanford University, CA), and mouse SNAP-25B/GST was supplied by Dr. Thomas C. Südhof. All three constructs have been described previously (18, 27). Syndet-GST was produced by inserting a *Bam*HI/*Sma*I polymerase chain reaction fragment, incorporating the entire Syndet coding sequence, into analogous sites of pGEX-4T-1. The sequence of the sense and antisense oligonucleotide primers used to generate the Syndet polymerase chain reaction fragment are as follows: 5' CGC GGA TCC CGA CTC ACC ATG GAT AAT CTG 3' and 5' GTT CCC GGG TTA ACT ATC AAT GAG TTT C 3', respectively.

When employed for far Western blotting, fusion proteins were dialyzed against PBS, after which sample volumes were reduced using Aquacide II (Calbiochem). When the purified cytoplasmic tails of VAMP2 and Syntaxin-4 were required, recombinant fusion proteins were prepared fresh but stored attached to glutathione-Sepharose beads at 4 °C. The cytoplasmic tails of VAMP2 and Syntaxin-4 were cleaved using 6 units/ml thrombin (Calbiochem, catalog number 605190) in 1–2 ml of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% β -mercaptoethanol, 2.5 mM CaCl₂. The cleaved proteins were separated from the glutathione-Sepharose beads by centrifugation and dialyzed against PBS. For surface plasmon resonance studies, full-length Syndet was cleaved from GST with thrombin as described above, dialyzed extensively against sodium acetate buffer (pH 4.0, 10 mM), over 8 h at 4 °C, and used immediately.

Partial Purification of p25 and p35—An ensemble of non-ionic detergents (including 1% Nonidet P-40, 1% Triton X-100, 60 mM β -octylglucoside (β -OG), 1% Tween 20, and 2% CHAPS), salt treatments, or various pH washes were initially tested to identify conditions that solubilized p25 and p35. 1% Triton X-100 and 60 mM β -OG detergent solubilization, followed by anion exchange chromatography, were eventually selected to purify p25 and p35, respectively, from plasma membranes isolated from 3T3-L1 adipocytes. Specifically, for p35 an aliquot of PM (2 mg) was resuspended in 2 ml of HES buffer (20 mM Hepes (pH 7.4), 1 mM EDTA, 250 mM sucrose) containing 1% Triton X-100 and incubated for 30 min on ice. Samples were centrifuged in a Beckman TLA100.3 rotor (135,000 \times g_{max} , 45 min, 4 °C) to remove insoluble material. The supernatant was chromatographed using a 7-ml Q-Sepharose anion exchange column (Amersham Pharmacia Biotech). The column was initially washed with 5 ml of Low Salt Buffer (20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% Triton X-100) and then 80 \times 250- μl fractions were collected over a 20-ml 0–1 M NaCl salt gradient in the presence of Triton X-100 (1%). p25 was purified in a similar manner with the following minor modifications. Prior to solubilization with detergent, plasma membranes were incubated in HES buffer containing 1 M NaCl at 4 °C for 30 min to remove peripheral membrane proteins. This procedure resulted in an immediate 3-fold enrichment of p25. Membranes were re-isolated by centrifugation using a Beckman TLA100.3 rotor (135,000 \times g_{max} , 45 min, 4 °C) and then resuspended in Low Salt Buffer containing 60 mM β -OG. Samples were incubated on ice for an additional 30 min, cleared of insoluble material by a second centrifugation step (as above), and frozen (–80 °C). This material was subsequently chromatographed using a 1-ml Mono Q HR5/5 (Amersham Pharmacia Biotech) FPLC column, connected to an LCC-501 Plus controlled FPLC system (Amersham Pharmacia Biotech). The sample was loaded onto the column in Low Salt Buffer containing 60 mM β -OG

and 40 \times 500- μl fractions were collected over a 0–1 M NaCl gradient in the presence of 60 mM β -OG.

Surface Plasmon Resonance—Surface plasmon resonance (SPR) studies were performed using the BIAcore System from Amersham Pharmacia Biosensor AB, Uppsala, Sweden. All coupling and operating reagents, except PBS, were prepared using proprietary chemicals. Full-length Syndet was immobilized on the surface of a carboxymethylated dextran CM5 chip via thiol disulfide exchange, according to the manufacturer's instructions. Briefly, an *N*-hydroxysuccinimide ester was introduced into the surface matrix of a CM5 chip by passing a solution (10 μl) of 50 mM *N*-hydroxysuccinimide and 200 mM *N*-ethyl-*N'*-(3-diethylaminopropyl) carbodiimide over the chip surface (a coupling flow rate of 5 $\mu\text{l}/\text{min}$ was employed at all times). A reactive, disulfide bond-containing group was then introduced by the nucleophilic displacement of *N*-hydroxysuccinimide by 2-(2-pyridinyldithio)ethaneamine. To accomplish this, 20 μl of 80 mM 2-(2-pyridinyldithio)ethaneamine in 0.1 M Borate Buffer (pH 8.5) was passed over the chip surface. Coupling of full-length Syndet was achieved by immediately passing Syndet (35 μl of a 190 $\mu\text{g/ml}$ solution in 10 mM sodium acetate (pH 4.0)) over the chip surface. The free thiol groups on Syndet underwent disulfide exchange with the thiopyridine ring of 2-(2-pyridinyldithio)ethaneamine. Unexchanged sites were then deactivated with 50 mM L-cysteine (20 μl) containing 1 M NaCl. The chip was subsequently washed extensively in Running Buffer (PBS/0.05% surfactant P20) and stored immersed in this buffer at 4 °C when not in use.

All binding experiments were performed at 25 °C in Running Buffer, at a flow rate of 1 $\mu\text{l}/\text{min}$. Binding of the cytoplasmic domains of Syntaxin-4 and/or VAMP2 to immobilized Syndet was measured as an increase in SPR and recorded in response units (RU). Samples were diluted in Running Buffer until binding values between 500 and 1000 RUs were recorded. Once sample injection was complete, the surface was regenerated by two 10- μl pulses of 0.5% SDS. Before loading the combination of Syntaxin-4 and VAMP2, both proteins were preincubated for 15 min at 4 °C to facilitate later formation of ternary SNARE complexes when passed over immobilized Syndet. To regenerate the chip surface after SNARE complex formation, SNARE complexes were allowed to diffuse off over a period of 48 h. Nonspecific binding of protein samples, to an unconjugated chip surface, was found to be irrelevant.

