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Syntaxin 4 in 3T3-L1 Adipocytes: Regulation by Insulin and Participation in Insulin-dependent Glucose Transport

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Syntaxins are thought to be membrane receptors that bind proteins of the synaptobrevin/vesicle-associated membrane protein (VAMP) family found on transport vesicles. Recently, we detected synaptobrevin II and cellubrevin on immunopurified vesicles containing the glucose transporter 4 (GLUT4) in insulin-responsive cells. In an effort to identify the plasma membrane receptors for these vesicles, we now examine the expression of syntaxins in the 3T3-L1 adipocyte cell line. Neither syntaxin 1A nor 1B was found, in keeping with the neuronal restriction of these isoforms. In contrast, syntaxins 2 and 4 were readily detectable. By subcellular fractionation and estimation of protein yields, 67% of syntaxin 4 was localized to the plasma membrane, 24% to the low-density microsomes, and 9% to the high-density microsomes. Interestingly, acute insulin treatment decreased the content of syntaxin 4 in low-density microsomes and caused a corresponding gain in the plasma membrane fraction, reminiscent of the recruitment of GLUT4 glucose transporters. In contrast, there was no change in the distribution of syntaxin 2, which was mostly associated in the plasma membrane. A fraction of the intracellular syntaxin 4 was recovered with immunopurified GLUT4-containing vesicles. Moreover, anti-syntaxin 4 antibodies introduced into permeabilized 3T3-L1 adipocytes significantly reduced the insulin-dependent stimulation of glucose transport, in contrast to the introduction of irrelevant immunoglobulin G, which was without consequence. We propose that either the plasma membrane and/or the vesicular syntaxin 4 are involved in docking and/or fusion of GLUT4 vesicles at the cell surface of 3T3-L1 adipocytes.

INTRODUCTION

Molecules presumed to be involved in docking and fusion of synaptic vesicles with the plasma membrane of presynaptic nerve terminals have been identified recently. These are the vesicular synaptobrevin II/*v*esicle *a*ssociated *m*embrane *p*rotein-2 (VAMP-2)[1] and the plasma membrane syntaxin 1 and synaptosome-associated protein 25 (SNAP-25), which together with the soluble proteins *N*-ethylmaleimide *s*ensitive *f*actor (NSF) and *s*oluble *N*SF-*a*ttachment *p*roteins (SNAPs) constitute a complex of 20S (Sollner *et al.*, 1993a,b; Sudhof *et al.*, 1993; Rothman, 1994). The membrane-bound proteins that bind the soluble components are collectively called *SNAP re*ceptors (SNAREs). A non-neuronal synaptobrevin, cellubrevin, has been found in a variety of cells (McMahon *et al.*, 1993), including muscle fibers (Volchuk *et al.*, 1994), adipocytes (Vol-

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[1] Abbreviations used: GLUT4, glucose transporter 4; HDM, high-density microsomes; LDM, low-density microsomes; NSF, *N*-ethylmaleimide-sensitive factor; PM, plasma membranes; SLO, streptolysin O; SNAP, soluble NSF attachment proteins; SNARE, SNAP receptors; VAMP, vesicle-associated membrane protein.

chuk *et al.*, 1995), and CHO fibroblasts in which it is involved in endosomal recycling (Galli *et al.*, 1994). In 3T3-L1 adipocytes, both synaptobrevin II and cellubrevin are associated with intracellular vesicles enriched in glucose transporter 4 (GLUT4) (Volchuk *et al.*, 1995). These vesicles are summoned to the plasma membrane in response to insulin (Birnbaum, 1989; James *et al.*, 1989; Zorzano *et al.*, 1989; Satoh *et al.*, 1993), increasing the cell-surface content of GLUT4, synaptobrevin II, and cellubrevin (Cain *et al.*, 1992; Volchuk *et al.*, 1995). The process, which is beginning to be understood, has properties of both the constitutive and the regulated pathways (James *et al.*, 1994) and is responsible for the increase in glucose uptake caused by the hormone.

In contrast to the wide distribution of synaptobrevins in non-neuronal cells, the mammalian syntaxin 1 (A or B forms) has been detected only in brain (Bennett *et al.*, 1992) and neurosecretory tissues (Jacobsson *et al.*, 1994; Gutierrez *et al.*, 1995). However, it is conceivable that non-neuronal homologues of syntaxin 1 participate in intracellular traffic in mammalian cells, in particular in the docking/fusion of GLUT4 glucose transporter-containing vesicles of insulin-sensitive cells. Additional members of the syntaxin family were recently detected in rat cDNA libraries and shown to code for related gene products denominated syntaxins 2, 3, 4, and 5 (Bennett *et al.*, 1993). We have recently detected the expression and distribution of syntaxin 4 in skeletal muscle (Sumitani *et al.*, 1995) and human neutrophils (Brumell *et al.*, 1995), but the function of this protein has not been explored. Because synaptobrevin II and cellubrevin were found in 3T3-L1 adipocytes, we surmised that these cells may have a cognate syntaxin. Here we report the expression of syntaxin 4 in 3T3-L1 adipocytes, its subcellular distribution and response to insulin, and its possible participation in insulin-stimulated glucose transport.

