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Syntaxin 4, VAMP2, and/or VAMP3/Cellubrevin Are Functional Target Membrane and Vesicle SNAP Receptors for Insulin-Stimulated GLUT4 Translocation in Adipocytes

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Introduction of the cytoplasmic domain of syntaxin 4, using either recombinant vaccinia virus or single-cell microinjection, resulted in an inhibition of insulin-stimulated GLUT4 but not GLUT1 translocation to the plasma membrane. This was specific for syntaxin 4, since neither the expression of syntaxin 3 nor the expression of a syntaxin 4 mutant in which the vesicle-associated membrane protein (VAMP) binding site was deleted had any significant effect. Consistent with the requirement for a functional VAMP binding site, expression of the cytoplasmic domains of VAMP2 or VAMP3/cellubrevin also resulted in an inhibition of insulin-stimulated GLUT4 translocation. In addition, immunoprecipitation of the expressed syntaxin 4 cytoplasmic domain resulted in an insulin-stimulated increase in the coimmunoprecipitation of GLUT4-containing vesicles. Together, these data demonstrate that syntaxin 4, VAMP2, and/or VAMP3/cellubrevin can function as target membrane and vesicle SNAP receptors, respectively, for insulin-responsive GLUT4 translocation to the plasma membrane.

Insulin increases glucose uptake in adipose tissue, skeletal muscle, and cardiac muscle primarily by regulating the intracellular trafficking of the GLUT4 glucose transporter isoform (3, 10, 23, 26, 27, 29, 31, 32). In the basal state, GLUT4 cycles continuously between the plasma membrane and one or more intracellular compartments, with the vast majority of the transporter residing within the cell interior (28, 39, 40, 46, 47, 62). Activation of the insulin receptor triggers a large increase in the rate of GLUT4 vesicle exocytosis and a smaller decrease in the rate of internalization by endocytosis (17, 28, 40, 62). The insulin-mediated increase in exocytosis is probably the limiting step for GLUT4 translocation, since complete inhibition of GLUT4 endocytosis does not result in plasma membrane GLUT4 accumulation to the same extent as does insulin stimulation (45, 51). This insulin-dependent shift in the cellular dynamics of GLUT4 vesicle trafficking results in a net increase of GLUT4 protein levels on the cell surface, thereby increasing the rate of glucose uptake (23). However, the precise molecular events and protein components responsible for GLUT4 vesicle cycling and the regulation of docking and fusion with the plasma membrane have remained enigmatic.

Recently, substantial progress has been made in our understanding of synaptic vesicle trafficking in the regulation of neurotransmitter release from the presynaptic membrane (for recent reviews, see references 8 and 48). In this process, protein complexes in the vesicle compartment (vesicle soluble NSF attachment protein [SNAP] receptors [v-SNAREs]) pair with their cognate receptor complexes at the target membrane (target membrane SNAP receptors [t-SNAREs]). Direct binding interactions between the t-SNARE and v-SNARE proteins in combination with the association of several accessory proteins (for example, synaptophysin and synaptotagmin) result in the formation of a 7S complex, which can be immunoprecipitated from detergent-solubilized brain membrane extracts (35, 49, 50). The 7S complex forms a receptor for the soluble NSF attachment protein (SNAP), which is one component of the general fusion machinery (13). When α-SNAP and the *n*-ethylmaleimide (NEM)-sensitive fusion factor (NSF) are added to solubilized brain membranes in the presence of MgATPγS, a 20S particle is formed (35, 49). Hydrolysis of ATP by NSF results in disruption of the 20S complex, which may facilitate the membrane fusion event (35, 49).