Microinjection of 3T3-L1 Adipocytes—Our procedure for microinjection of 3T3-L1 adipocytes, and quantitation of insulin stimulated GLUT4 translocation to the plasma membrane, by confocal microscopy, has been described in detail elsewhere (28). Briefly, purified IgG antibodies were microinjected using a Zeiss automated injection system coupled to an Eppendorf microinjector and GLUT4 translocation in response to insulin addition assessed using the plasma membrane lawn assay (4). For these experiments, antisera (200 μl), against full-length Syndet, or preimmune serum, was affinity purified over a 1-ml Protein-G Sepharose column. Purified IgG was used at a concentration of 1 mg/ml in a buffer consisting of 5 mM Na₃PO₄ (pH 7.2) and 100 mM KCl. At least 300 cells per condition were microinjected, and three conditions were tested with or without insulin stimulation. The tested conditions were mock-injected, anti-Syndet IgG-injected, and irrelevant IgG-injected.

Peptide Inhibition Studies—Syndet C-terminal peptide (NH₂)-TEK-ADTNKNRIDIANTRAKKLIDS-(OH) and a randomized control peptide (NH₂)-YNFTNKKISVQRLASYRRITSSK-(NH₂) were synthesized by Chiron Mimotopes (Melbourne, Australia) to >90% purity. For SPR studies, residual trifluoroacetic acid contamination was first removed from each peptide preparation by two cycles of lyophilization from 10 mM HCl and then a final cycle from H₂O. Both peptides were then used at a final working concentration of 100 μM . To determine the effect of both peptides on the insulin-stimulated translocation of GLUT1 and GLUT4 to the PM, 3T3-L1 adipocytes were first permeabilized with the bacterial toxin streptolysin O as described previously (4) but with the following modifications. Cells were incubated for 10 min in the presence of 0.5 $\mu\text{g/ml}$ streptolysin O dissolved in ice-cold Intracellular Buffer (ICB) (140 mM potassium glutamate, 20 mM Hepes (pH 7.2), 5 mM MgCl₂, 5 mM EGTA, 5 mM NaCl). They were then washed in ice-cold ICB (3 \times 2 ml) and subsequently incubated at 37 °C in the presence or absence of test peptide for an additional 10 min in modified ICB (ICB, 1 mg/ml bovine serum albumin, 1 mM dithiothreitol, an ATP-regeneration system (40 IU/ml creatine phosphokinase, 5 mM creatine phosphate, and 1 mM ATP)). Cells were quickly transferred into fresh modified ICB containing the test peptide +/- insulin (4 $\mu\text{g/ml}$ final) for 15 min at 37 °C. They were subsequently washed 3 times in ice-cold PBS and then processed for GLUT1 or GLUT4 translocation using the plasma membrane lawn assay followed by confocal image analysis as

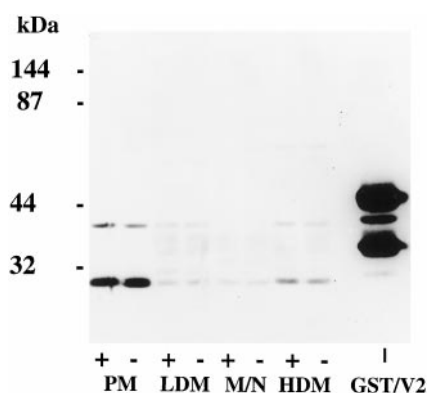


FIG. 1. Two VAMP2 binding proteins are detectable in the plasma membrane of 3T3-L1 adipocytes by far Western blotting. 3T3-L1 adipocyte membrane fractions were prepared from insulin- (+) and non-insulin (-)-stimulated cells. 80 μ g of each fraction was subjected to SDS-PAGE using a 10% resolving gel and transferred to PVDF membranes. Membranes were then incubated with VAMP2 _{$\alpha\alpha$ 1-94}/GST, and labeled bands were identified by immunoblotting with an anti-GST primary antibody. Samples correspond to plasma membranes (PM), low density microsomes (LDM), mitochondria/nuclei (M/N), high density microsomes (HDM) and recombinant VAMP2 _{$\alpha\alpha$ 1-94}/GST (5 μ g, GST/V2). The relative positions of molecular mass markers (kDa) are displayed on the left of the figure. Two major breakdown products are present in the VAMP2 _{$\alpha\alpha$ 1-94}/GST control lane.

described previously (4). Multiple random images of each condition, performed in duplicate, were captured and quantitated using NIH Image Analysis software.

Statistical Analyses—To assess the degree of GLUT1 or GLUT4 translocation under each condition tested, basal GLUT1 and GLUT4 levels, respectively, were first subtracted and the resulting value expressed as a relative percentage of the control insulin response. Three (GLUT1) or four (GLUT4) independently obtained sets of data were then averaged for each condition and are presented as the mean \pm S.E. The significance of each treatment was determined using analysis of variance with a Turkey-Kramer multiple comparison *post hoc* test of significance.