MATERIALS AND METHODS

Reagents

An anti-syntaxin 4 polyclonal antiserum (designated αSy4) was generated from a rat syntaxin 4 cDNA (822 base pairs [bp] corresponding to amino acids 1–274, which include the cytoplasmic domain). The cDNA was kindly provided by Dr. R. Scheller (Howard Hughes Medical Institute, Stanford University Medical Center, Stanford, CA) and then subcloned into a pGEX2TK glutathione-S-transferase (GST) expression vector for transformation of DH5α-competent *Escherichia coli*. The recombinant GST-syntaxin 4 fusion protein was purified and inoculated into New Zealand white rabbits. The resulting immune serum was supplemented with protease inhibitors (1 mM phenanthroline, 1 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, and 5 mM EDTA) and affinity-purified by binding for 16 h at 4°C to GST-syntaxin 4 immobilized on a nitrocellulose filter presaturated with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The filter was washed in PBS, and the bound antibody was eluted with 0.2 M glycine-HCl pH 2.2 for 3 min followed by neutralization with 1 M Tris-base. The antibody did not recognize recombinant syntaxins 1A, 2, or 3 but reacted clearly with recombinant syntaxin 4. Recombinant syntaxins 1A, 2, and 3 were prepared as GST fusion proteins, as previously described (Calakos *et al.*, 1994). A rabbit polyclonal antibody directed against recombinant syntaxin 2 (amino acids 1–267) was produced by Babco (Richmond, CA). The anti-syntaxin 2 antiserum was affinity purified on a syntaxin 2 column (Affi-Gel; Bio-Rad, Hercules, CA) according to standard procedures. The following antibodies were used for immunoblotting: anti-GLUT4 antiserum (East Acres Biologicals, Southbridge, MA); anti-α1 Na^+/K^+-ATPase monoclonal antibody 6H (gift from Dr. M. Caplan, Yale University, New Haven, CT); anti-syntaxin 1A and 1B monoclonal antibodies SPM-1 (gift from Dr. T. Abe, Niigata University, Japan) and HPC-1 (Sigma Chemical, St. Louis, MO). Syntaxin 1A and syntaxin 1B cDNAs were gifts from Dr. R. Scheller (Howard Hughes Medical Institute, Stanford University, Stanford, CA).

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from rat brain and 3T3-L1 adipocytes by the single-step RNA isolation procedure with acid guanidinium thiocyanate-phenol-chloroform (Chomczynski and Sacchi, 1987). RNA was resolved under denaturing conditions in 1.2% (w/v) agarose gels containing 8% (v/v) formaldehyde, transferred onto nytron filters, baked for 90 min at 80°C, and prehybridized overnight at 42°C with 200 μg/ml salmon sperm DNA in 6× SSPE (1× SSPE: 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.4), 10× Denhardt's solution, and 0.5% SDS. Hybridization was performed in 50% formamide, 6× SSPE, 0.5% SDS, 5% dextran sulfate, and 100 μg/ml salmon sperm DNA for 48 h at 42°C with [α-^{32}P]dCTP-labeled syntaxin 1A cDNA (4.7×10^6 cpm/ml; 4.73×10^7 cpm/μg), syntaxin 1B cDNA (3.5×10^6 cpm/ml; 1.75×10^8 cpm/μg) or syntaxin 4 cDNA (2.2×10^6 cpm/ml; 2.2×10^7 cpm/μg), labeled by the random primer method. After hybridization, the filters were washed three times for 5 min in 1× SSC, 0.1% SDS at room temperature, and two times for 1 h with 0.1× SSC, 0.5% SDS at 65°C, before exposure to autoradiographic film.

Subcellular Fractionation

3T3-L1 fibroblasts were induced to differentiate into adipocytes, as described previously (Student *et al.*, 1980). Confluent adipocytes (8–10 d post differentiation) grown on 10-cm-diameter dishes were serum deprived for 3 h before stimulation with 100 nM insulin for 20 min at 37°C. Subcellular fractions from control and insulin-stimulated cells were prepared as previously described (Piper *et al.*, 1991). Briefly, cells were rinsed twice with HEPES-saline (20 mM HEPES pH 7.4, 140 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, and 1.0 mM $CaCl_2$) and once with homogenization buffer (20 mM HEPES, pH 7.4, 255 mM sucrose, and 1.0 mM EDTA). The cells were scraped in homogenization buffer containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 μM leupeptin, and 10 μM E-64) and homogenized by 30 strokes with a Teflon-on-glass homogenizer at 1200 rpm. The homogenate was centrifuged at 19,000 *g* for 20 min to yield pellet P1 and supernatant S1. S1 was centrifuged at 41,000 *g* for 20 min to obtain the high-density microsomes (HDM). The resulting supernatant, S2, was centrifuged at 195,000 *g* for 75 min to sediment the low-density microsomes (LDM). P1 was layered on a 1.12 M sucrose cushion and centrifuged at 100,000 *g* for 60 min. The interface material was collected and pelleted at 50,000 *g* for 20 min to yield the plasma membranes (PM). Protein concentration was determined by the Bradford procedure (Bio-Rad). Total membranes from fibroblasts and adipocytes were prepared as described earlier (Volchuk *et al.*, 1995). Crude brain microsomes were prepared according to Nagamatsu *et al.* (1992).