Several studies, based largely on the use of *Clostridium botulinum* neurotoxins, have demonstrated that the v-SNAREs responsible for neurotransmitter release are members of the synaptobrevin or vesicle-associated membrane protein (VAMP) family (41–43, 59). The synaptobrevins are approximately 18-kDa membrane proteins having their carboxyl terminus oriented toward the cytoplasm and their amino-terminal domain spanning the membrane facing the vesicle lumen (1, 19, 52, 56). Similarly, the syntaxin isoforms are approximately 35-kDa membrane proteins that have functional properties of t-SNAREs due to their localization to the plasma membrane and, in the case of syntaxin 1 and 4, bind to the VAMP/synaptobrevin proteins (7, 35). A second t-SNARE is SNAP-25, a 25-kDa hydrophilic protein which associates with the plasma membrane via several palmitoylated cysteine residues (21). Both the syntaxins and SNAP-25 are specific targets for specific neurotoxins which are known to block neurotransmitter release (4, 5). Together, a core complex consisting of synaptobrevin, syntaxin, and SNAP-25 bridge synaptic vesicles with the plasma membrane while forming a high-affinity binding site for α-SNAP (33).

In adipocytes, the insulin-stimulated trafficking of the GLUT4 vesicle has several features common to the regulated exocytosis pathway of secretory vesicles, including the presence of specialized vesicular proteins. For example, members of the VAMP/synaptobrevin family have been detected in membrane fractions containing the insulin-sensitive GLUT4 protein (6,

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57). The presence of VAMPs in intracellular GLUT4 vesicles suggests that specific targeting of GLUT4 to the plasma membrane may occur by a mechanism similar to that proposed for synaptic vesicle transport. Based upon this paradigm, we have investigated the potential role of syntaxin protein isoforms as functional plasma membrane t-SNAREs and the VAMP isoforms as potential v-SNAREs for insulin-stimulated GLUT4 translocation. Our data demonstrate that syntaxin 4, VAMP2, and VAMP3/cellubrevin are required functional t-SNAREs and v-SNAREs for the insulin-stimulated GLUT4 translocation to the plasma membrane.

MATERIALS AND METHODS

Tissue culture. 3T3L1 preadipocytes were obtained from the American Type Tissue Culture repository and were cultured at 37°C in an 8% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and 10% calf serum. Confluent cultures were induced to differentiate by incubation of the cells with DMEM containing 25 mM glucose, 10% fetal bovine serum, 1 μg of insulin per ml, 1 μM dexamethasone, and 0.5 mM isobutyl-1-methylxanthine. After 4 days, the medium was changed to DMEM containing 25 mM glucose, 10% fetal bovine serum, and 1 μg of insulin per ml, and the incubation was continued for an additional 4 days. The medium was then changed to DMEM containing 25 mM glucose and 10% fetal bovine serum. Under these conditions, more than 95% of the cell population morphologically differentiated into adipocytes. The adipocytes were maintained for an additional 4 to 8 days prior to use.

Northern blotting. Total cellular RNA was isolated from 3T3L1 fibroblasts, 3T3L1 adipocytes, or rat epididymal fat pads by the isothiocyanate-CsCl method (12). RNA was quantified spectrophotometrically by measurement of the absorbance at 260 nm and stored as an ethanol precipitate at −70°C. A 10-μg portion of total RNA was fractionated by 1% agarose–formaldehyde electrophoresis. After electrophoresis, the RNA was transferred to 0.45-μm-pore-size Nytran filters (Schleicher & Schuell, Inc.), and the filters were prehybridized for 1 h at 60°C in a solution consisting of 50% deionized formamide, 5× Denhardt's reagent, 1.0% sodium dodecyl sulfate and 200 μg of sheared salmon sperm DNA per ml. The filters were incubated overnight at 60°C with 2×10^6 cpm of radiolabeled antisense RNA per ml derived from full-length cDNAs corresponding to rat syntaxin 2, 3, or 4. The filters were washed, as specified by the manufacturer, by a high-stringency wash with RNase A (10 μg/ml in 2× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.7] at 37°C for 15 min) and then exposed to Hyperfilm (Amersham) at −70°C.