RESULTS

Far Western Blotting Identifies two VAMP2-binding Proteins in 3T3-L1 Adipocyte Plasma Membrane—To identify potential VAMP2 binding proteins in 3T3-L1 adipocytes, we employed far Western blotting. A GST fusion protein encoding the cytoplasmic domain of VAMP2 (VAMP2 _{$\alpha\alpha$ 1-94}/GST) was used to probe various 3T3-L1 adipocyte subcellular fractions. Two proteins of average molecular mass of 25 and 35 kDa were specifically labeled with the VAMP2 probe (Fig. 1). These proteins were highly enriched in the plasma membrane fraction with lower amounts detected in other fractions. There was no effect of insulin on the distribution of either p25 or p35. These proteins were not labeled using GST alone (data not shown). Based on their migration in SDS-PAGE and their enrichment in the plasma membrane fraction, we surmised that p25 and p35 may be homologues of SNAP-25 and Syntaxin, respectively.

p35 Identified as Syntaxin-4—To elucidate the identity of p25 and p35, we adopted two strategies. p35 was predicted to be a Syntaxin homologue based on its molecular mass and its specific interaction with VAMP2 (Fig. 1). It has previously been shown, using *in vitro* binding studies with recombinant fusion proteins, that VAMP2 binds to Syntaxin-1 and Syntaxin-4 but not to Syntaxin-2 and -3 (27). Also, we have previously shown from a cDNA library screen and subsequent immunolocalization studies that Syntaxin-4, but not Syntaxin-1, is expressed in high abundance in the plasma membrane of 3T3-L1 adipocytes (21). To determine if p35 and Syntaxin-4 were the same protein, the filter used for far Western blotting (Fig. 1) was stripped and re-probed with an antibody specific for Syntaxin-4. The Syntaxin-4 antibody immunolabeled a band of

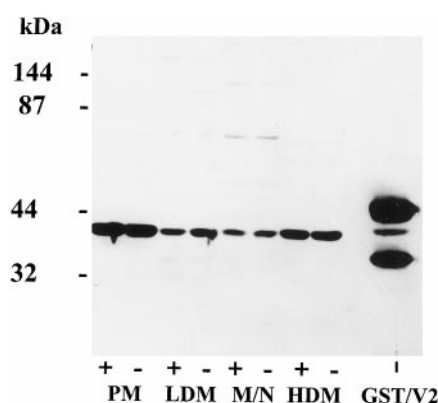
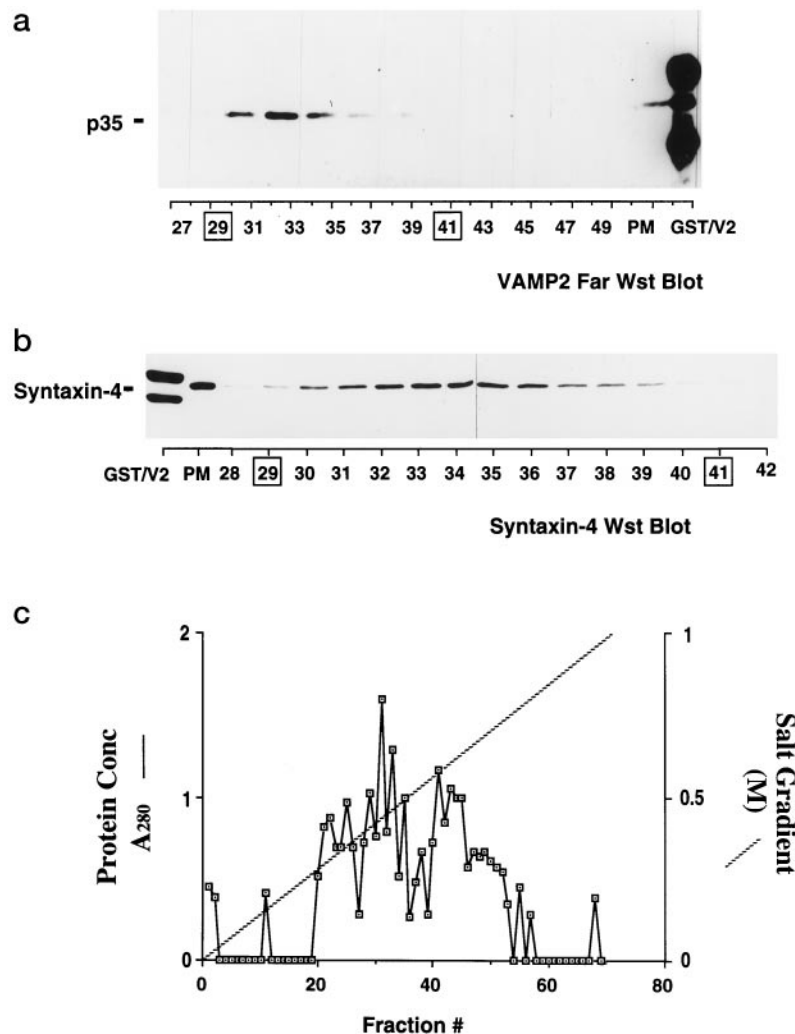


FIG. 2. Syntaxin-4 and p35 have identical subcellular distributions in 3T3-L1 adipocytes. The PVDF membrane used in Fig. 1 was stripped and re-probed with an antibody specific for Syntaxin-4. The recombinant VAMP2 _{$\alpha\alpha$ 1-94}/GST fusion protein, which was loaded onto the gel as a control (GST/V2), is labeled by this antibody because it was raised against a GST-Syntaxin-4 recombinant fusion protein (21) and hence contains anti-GST antibodies. M/N, mitochondria/nuclei.

identical molecular mass and subcellular distribution to p35 (Fig. 2). To confirm further the identity of p35 as Syntaxin-4, plasma membranes were harvested from adipocytes, detergent-solubilized, and then subjected to ion exchange chromatography. p35 was resolved into a single peak (fractions 30–39), and this peak corresponded exactly with the peak of immunoreactive Syntaxin-4 (Fig. 3). A comparison of the p35 signal intensity to total protein ratio, between fraction 34 and crude adipocyte homogenate, revealed that p35 had been purified over 1000-fold (data not shown). Such an enrichment underscores the utility of this procedure for comparing p35 distribution with Syntaxin-4 immunoreactivity. Hence, based on the observations that Syntaxin-4 and p35 have identical subcellular distributions, analogous mobilities in SDS-PAGE and indistinguishable chromatographic separations by ion exchange, coupled with the fact that Syntaxin-4 binds VAMP2 *in vitro* and VAMP2-GST bound p35, we conclude that p35 and Syntaxin-4 are the same protein.