Immunoisolation of GLUT4-containing Vesicles

GLUT4-containing vesicles were immunoisolated as described previously (Volchuk *et al.*, 1995). Briefly, unstimulated 3T3-L1 adipo-

cytes were scraped in homogenization buffer (5 ml per 10-cm-diameter dish), and S2 was prepared as described above. Five hundred microliters were used per immunoprecipitation (containing ~350 μg of LDM plus cytosolic protein), adjusted to 100 mM potassium phosphate and added to magnetic beads conjugated to sheep anti-rabbit IgG (M-280 Dynabeads; Dynal, Great Neck, NY) that had been coated with either anti-GLUT4 or rabbit nonimmune serum. The mixture was incubated for 14–16 h at 4°C with gentle end-over-end rotation. The supernatant was collected, the beads were washed three times in 100 mM potassium phosphate (pH 7.4), the soluble washes pooled with the supernatant and centrifuged at 200,000 *g* for 60 min to sediment the membrane proteins. The immune pellets (P) and the sedimented, nonadsorbed supernatant proteins (S) were resuspended in 2× Laemmli sample buffer (Laemmli, 1970) containing 8 M urea, but they were not boiled to avoid dissociation of the heavy and light chains of the IgG. The entire volume of pellet and supernatant fractions from individual immunoprecipitations was resolved by SDS-PAGE and immunoblotted with specific antibodies. Subcellular fractions or immunoprecipitates were resolved by SDS-PAGE on 10 or 13% polyacrylamide gels, as described in the figure legends, by using reducing conditions without boiling. Scanning of immunoblots was done in the linear range of signals, with either a Molecular Dynamics PhosphorImager (Sunnyvale, CA) or a pdi model DNA35 scanner with version 1.3 of the Discovery Series one-dimensional gel-analysis software.

3T3-L1 Adipocyte Permeabilization and Glucose Transport

3T3-L1 adipocytes were permeabilized with streptolysin O (SLO) and subjected to measurement of 2-deoxyglucose uptake essentially as described by Clarke *et al.* (1994). Briefly, 3T3-L1 adipocytes grown in 12-well plates were treated with freshly activated 0.625 units/ml SLO (Difco, Detroit, MI) in isotonic potassium glutamate buffer (138 mM glutamic acid, 0.1% BSA, 1 mM EGTA, 0.285 mM CaCl2, 8 mM MgCl2, and 20 mM HEPES adjusted to pH 7.15 with KOH, containing a creatine phosphokinase-based ATP regenerating system). After 5 min at 37°C the SLO was aspirated, and the cell monolayers were incubated in potassium glutamate buffer for 15 min at 37°C with affinity-purified αSy4 (75 μg/ml) or equivalent concentrations of other IgG. Insulin (100 nM), where present, was added for a subsequent 20 min in the continuous presence of the antibodies. Uptake of [^{3}H]2-deoxyglucose and [^{14}C]sucrose was measured by adding them to cells at a final concentration of 10 and 2 μM, respectively. After 5 min, the solutions were aspirated, and radioactivity associated with the cells was determined by subtracting the diffusion-dependent uptake and accounting for radioactive spillover, as described earlier (Clarke *et al.*, 1994; Herbst *et al.*, 1995). Assessment of cell permeabilization was done by propidium iodide staining of nuclei.

RESULTS

Expression and Distribution of Syntaxin 4 in 3T3-L1 Adipocytes

Although the mammalian syntaxin 1 has been detected only in neuronal cells, not all tissues have been sampled, and indeed the *Drosophila* syx-1A is known to play a key role in non-neuronal secretion (Schulze *et al.*, 1995). Therefore, we explored the possible expression of syntaxin 1 in differentiating 3T3-L1 adipocytes with two specific monoclonal antibodies, SPM-1 (Horikawa *et al.*, 1993) and HPC-1 (Akagawa and Barnstable, 1986). Although these antibodies detected syntaxin 1 readily in crude brain microsomes, no signal was observed in total membranes or purified LDM and PM fractions of 3T3-L1 adipocytes.