Vaccinia virus infection. To efficiently express cDNAs in differentiated 3T3L1 adipocytes, we used the recombinant vaccinia virus system (34). The cDNAs for proteins to be expressed were cloned into the recombination vector plasmid, pSC65. This plasmid encodes the *Escherichia coli lacZ* gene expressed from the p7.5 vaccinia virus promoter and contains a polylinker downstream from a strong synthetic early-late promoter in the opposite orientation. Nucleotide sequences encoding the cytoplasmic domains of syntaxin 4 (amino acids 1 through 273; upstream primer, ccgtcgacATGCGCGACAGGACCC; downstream primer, ccc tcgagCTTTTTCTTCCTCGCC) and syntaxin 3 (amino acids 1 through 263; upstream primer, gcgtcgacATGAAGGACCGACTGGAGC; downstream primer, gcctcgagTTTCTTTCGAGCCTGACCC) were amplified by PCR. These oligonucleotides were designed to contain *Sal*I and *Xho*I restriction sites at the 5′ and 3′ ends, respectively. A deletion of the region of the syntaxin 4 cytoplasmic domain which is highly homologous to the region responsible for protein complex binding in syntaxin 1A was generated by deleting amino acids 205 through 273 of the cytoplasmic domain of syntaxin 4. This was accomplished by digestion with *Bgl*II and *Xho*I and insertion of a *Bgl*II-*Xho*I linker which maintained the *myc* tag in frame with the truncated syntaxin protein. Similarly, the cytoplasmic domains of VAMP2 (amino acids 1 through 94; upstream primer, ccgtcgacATG TCGGCTACCGCTGCC; downstream primer, cgctcgagCTTGAGGTTTTTCC ACCA) and VAMP3/cellubrevin (amino acids 1 through 81; upstream primer, ccgtcgacATGTCTACAGGGGTGCC; downstream primer, cgctcgagCTTGCAG TTCTTCCACC) were amplified by PCR. These oligonucleotides were designed to contain *Sal*I and *Xho*I restrictions sites at the 5′ and 3′ ends, respectively. The amplified fragments were subcloned into the *Sal*I-*Xho*I site of a pBluescript SK− (Stratagene) vector which was modified to include sequences encoding the *myc* epitope (EEQKLISEEDLL) between the *Xho*I and *Kpn*I sites of the vector. The *Sal*I/*Kpn*I fragments encoding the cytoplasmic domains of either syntaxin 3, syntaxin 4, VAMP2, or VAMP3/cellubrevin fused to the *myc* epitope were then subcloned into these sites of the vaccinia virus recombination plasmid, pSC65. The nucleotide fidelity of these constructs was verified by sequencing in each direction across the entire subcloned region. The empty vaccinia virus recombination cloning vector was also used to generate a recombinant control virus.

Homologous recombination of the plasmids and wild-type vaccinia virus was carried out in RK13 cells. Recombinant virus was selected by plaque assays of virus-infected thymidine kinase-negative (TK-143) cells. Recombinant viruses were plaque purified three times, and high-titer stocks were prepared in HeLa cells. Expression of the cytoplasmic domains of syntaxin 3, syntaxin 4, VAMP2, and VAMP3/cellubrevin containing the *myc* epitope was confirmed by immunoblot analysis of infected-cell lysates with the 9E10 monoclonal antibody directed against the *myc* epitope (Santa Cruz Biologicals). Quantitative infection of differentiated 3T3L1 adipocytes was performed by infecting 35-mm plates of cultured 3T3L1 adipocytes at a multiplicity of infection of 10 PFU/cell for 4 h in DMEM containing 25 mM glucose and 0.5% bovine serum albumin. Infection of 100% of the cell population was confirmed by an in situ assay for β-galactosidase activity (60). At 4 h postinfection, the cells were either left untreated or treated with 100 nM insulin for 15 min at 37°C. The cells were then used for the preparation of plasma membrane sheets and subsequent analysis by immunoblotting as described below.

Plasma membrane sheet assay. Preparation of plasma membrane sheets from differentiated 3T3L1 adipocytes was performed essentially by the method of Robinson et al. (38). Briefly, cells cultured on 35- or 60-mm dishes, following the appropriate treatment as described in each figure legend, were rinsed once in ice-cold phosphate-buffered saline (PBS) and incubated with 0.5 mg of poly-D-lysine (Sigma) per ml for 30 s. The cells were then swollen in a hypotonic buffer (23 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 1 mM EGTA [pH 7.5]) by three successive rinses. The swollen cells were sonicated for 5 s at power setting 5 with a model 550 Fisher sonic dismembrator fitted with a 5-mm microtip set 1 cm above the surface of the cell monolayer in 18 ml of sonication buffer (70 mM KCl, 30 mM HEPES, 5 mM $MgCl_2$, 3 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride [PMSF] [pH 7.5]). The bound plasma membrane sheets were washed three times in sonication buffer and used for either indirect immunofluorescence or immunoblot analysis as described below.