p25 Identified as the SNAP-25 Homologue Syndet—It has previously been shown that Syntaxin binds to both its cognate v-SNARE in the transport vesicle (see above) and to another t-SNARE in the corresponding target membrane. In the case of neurons the latter is a protein known as SNAP-25 (13). Based on the above studies implicating Syntaxin-4 as one of the VAMP2-binding proteins (p35) in adipocytes (Figs. 1 and 2), we reasoned that if p25 is also a part of this complex then this species should also interact with Syntaxin-4. To confirm this, a recombinant fusion protein comprising the cytoplasmic tail of Syntaxin-4 fused to GST specifically labeled a protein of molecular mass of 25 kDa in adipocytes that had a similar subcellular distribution to p25. Most notably, this protein was highly enriched in the plasma membrane fraction (Fig. 4a). It is also noteworthy that GST-Syntaxin-4 did not label the p35 species. Based on the neuronal model (13), we initially surmised p25 was SNAP-25. We were, however, unable to detect SNAP-25 in adipocyte plasma membranes using an antibody specific for the C terminus of neuronal SNAP-25 (Fig. 5a). Furthermore, this antibody did not cross-react with the highly purified adipocyte p25 protein (#17, Fig. 5a) suggesting that these are distinct molecular entities. Thus, these data suggested that p25 was a novel SNAP-25-like protein. A SNAP-25 homologue called Syndet was recently described in 3T3-L1 adipocytes (20). This protein was highly enriched in the plasma membrane of these cells (20) (Fig. 5b). A related protein referred to as SNAP-23 has also been characterized in human

FIG. 3. p35 and Syntaxin-4 resolve as indistinguishable peaks by anion exchange chromatography. Plasma membranes were isolated from 3T3-L1 adipocytes, solubilized with 1% Triton X-100, and centrifuged to remove insoluble material. This procedure resulted in an immediate 10-fold enrichment of p35 (data not shown). The Triton X-100 supernatant was chromatographed using a 7-ml Q-Sepharose anion exchange column and 80 × 250- μ l fractions collected along a 20-ml 0–1 M NaCl gradient. Indicated gradient fractions, were used for far Western blotting using a VAMP2 _{α 1–94}/GST probe (a), immunoblotting with an anti-Syntaxin-4 antibody (b), and protein estimation (c). As in Fig. 2, the recombinant VAMP2 _{α 1–94}/GST fusion protein (GST/V2) serves as a control for blotting and molecular mass. *Wst blot*, Western blot.



lymphocytes (29). This protein exhibits 86% identity with Syndet at the amino acid level but may simply represent a species-specific homologue. Syndet has been shown to bind Syntaxin-4 using the yeast two-hybrid system (30), making it a good p25 candidate.

To confirm the identity of p25 as Syndet, we performed similar studies to those described above for the p35 protein. Immunoblotting with a Syndet-specific antibody confirmed that Syndet has an identical molecular mass and subcellular distribution to p25 (Fig. 4). We then employed anion exchange chromatography to independently confirm the equivalence of both proteins. In this instance, peripheral membrane proteins were first stripped from 3T3-L1 adipocyte plasma membranes using high ionic strength. Salt-stripped membranes were then solubilized with β -OG and subjected to ion exchange chromatography using a Mono Q column. Interestingly, this protocol resolved two peaks of p25 (as determined by far Western blotting with the cytoplasmic tail of Syntaxin-4), both of which coincided identically with immunoreactive Syndet (Fig. 6, a and b).

To understand further the nature of the two p25/Syndet peaks, we stripped and reprobbed the Syndet filter with antisera specific for Syntaxin-4. Interestingly, Syntaxin-4 co-chromatographed identically with the second Syndet peak but was undetectable in the first (Fig. 6c). This suggests that the second peak may represent a Syndet/Syntaxin-4 heterodimer, and the first represents monomeric Syndet. In summary then, based on the observations that p25 and Syndet have identical subcellu-

lar distributions as well as analogous mobilities in SDS-PAGE, coupled with their identical resolution using anion exchange chromatography, we conclude that p25 is Syndet.

VAMP2, Syntaxin-4, and Syndet Form an SDS-resistant SNARE Complex in Vitro—Syntaxin-1A, VAMP2, and SNAP-25 form a stable SDS-resistant ternary complex *in vivo* (31). It is considered that the formation of this high affinity intermediate serves as a fundamental step in facilitating the specificity with which a particular vesicle docks with its appropriate target membrane. Thus, in order to determine if VAMP2, Syntaxin-4, and Syndet might fulfil a similar function in enabling GLUT4 vesicles to dock and fuse with the plasma membrane of adipocytes, we attempted to reconstitute the formation of this ternary complex using surface plasmon resonance. By immobilizing Syndet in a conformation thought to mimic its proposed orientation with respect to the lipid bilayer *in vivo*, we were able to show that Syndet binds both Syntaxin-4 and VAMP2 with high individual affinity (Fig. 7). Both interactions were completely disrupted with 0.5% SDS. However, co-incubation of all three proteins simultaneously on the sensor chip resulted in the formation of a ternary complex that was resistant to disruption by 0.5% SDS at 25 °C (Fig. 7), as well as other denaturants including 8 M urea, 0.1 M phosphoric acid, and 6 M guanidinium HCl (data not shown). During the course of our studies, we found that preincubation of VAMP2 and Syntaxin-4, prior to addition to the chip surface, substantially increased the number of ternary SDS-resistant complexes formed. Such a treatment presumably increases the