However, to examine more specifically the possibility that syntaxin 1 might be expressed in 3T3-L1 adipocytes, total RNA was isolated and probed with syntaxin 1A and 1B cDNAs (Figure 1, A and B). Syntaxins 1A and 1B were readily detectable in brain RNA but not in RNA from 3T3-L1 cells. Therefore, it is unlikely that syntaxin 1 is expressed in these cells. Because syntaxin 4 mRNA is expressed at high levels in heart and skeletal muscle (Bennett *et al.*, 1993), tissues with insulin-regulated traffic, and recombinant syntaxin 4 associates with synaptobrevin II in vitro (Calakos *et al.*, 1994), we investigated the possible expression and distribution of this syntaxin isoform in the insulin-responsive 3T3-L1 adipocytes. Total RNA from 3T3-L1 adipocytes and rat brain was subjected to Northern blot analysis with a syntaxin 4-cDNA probe. Figure 1C shows that a single transcript of 2.0 kb, corresponding to the syntaxin 4 transcript, was detected in both samples.

To study the syntaxin 4 protein, a polyclonal antibody was generated in rabbits immunized with a GST fusion protein encoding the majority of the rat syntaxin 4 sequence, missing only the C-terminal putative membrane-spanning domain. The affinity-purified syntaxin 4 antibody (αSy4) recognized a single major band of the expected molecular weight for syntaxin 4 (36 kDa) in total membranes from 3T3-L1 adipocytes. Upon subfractionation of 3T3-L1 adipocytes, syntaxin 4 was found to be enriched in the PM, with lower concentrations found in the LDM and HDM fractions (Figure 2 left panel). The results of five independent

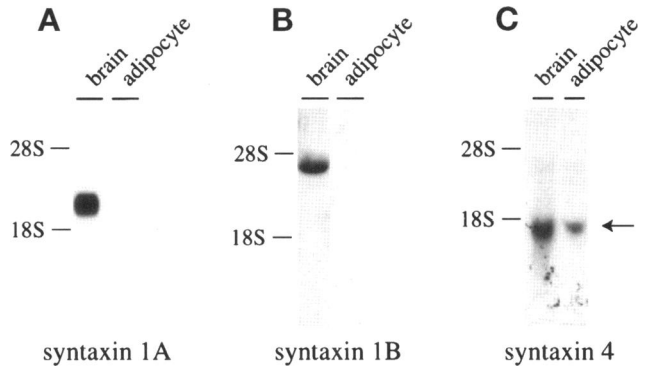

Figure 1. Northern blot analysis of syntaxin 1 and 4 transcripts. Total RNA was isolated from rat brain and 3T3-L1 adipocytes, as described in MATERIALS AND METHODS. Five micrograms of rat brain RNA and 20 μg of adipocyte RNA were separated on agarose gels and probed with cDNAs for syntaxin 1A (A) or syntaxin 1B (B), as described in MATERIALS AND METHODS. A 2.7 kb syntaxin 1A transcript and a 4.5 kb syntaxin 1B transcript were detected only in brain RNA. In (C), 20 μg of RNA from rat brain and 3T3-L1 adipocytes were probed with syntaxin 4 cDNA. Equal RNA loading was confirmed by ethidium bromide fluorescence of rRNA. A single transcript of 2.0 kb was detected in both samples (arrow). The positions of migration of the 28S and 18S rRNAs are indicated.

experiments, summarized in the right panel, confirm that syntaxin 4 is concentrated in the PM>HDM> LDM when expressed per microgram of protein. The net amount of syntaxin 4 in the isolated fractions was also calculated by multiplying the concentration of syntaxin 4 by the protein recovered in each fraction. By this account, the yield of syntaxin 4 in the LDM is found to be substantial (24%), although the majority of the protein is still present in the PM (67%) and only a small fraction is recovered in the HDM (9%). It is unlikely that a significant amount of syntaxin 4 in the LDM arises from contamination of this fraction by PM, because the presence of the plasma membrane marker α1 Na^+/K^+-ATPase was comparatively lower in this fraction (Figure 3A).

In contrast to the demonstrable presence of syntaxin 4 in these cells, syntaxin 3 could not be detected with an isoform-specific polyclonal antibody. Syntaxin 2, on the other hand, was detectable, as shown below.

Insulin Causes Subcellular Redistribution of Syntaxin 4 but not Syntaxin 2

To test whether the subcellular distribution of syntaxin 4 is affected by insulin, we subfractionated 3T3-L1 adipocytes treated with or without 100 nM insulin for 20 min, and the fractions were immunoblotted with αSy4 or other specific antibodies. The α1 Na^+/K^+-ATPase isoform, which in the basal state is almost exclusively localized in the PM, showed no change in distribution after hormonal stimulation (Figure 3A, left panel). In contrast, the concentration of GLUT4 in the LDM diminished with insulin treatment concomitant with an elevation of GLUT4 protein concentration in the PM (Figure 3A, left panel). Like the α1 Na^+/K^+-ATPase, syntaxin 2 was present almost exclusively in the PM of 3T3-L1 adipocytes (Figure 3A, right panel). The distribution of this protein was not altered in response to insulin in five separate experiments. In contrast, the concentration of syntaxin 4 diminished in the LDM fraction after insulin stimulation, and a small gain in syntaxin 4 was detected in the PM fraction. This is better appreciated in the final row in Figure 3A, right panel, which shows a lower exposure of the film.