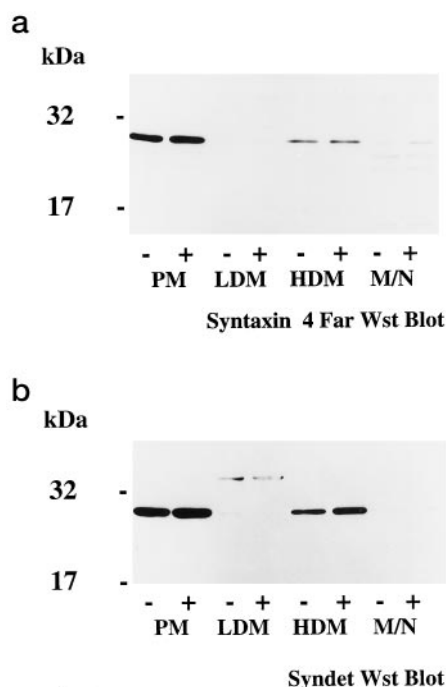


FIG. 4. Syndet and p25 have an identical subcellular distribution in 3T3-L1 adipocytes. Plasma membranes (*PM*), low density microsomes (*LDM*), high density microsomes (*HDM*), and mitochondria/nuclei (*M/N*) were obtained from adipocytes incubated in the absence (–) or presence (+) of insulin, subjected to SDS-PAGE, and transferred to a sheet of PVDF membrane. This membrane was then incubated with recombinant Syntaxin-4_{αα 5–274}/GST and one high intensity band was detected (*a*). This band was absent from a control experiment using GST alone (data not shown). The membrane used in *a* was subsequently stripped and reprobed with an antibody specific for the N terminus of Syndet (*b*). The relative positions of molecular mass markers are shown at the left (kDa). *Wst blot*, Western blot.

number of Syntaxin-4/VAMP2 heterodimers that are available to compete with the free monomers for Syndet-binding sites. It also suggests that the heterodimeric species is important for SDS-resistant ternary complex formation. Based on the above data, we propose that the adipocyte SNAREs Syndet, Syntaxin-4, and VAMP2 form a stable complex *in vivo* that constitutes a functional docking intermediate facilitating the specific targeting of GLUT4 vesicles to the cell surface in response to insulin.

Functional Role of Syndet in GLUT4 Trafficking—To determine if Syndet plays a role in the insulin-dependent trafficking of GLUT4 to the plasma membrane in 3T3-L1 adipocytes, we adopted two strategies. First, 3T3-L1 adipocytes were microinjected with affinity purified Syndet-specific antibodies to determine the effect on insulin-stimulated GLUT4 translocation. Plasma membrane GLUT4 labeling in microinjected cells was determined using the plasma membrane lawn assay. As shown in Fig. 8*a*), this antibody inhibited insulin-stimulated GLUT4 translocation to the plasma membrane by ~40%. In contrast, microinjection of IgG, purified from preimmune serum, had no significant effect. The second approach was based on recent studies by Gutierrez *et al.* (32) who showed that digitonin-mediated delivery of a peptide encoding the last 20 amino acids of SNAP-25 into chromaffin cells inhibited the calcium-stimulated, slow ATP-dependent component of chromaffin granule exocytosis by ~60–80%. A synthetic peptide comprising an identical domain in Syndet was produced and incubated with streptolysin O-permeabilized 3T3-L1 adipocytes. In permeabilized cells incubated with insulin alone, we observed an 11-fold increase in GLUT4 labeling at the cell surface compared with non-stimulated cells (data not shown). The Syndet C-terminal

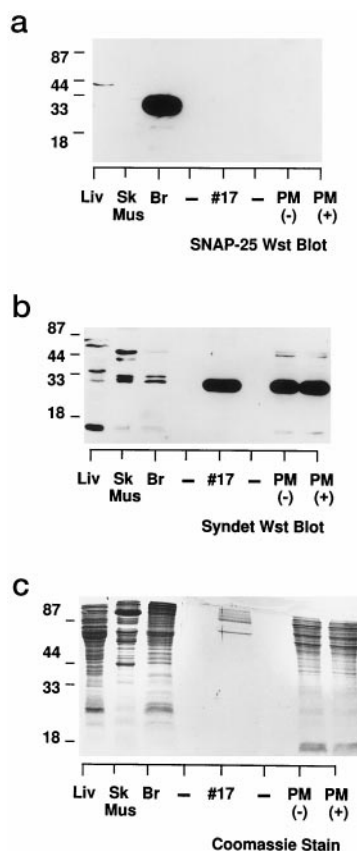


FIG. 5. Distribution of SNAP-25 and Syndet among different tissue and membrane fractions. Aliquots of rat liver membranes (*Liv*), rat skeletal muscle membranes (*Sk Mus*), whole rat brain membranes (*Br*), fraction 17 (#17) from the anion exchange column (see Fig. 6), and 3T3-L1 adipocyte plasma membranes isolated from cells incubated in the absence or presence of insulin (*PM*(–/+)) were immunoblotted with antibodies specific for the C terminus of mouse SNAP-25 (*a*), or the N terminus of the mouse SNAP-25 homologue, Syndet (*b*). A Coomassie stain of the same fractions is presented in *c* to illustrate the relative enrichment of Syndet obtained using anion exchange chromatography (compare #17 to *PM*). The relative positions of molecular mass markers (kDa) are illustrated on the left-hand side of the figure. *Wst blot*, Western blot.

peptide had no significant effect on cell-surface GLUT4 levels in the basal state. However, when introduced in the presence of insulin, this peptide blocked insulin-stimulated GLUT4 translocation by ~35% in a dose-dependent manner. Concentrations higher than 100 μM were not tested, but it is apparent from Fig. 8*b*) that inhibition was approaching an apogee. Inhibition was specific because a scrambled peptide did not impair GLUT4 translocation. The inhibition observed in these experiments was comparable to that observed following microinjection of the anti-Syndet antisera and corresponded well with the inhibitory concentrations required for the SNAP-25 C-terminal 20-mer to inhibit chromaffin granule exocytosis (32).