The results of several independent experiments on the effect of insulin on the distribution of these proteins were analyzed densitometrically by scanning the films in the linear range (Figure 3B). The results are expressed as fractional changes in the concentration of these proteins within each membrane, i.e., (Insulin − Control)/Control. Values above the line represent relative gains, and values below the line represent relative losses. Neither α1 Na^+/K^+-ATPase nor syntaxin 2 showed any significant change in the LDM or PM in response to the hormone. In contrast, there was a sharp reduction in GLUT4 in the LDM and a marked increase in the PM, as expected. A qualitatively similar change was also observed in the distribution of syntaxin 4. Insulin caused a substantial and statistically significant ($p < 0.001$) fold reduction in syntaxin 4 in the LDM (from zero to $^{-}0.32 \pm 0.02$), as well as a significant ($p < 0.025$) fold increase in the PM (from zero to $^{+}0.17 \pm 0.05$) and no significant effect in the HDM. The fractional changes caused by insulin are quantitatively different for GLUT4 and syntaxin 4, because GLUT4 is concentrated originally in the LDM and syntaxin 4 in the PM.

Taking into account the protein yield of each fraction and the densitometric reading of syntaxin 4 per fraction, it can be calculated that when the PM recovered from each dish of cells experienced a net gain of 10 arbitrary units of syntaxin 4, the LDM experienced a net loss of 8.1 units, and the HDM experienced a net loss of 1.4 units (the latter, however, was not statistically significant). To the extent that the yield of membranes can represent the net cellular amounts of each

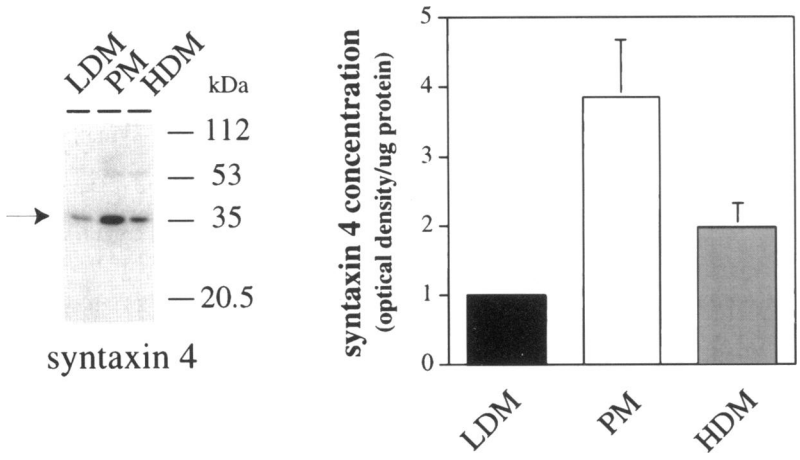

Figure 2. Syntaxin 4 expression and distribution in 3T3-L1 cells. 3T3-L1 adipocytes were fractionated as described in MATERIALS AND METHODS. Equal protein concentrations (20 μg) of low-density microsomes (LDM), plasma membranes (PM), and high-density microsomes (HDM) were resolved by SDS-PAGE (13% polyacrylamide) and immunoblotted with affinity-purified αSy4. The left panel shows a representative immunoblot, and the right panel shows quantitated results of densitometric quantification of five independent experiments. The relative concentration of syntaxin 4 in the three fractions has been normalized to the value in the LDM.

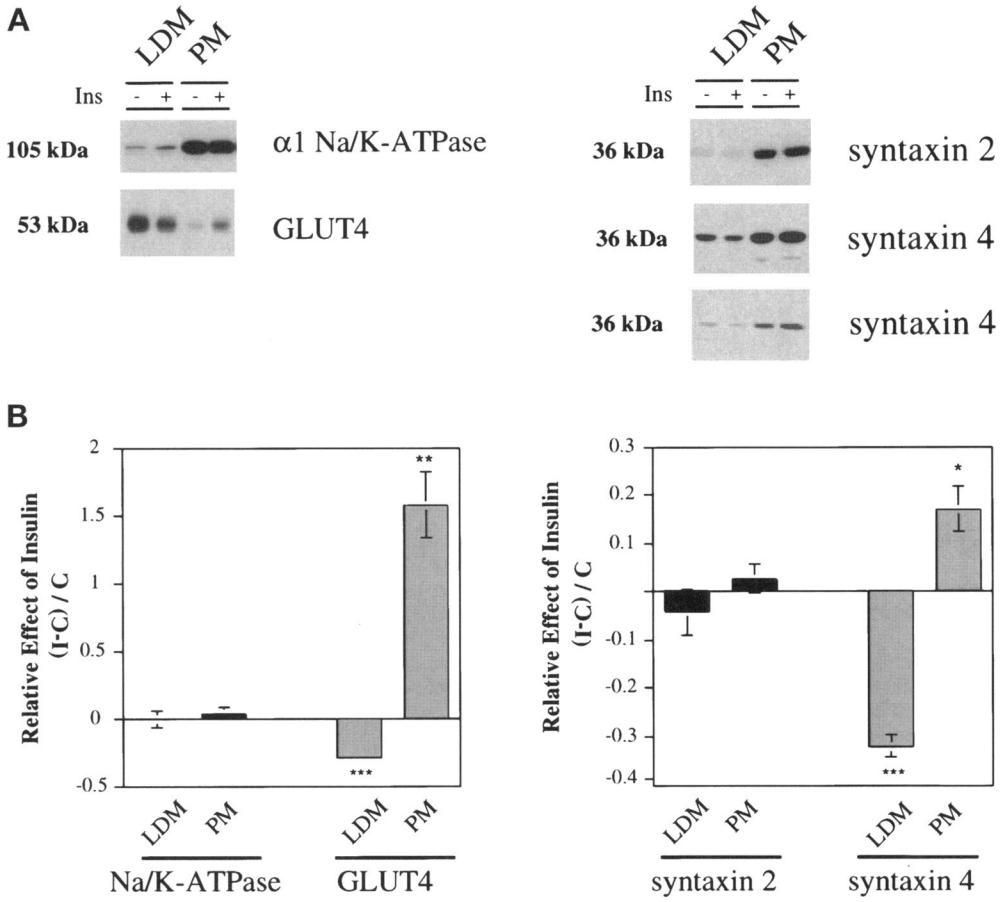

Figure 3. Effect of insulin on the distribution of syntaxin 4 and other membrane proteins. Twenty micrograms of 3T3-L1 adipocyte LDM and PM from control (−) and insulin-stimulated (100 nM for 20 min) (+) cells were resolved by SDS-PAGE on 13% polyacrylamide gels and immunoblotted with monoclonal antibody to the α1 Na^+/K^+-ATPase, anti-GLUT4 antiserum, anti-syntaxin 2 antibody, or affinity-purified anti-syntaxin 4 antibody (αSy4). Immunoreactivity was detected either by [^{125}I]-labeled sheep anti-mouse IgG or [^{125}I]-labeled protein A. (A) Representative immunoblots detecting each protein. High and low exposures of the film are shown for syntaxin 4 to emphasize the changes in the LDM and PM. (B) Averaged results of the scans of five independent experiments similar to those shown in A. The results are expressed as the fractional changes caused by insulin in each membrane, i.e., (Insulin − Control)/Control. Values above the line represent gains; values below the line represent losses. The SE value for the change in GLUT4 in the LDM fraction (± 0.024) is too small to show above/below the bar. *$p < 0.025$; **$p < 0.005$; ***$p < 0.001$ for Student's *t* test for paired data.

compartment, these calculations suggest that the gain of syntaxin 4 in the PM can be closely accounted for by the loss of this protein from the LDM.

Presence of Syntaxin 4 in GLUT4-containing Vesicles

To assess the possibility that the insulin-induced redistribution of syntaxin 4 is associated with the movement of the GLUT4, we examined whether a fraction of the syntaxin 4 in the LDM would copurify with GLUT4-containing vesicles. GLUT4-containing vesicles were immunoisolated from a fraction containing LDM plus cytosolic proteins (supernatant from the 41,000 × *g* centrifugation), which is largely devoid of PM or HDM. Nonimmune serum precipitated only a minute amount of the GLUT4 present in the supernatant, whereas the majority of this protein was precipitated by the anti-GLUT4 antibody (Figure 4). The immunoblots were densitometrically scanned, and the amount of GLUT4 brought down by the nonimmune serum was routinely subtracted from that precipitated by the anti-GLUT4 antibody. In six independent experiments, 73 ± 5% of the total GLUT4 protein was specifically precipitated in vesicular form. When these GLUT4-containing vesicles were probed with αSy4, it was found that 25 ± 5% of the total syntaxin 4 present in the LDM cosedimented with the GLUT4-containing vesicles. The syntaxin 4 present in the immunoprecipitated GLUT4 vesicles does not originate from contaminating PM, because a bona fide PM marker, the α1 subunit of the Na/K-ATPase, did not copurify with the vesicles (Figure 4, top). This panel is overexposed, compared with Figure 3A, to increase the signal of the α1 polypeptide that is present in very low levels in the S2 sample, likely due to residual contamination with PM and endoplasmic reticulum.

A similar presence of syntaxin 4 in GLUT4 vesicles was observed when the vesicles were immunopurified with the monoclonal antibody 1F8, followed by immunoblotting of the SDS-PAGE–separated immunoprecipitated proteins with polyclonal anti-GLUT4 and αSy4 antibody.