Insulin-stimulated GLUT1 Trafficking Remains Unaltered in the Presence of the Syndet Peptide—The continual flux of GLUT4 between its storage compartment, the plasma membrane, and the endosomal system under fasting conditions implies that a proportion of GLUT4 is always present in the constitutively recycling endosomal compartment. As much as 40% of GLUT4 has been estimated to exist in this compartment (17). Insulin has been shown to stimulate the movement of constitutively recycling endosomal proteins, such as the transferrin receptor and GLUT1, to the plasma membrane by a factor of approximately 2–3 (33). To determine whether Syndet performs a direct and specific role in the insulin-dependent

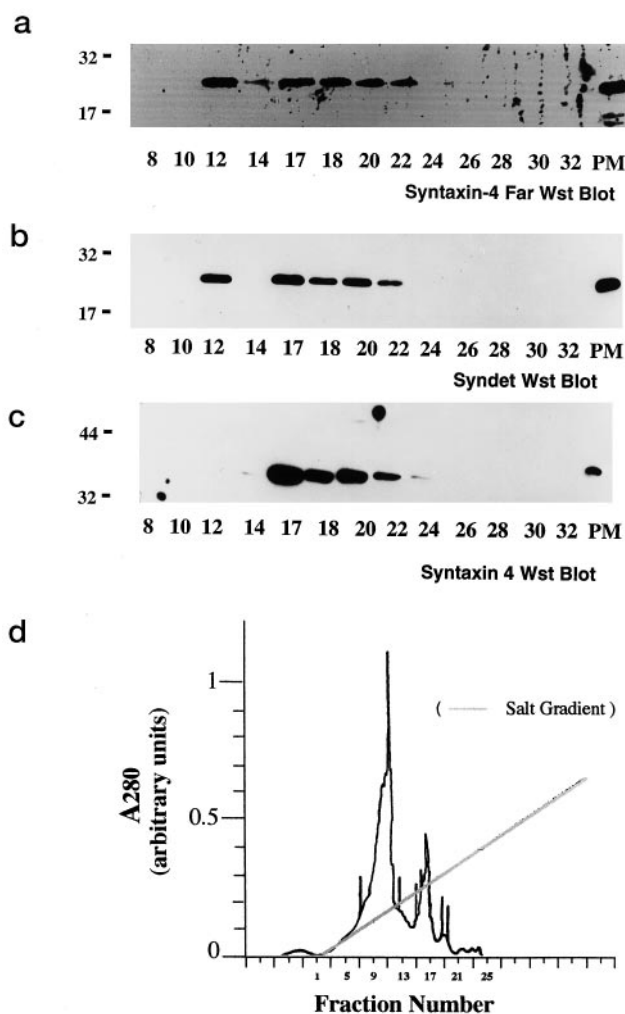


FIG. 6. p25 and Syndet exhibit identical anion exchange elution profiles. Plasma membranes were isolated from 3T3-L1 adipocytes, stripped of peripheral membrane protein, and then solubilized with 60 mM β -OG and centrifuged to remove insoluble matter. The β -OG supernatant was chromatographed using a 1-ml Mono Q anion exchange column and 40×500 - μ l fractions collected along a 20-ml 0–1 M NaCl gradient. Every second fraction was used for far Western blotting (*Far Wst Blot*) using a Syntaxin-4_{αα1–274}/GST probe (a), immunoblotting using an N-terminal specific, anti-Syndet antibody (b), immunoblotting using an anti-Syntaxin-4 antibody (c), and protein estimation (d). b, low levels of Syndet were detectable in lane 14 upon longer exposure (data not shown). For c, the PVDF membrane used to generate b was stripped and reprobed. The relative positions of molecular mass markers are shown on the left (kDa).

trafficking of GLUT4 to the plasma membrane or, rather, performs a more perfunctory role in constitutive trafficking, we incubated streptolysin O-permeabilized 3T3-L1 adipocytes with the Syndet C-terminal peptide and measured GLUT1 translocation in the presence or absence of insulin using the plasma membrane lawn assay. At 100 μ M final concentration, where inhibition of insulin-induced GLUT4 translocation was maximal, we were unable to discern any significant effect on GLUT1 trafficking both in the basal (not shown) or insulin-stimulated states (Fig. 8c). As expected, the scrambled control peptide was without effect. These data suggest that a substantial proportion of GLUT4 trafficking to the PM under the action of insulin probably does so independently of the constitutively recycling machinery. In addition, these data lend further support to the notion that GLUT4 resides, and responds to insulin, in discrete GSVs.

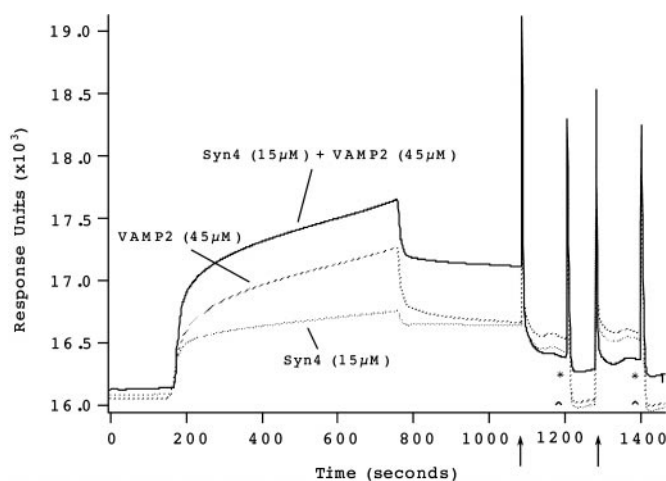


FIG. 7. VAMP2, Syntaxin-4, and Syndet form an SDS-resistant SNARE complex *in vitro*. Surface plasmon resonance was used to study the interactions of recombinant VAMP2, Syntaxin-4, and Syndet. Full-length Syndet_{αα1–210} was immobilized onto the surface of a carboxymethyl dextran chip via disulfide bridging with Syndet's five internal cysteines. Syntaxin-4, VAMP2, or a combination of both proteins were passed over the surface of the chip at the concentrations shown, and binding was measured as an alteration of surface plasmon resonance (recorded as *Response Units*). The effects of 0.5% SDS on these interactions were studied by pulsing the chip twice with SDS after the proteins had bound. The arrows denote the start of each pulse. Syntaxin-4 and VAMP2, when added on their own, were completely dissociated from the sensor chip after incubation with SDS, as indicated by the base-line plateau achieved and marked by the chevron () shown on the right of the trace. However, when Syntaxin-4 and VAMP2 were incubated on the chip together, an SDS-resistant complex was evident (denoted by the asterisk) because it was not possible to achieve the original base line observed prior to binding.