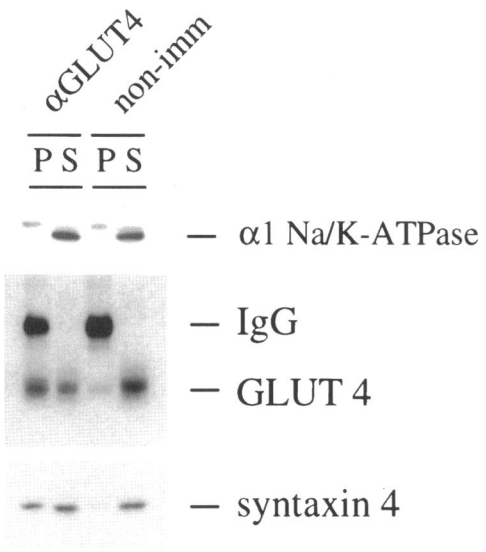

Figure 4. Presence of syntaxin 4 on GLUT4-containing vesicles of 3T3-L1 adipocytes. GLUT4-containing vesicles were immunoisolated with anti-GLUT4 antiserum (αGLUT4), as described in MATERIALS AND METHODS. Control immunoprecipitations were done in parallel using rabbit nonimmune serum (non-imm). Immunoprecipitated pellets (P) and remaining supernatant membranes (S) were resolved by SDS-PAGE on 10% polyacrylamide gels and immunoblotted with antibodies to the α1 subunit of the Na^+/K^+-ATPase, GLUT4, or syntaxin 4 (affinity-purified αSy4). Quantitated results from six independent experiments appear in the text.

Anti-Syntaxin 4 Antibodies Diminish Insulin-dependent Stimulation of Glucose Transport

Incubation of 3T3-L1 adipocytes with SLO under controlled conditions creates pores that allow entry of antibodies without substantial loss of intracellular contents within ~45 min (Robinson *et al.*, 1992; Clarke *et al.*, 1994). The cells retain insulin responsivity, and glucose uptake can be measured in permeabilized cells, provided that the nonspecific diffusion is subtracted (Clarke *et al.*, 1994; Herbst *et al.*, 1995). Using this experimental paradigm, we assessed the effect of αSy4 on the ability of insulin to stimulate glucose uptake. The results of three experiments, illustrated in Figure 5, show that insulin caused a 3.7-fold stimulation of glucose uptake in these permeabilized cells, consistent with the two previous studies (Clarke *et al.*, 1994; Herbst *et al.*, 1995). Importantly, the insulin-stimulated glucose uptake was significantly lower ($p < 0.05$, analysis of variance) in the presence of αSy4 (14.01 ± 0.67 pmol'min'mg protein) than either in the absence of any additions (25.12 ± 3.59 pmol'min'mg protein) or in the presence of irrelevant IgG (22.95 ± 2.65 pmol'min'mg protein). Moreover, neither αSy4 nor irrelevant IgG affected the rate of basal glucose uptake. In two additional experiments, antibodies directed to the C terminus of GLUT4, which bind to GLUT4 vesicles did not alter the insulin-dependent stimulation of glucose uptake (3.7-fold stimulation), highlighting the fact that antibody binding to GLUT4 vesicles is not sufficient per se to inhibit insulin action.

DISCUSSION

The syntaxins belong to a recently described family of proteins (Bennett *et al.*, 1993). To date, only syntaxin 1 has been reported to participate in the 7S and 20S complexes believed to mediate the attachment and fusion of synaptic vesicles to the plasma membrane (Sollner *et al.*, 1993a,b). In contrast, there have been very few studies detecting the presence, subcellular distribution, or regulation of endogenous syntaxins 2–5. We have recently detected syntaxin 4 in membranes of rodent skeletal muscle (Sumitani *et al.*, 1995) and human neutrophils (Brumell *et al.*, 1995). In the present study we searched for the syntaxin isoforms expressed in 3T3-L1 adipocytes, given that these cells express VAMP isoforms and are recognized for their regulated intracellular traffic, in particular for their ability to respond to insulin with mobilization of intracellular vesicles containing GLUT4.

In 3T3-L1 adipocytes, 67% of the syntaxin 4 was associated with purified PM. This is consistent with a role of this protein as an acceptor t-SNARE for incoming organelles. However, the function of syntaxin 4 in 3T3-L1 adipocytes may extend beyond serving as a

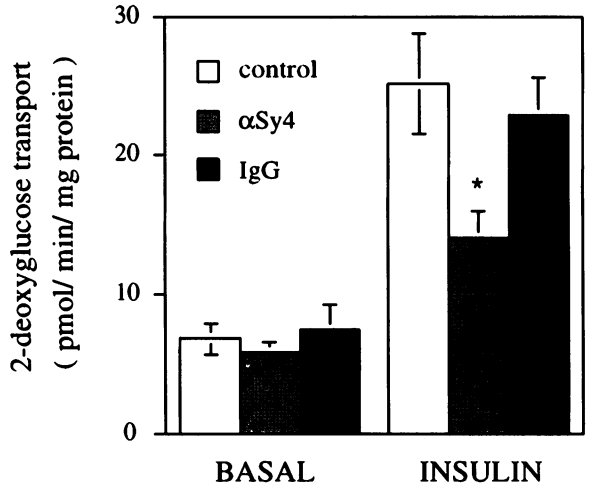

Figure 5. Effect of anti-syntaxin 4 antibody on insulin-dependent stimulation of glucose transport. 3T3-L1 adipocytes were permeabilized with SLO, as described in MATERIALS AND METHODS, and incubated for 15 min without (Control) or with 75 μg/ml affinity-purified anti-syntaxin 4 antibody (αSy4) or irrelevant IgG (IgG). Insulin (100 nM) was then added for 20 min before measurement of carrier-mediated glucose uptake. Results are of three independent experiments, each performed in triplicate. *$p < 0.05$ for insulin stimulation in SLO-permeabilized cells exposed to αSy4 relative to SLO-permeabilized cells with or without irrelevant IgG (analysis of variance).

plasma membrane t-SNARE. Indeed, we detected 24% of the cellular content of syntaxin 4 in LDM and 9% in HDM obtained after subcellular fractionation. Of the syntaxin 4 recovered in the LDM, 25% was recovered when 73% of the GLUT4-containing vesicles was immunopurified. If this fraction of GLUT4-containing vesicles is a representative sample of the whole population, then it can be calculated that 34% of the LDM syntaxin 4 is associated with GLUT4-containing structures. By further calculation it can be estimated that ~8% of the cellular syntaxin 4 colocalizes with the intracellular glucose transporters. This physical association is preserved most likely through the recruitment of the vesicles to the cell surface in response to insulin, insofar as the concentration of syntaxin 4 in the LDM decreased in insulin-treated cells and increased concomitantly in the PM (Figure 3). This behavior was specific, because the other syntaxin isoform expressed in these cells, syntaxin 2, did not change its distribution in response to the hormone. The redistribution of syntaxin 4 is consistent with the interpretation that insulin causes recruitment of vesicles containing syntaxin 4 but does not differentiate whether this protein is mobilized solely along with GLUT4 or whether its traffic includes vesicles devoid of GLUT4 protein. The results in Figure 4 clearly show that a significant fraction of the syntaxin 4 present in the LDM is associated with GLUT4-containing vesicles.

The recruitment of syntaxin 4 to the plasma membrane suggests that it may participate in the formation of a complex with complementary SNAREs in what could be considered "reverse" directionality. Consistent with this scenario, a fraction of the synaptobrevins is found on the cell surface (Cain *et al.*, 1992; Volchuk *et al.*, 1994, 1995) in addition to their abundance in the LDM. Alternatively, it is conceivable that the presence of syntaxin 4 on the translocating GLUT4-containing vesicle is required for capturing incoming vesicles. Following the two-pool model of glucose transporters (James *et al.*, 1994; Volchuk *et al.*, 1995), syntaxin 4 could be a resident of the endosomal recycling pool, potentially binding incoming vesicles from the regulated exocytic pool or from the TGN during protein sorting. It is less likely that the presence of syntaxin 4 on the GLUT4-containing vesicle is merely a reflection of the bulk flow of membrane that accompanies the continuous recycling of the glucose transporter, because neither syntaxin 2 nor $\alpha 1$ Na^+/K^+-ATPase copurify with the GLUT4 vesicles. Accordingly, only syntaxins 1 and 4, but not 2 or 3, bind synaptobrevin (Calakos *et al.*, 1994). Thus, of the syntaxins expressed in 3T3-L1 adipocytes, only syntaxin 4 fulfills the current criteria of a SNARE protein.

Clostridial toxins have proven useful in discerning the role of distinct v- and t-SNAREs in synaptic secretion (Niemann *et al.*, 1994) by virtue of their ability to selectively hydrolyze specific peptide bonds in each SNARE. There is currently no known toxin that can cleave syntaxin 4, because recent studies have demonstrated that botulinum toxin C1, which cleaves syntaxins 1, 2, and 3, does not affect syntaxin 4 (Schiavo *et al.*, 1995). For this reason, we resorted to using the anti-syntaxin 4 antibody to assess the possible function of syntaxin 4 in insulin-dependent stimulation of glucose transport. The results in Figure 5 indicate that addition of affinity-purified αSy4 reduces significantly the insulin stimulation of glucose uptake, implicating syntaxin 4 in the molecular mechanism of this action of the hormone. Whether it is the vesicular syntaxin 4 and/or the PM one that participates in the process remains to be determined. In this regard it is particularly notable that syntaxin 1 is localized to synaptic vesicles (Koh *et al.*, 1993; Schulze *et al.*, 1995; Walch-Solimena *et al.*, 1995) and chromaffin granules (Tayaga *et al.*, 1995) in addition to the surface membrane of neuroendocrine cells, and it is the vesicular syntaxin 1 that is preferentially cleaved by botulinum toxin C1 (Walch-Solimena *et al.*, 1995).

In summary, in 3T3-L1 adipocytes syntaxin 4 is present at the cell surface and in intracellular membranes, including the GLUT4-containing vesicles. Insulin causes partial redistribution of syntaxin 4, and an antibody to syntaxin 4 diminishes the stimulation of glucose uptake by the hormone. These results suggest that this recently described protein participates in the translocation of the GLUT4 that culminates in the stimulation of glucose transport.

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