DISCUSSION

Substantial evidence suggests that VAMP2 acts as a v-SNARE in the insulin-induced translocation of GLUT4 to the plasma membrane of adipocytes. Morphologically, there is a high degree of co-localization between VAMP2 and GLUT4 in an intracellular compartment distinct from recycling endosomes which has been referred to as GSVs (17, 34). Tetanus toxin, botulinum neurotoxin, and IgA protease (35, 36), each of which cleaves VAMP2, inhibit insulin-stimulated GLUT4 translocation in adipocytes. Microinjection of synthetic peptides comprising the unique N terminus of VAMP2 also have a specific effect on GLUT4 trafficking in adipocytes (19). Furthermore, overexpression, microinjection, or permeabilized entry of recombinant proteins comprising the VAMP2 cytoplasmic tail inhibit insulin-stimulated GLUT4 translocation by as much as 50% (28, 35, 37). In the present study we hypothesized that if GSVs fuse directly with the cell surface in response to insulin then, as predicted from the SNARE hypothesis, the adipocyte plasma membrane should be enriched in VAMP-2-specific t-SNAREs. Furthermore, these adipocyte t-SNAREs should bear some resemblance to the t-SNAREs that regulate docking of synaptic vesicles in the neuron because this process is also mediated by VAMP2.

To identify such proteins we have employed far Western blotting using the cytoplasmic domain of VAMP2 and isolated two proteins of relative molecular mass 25 and 35 kDa. By using a series of sequential purification techniques, we have enriched for both of these proteins more than 1000-fold and showed that they are indistinguishable from immunoreactive Syndet and Syntaxin-4, respectively. In the case of Syndet we present functional data to support a role for this protein in the insulin-regulated trafficking of GLUT4. Introduction of either antibodies or a peptide to the C terminus had a significant inhibitory effect. In both cases we observed a 35–40% inhibi-

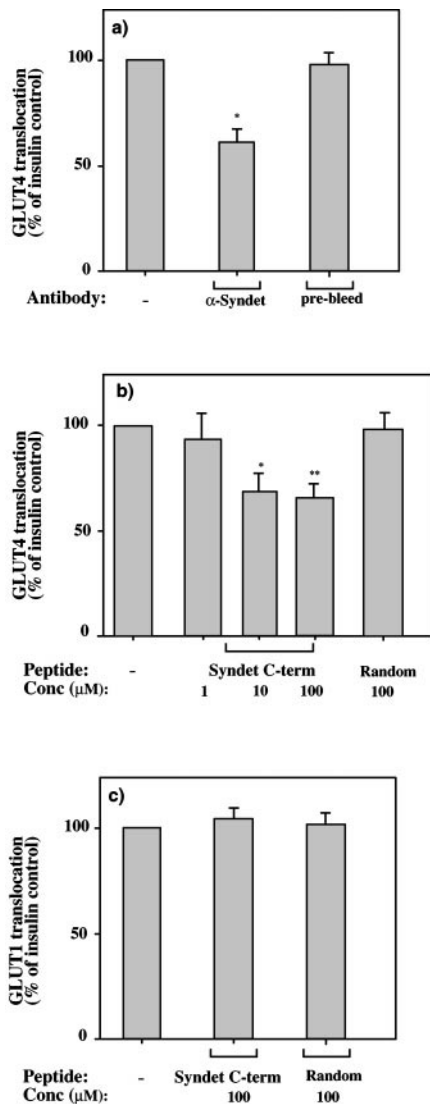


FIG. 8. Syndet-directed antibodies, and a C-terminal Syndet peptide, inhibit insulin-induced GLUT4 translocation in 3T3-L1 adipocytes; GLUT1 trafficking remains unaffected. Cells were grown on glass coverslips and allowed to reach basal conditions. *a*, antibodies generated against full-length Syndet, or preimmune control antibodies, were microinjected into 3T3-L1 adipocytes. Cells were then incubated in the absence or presence of insulin, and cell-surface GLUT4 levels were measured using the PM lawn assay (4). *b* and *c*, 3T3-L1 adipocytes were permeabilized with streptolysin (4) as described and then incubated with a peptide comprising either the last 24 amino acids of Syndet (1, 10, and 100 μM final concentration), a randomized control peptide (100 μM), or with no additions (-) for 10 min. Cells were then incubated in the additional presence or absence of insulin for a further 15 min, and cell surface GLUT4 (*b*) or GLUT1 (*c*) levels were measured using the PM lawn assay. Individual conditions within each experimental set were expressed as a relative percentage of the insulin control (set to 100%) following subtraction of either basal GLUT4 or GLUT1 levels, respectively. All data represent the mean \pm S.E. of a minimum of three separate experiments. Data were significantly different from insulin control values at the level of * $p < 0.05$; ** $p < 0.001$.

tion of GLUT4 translocation (Fig. 8, *a* and *b*). In similar studies, we have observed a comparable degree of inhibition using either the cytoplasmic tail of VAMP2 or a VAMP2-specific peptide (19). Syndet appears to perform a very specific role in the insulin-dependent trafficking of GLUT4 to the cell surface, since even under conditions that maximally inhibited GLUT4 translocation, no inhibition of either the basal or insulin-stimulated trafficking of the constitutively recycling glucose transporter, GLUT1, could be detected (Fig. 8*c*). Furthermore, no

effect on the basal trafficking of GLUT4 could be discerned. Studies similar to those we have presented here also implicate a role for Syntaxin-4 in the insulin-induced movement of GLUT4 to the cell surface of adipocytes (28, 35, 37).

It has previously been shown that SNAREs play an important role in intracellular vesicular transport reactions at least in part by forming stable ternary complexes. Such complexes are resistant to denaturation by SDS and can be formed even under *in vitro* conditions using recombinant proteins (31, 38). We sought to determine if Syndet, Syntaxin-4, and VAMP2 could also form such complexes. To achieve this aim we employed SPR. Interestingly, using this technique we found that we were only able to establish such an interaction by immobilizing Syndet onto the chip surface. Our immobilization strategy took advantage of the five cysteines present in the mid-portion of the molecule. We surmise that our ability to form SNARE complexes using this strategy probably reflects the fact that under these conditions Syndet is being preserved in a topology on the chip surface that is close to that normally adopted under its native conditions. This conclusion is supported by studies on the related t-SNARE SNAP25, which appears to be palmitoylated midway between the SNARE binding domains located at its N and C termini (39). Based on sequence comparisons, similar SNARE binding domains are present in Syndet (20, 40). The reconstitution of a ternary SNARE complex on the SPR sensor chip will facilitate the identification of proteins that bind SNAREs at different stages of the assembly process and possibly aid in the elucidation of how these interactions might be modulated by phosphorylation, GTPases, and other factors. In order to implicate a role for Syndet, Syntaxin-4, and VAMP2 in GLUT4 trafficking, it will be necessary to demonstrate that they form a ternary complex *in vivo*, and this is a major direction of our current work.

One of the curious findings in the present study was that in our functional analyses of Syndet in GLUT4 trafficking, we observed at most a 40% inhibition of insulin-stimulated GLUT4 translocation. It is of interest that this is in line with the effects of reagents that disrupt the function of either VAMP2 or Syntaxin-4 on GLUT4 trafficking (19, 28). On the one hand this may simply reflect the experimental limitations of our approach where peptides or recombinant fusion proteins have been incubated with permeabilized cells. It is more likely, however, that this lack of complete inhibition reflects the function of Syndet in adipocytes. One interpretation is that only 40% of intracellular GLUT4 is susceptible to the actions of the Syndet peptide. A similar explanation has been advanced in studies on SNAP25 in neuroendocrine cells where it was shown that a peptide, analogous to that used in this study, specifically inhibited the fusion of only undocked and not pre-docked vesicles, the former constituted only 30% of the total releasable pool (32). Such an explanation would appear to be unlikely for adipocytes, however, as there is no morphological evidence for pre-docked GLUT4-containing vesicles (7). An alternative possibility therefore, as indicated by recent studies using a horseradish peroxidase conjugate of transferrin (17, 19), is that GLUT4 exists within at least two intracellular compartments, one is endosomal and thus co-localizes with transferrin-conjugated horseradish peroxidase, and the other is a post-endocytic compartment that is inaccessible to transferrin-conjugated horseradish peroxidase. Up to 40% of intracellular GLUT4 has been calculated to be present in the former compartment. We suggest that it is only the latter compartment that is regulated by Syndet, and in turn sensitive to the presence of our Syndet blocking reagents. Consistent with this suggestion is the observation that GLUT1, a molecule believed to traffic via endosomes, is completely unaffected in our GLUT4 trafficking as-

says. It is worth mentioning that there is no direct evidence that GLUT4 in this post-endocytic compartment traffics directly to the cell surface rather than merging with endosomes en route. If the latter is true, a role for Syndet at an intracellular location needs to be considered as an additional possibility. We cannot exclude this possibility, particularly since we do observe some Syndet and Syntaxin-4 in the HDM fraction and, although contaminated to some extent with plasma membranes, this fraction also contains fluid phase endosomal markers. Against such a proposition, however, is the observation that the majority of Syndet and Syntaxin-4 is found at the cell surface. Hence, it is quite conceivable that the Syndet peptide and antibody used in this study have quite a significant effect on the ability of vesicles derived from the post endocytic compartment to dock and fuse with the plasma membrane.

Whereas we have proven a role for Syndet in GLUT4 trafficking, the function of this protein in this process remains unknown. In an attempt to determine if the Syndet C-terminal peptide was interfering with the formation of a functional Syntaxin-4, Syndet, and VAMP2 SNARE complex, we assessed the effect of this peptide on the formation of this complex *in vitro* using our SPR assay. Paradoxically, we were unable to detect any measurable effect using this strategy (data not shown). Suffice to say, this observation does not preclude the possibility of an effect of this peptide on SNARE assembly *in vivo*. It is conceivable that in certain micro-environments the Syndet peptide might undergo a major conformational rearrangement. It is believed, for example, that during vesicle fusion the activation of lipid kinases might alter the microacidity surrounding the fusion site. Limitations of the SPR carboxy dextran chip surface at low pH precluded our ability to test this hypothesis. More likely, however, are the possibilities that the Syndet peptide functions to inhibit a step just after individual SNARE complexes have formed. If the C terminus of Syndet, for example, functions as an oligomerization domain, but only after ternary SNARE complexes have formed, then the effect of the Syndet peptide would have gone unrecognized on the SPR chip surface. Higher order SDS-resistant ternary SNARE complexes have already been described *in vitro* (31). Interestingly, several groups have also implicated a role for the C terminus of SNAP-25 in a post-docking, fusion-catalyzing reaction (41–43). Some evidence exists to suggest the last 20 amino acids of this protein may play a role in Rab binding (44) and/or in activation of lipid-modifying reactions (45). The similarity between the multimer forming fusion peptides of viral fusogenic proteins and the C termini of SNAP-25-like proteins is also very interesting (46).

In conclusion, we have provided direct evidence that Syndet represents a major t-SNARE in 3T3-L1 adipocytes that mediates GLUT4 mobilization to the plasma membrane in response to insulin. Furthermore, the demonstration that Syndet along with Syntaxin-4 can form a stable SDS-resistant ternary complex with VAMP2, a major v-SNARE expressed in adipocytes, provides support not only for the GSV model of GLUT4 trafficking in response to insulin but also for the generality of the SNARE mechanism in transport vesicle reactions. It remains to be seen if these molecules are targets of insulin action. It is easily envisaged that the precise interaction of Syndet, Syntaxin-4, and VAMP2 could stand as one of the last steps in preventing or allowing GLUT4 to be integrated into the cell surface.

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