Electrophoresis and immunoblotting. Plasma membrane sheets were scraped into a buffer containing 250 mM sucrose, 10 mM Tris (pH 7.4), and 2 mM EGTA and pelleted by centrifugation at 200,000 × *g* for 1 h at 4°C. The pelleted fraction was solubilized in 100 μl of Laemmli buffer, and 15 μl of the total solubilized fraction was separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and transferred to nitrocellulose membranes. The nitrocellulose membranes were immunoblotted with either GLUT4 polyclonal antibody (IRGT; East Acres Biologicals) or a GLUT1 polyclonal antibody (East Acres Biologicals) by using an enhanced chemiluminescence detection kit (Amersham). To confirm equal loading of samples, the total amounts of protein and glycoproteins in each lane were visualized by the DIG glycan/protein double-labeling kit (Boehringer-Mannheim).

Preparation of total-cell extracts. Total-cell extracts were prepared from 10-cm plates of vaccinia virus-infected adipocytes which had been treated with 100 nM insulin for 15 min or left untreated. Cells from one plate were washed three times with ice-cold PBS and scraped into 1 ml of homogenization buffer (250 mM sucrose, 10 mM Tris [pH 7.4], 2 mM EGTA, 1 mM sodium pyrophosphate, 10 mM NaF, 100 μM Na_3VO_4, 1 mM PMSF, 5 μg of aprotinin per ml, 5 μg of leupeptin per ml). The cells were homogenized by 25 strokes of a Dounce homogenizer with a tight-fitting pestle. Nuclei and unbroken cells were removed by centrifugation at 1,000 × *g* for 4°C for 10 min, and the resultant supernatant, containing total membranes and cytosolic proteins, was used for immunoprecipitation.

Immunoprecipitation of syntaxin 4. A syntaxin 4-specific antibody was prepared in sheep by injection of a glutathione-*S*-transferase (GST) fusion protein containing the cytoplasmic domain of syntaxin 4 (Elmira Biologicals, Iowa City, Iowa). A 10-μl sample of either sheep preimmune serum or syntaxin 4 antiserum was preloaded onto 10-μl of protein G-Sepharose beads (Sigma) for 2 h at 4°C in 500 μl of TBS (10 mM Tris [pH 7.4], 150 mM NaCl). The cell supernatants were added to either the control serum or anti-syntaxin 4-loaded beads for 2 h at 4°C. The beads were washed five times by microcentrifugation and resuspension in a buffer containing 50 mM HEPES (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1 mM sodium pyrophosphate, 10 mM NaF, 100 μM Na_3VO_4, 1 mM PMSF, 5 μg of aprotinin per ml, and 5 μg of leupeptin per ml. Similarly, the monoclonal *myc* epitope-specific antibody covalently linked to agarose beads (9E10-AC; Santa Cruz) was used in an analogous manner for immunoprecipitation.

Preparation of fusion proteins and single-cell microinjection. GST fusion proteins were generated by cloning the cytoplasmic fragments of syntaxin 3 and syntaxin 4 (as described above) into pGEX-3X or pGEX-4T2 cloning vectors (Pharmacia) in frame with GST. The fusion proteins were expressed in *E. coli* and isolated on glutathione-coated beads (Pharmacia). The fusion proteins were eluted from the beads with 10 mM reduced glutathione and dialyzed against three or four changes of PBS (5 mM Na_2HPO_4 [pH 7.4], 150 mM NaCl). The eluted proteins were concentrated to 2 mg/ml by centrifugation through 10-kDa-cutoff microconcentrators (Amicon).

3T3L1 adipocytes used for microinjection were grown on 60-mm tissue culture dishes. Prior to microinjection, the cells underwent a medium change to Leibovitz's L-15 medium containing 10% fetal bovine serum and placed on a 37°C heating stage of a Leica phase-contrast microscope. Microinjecting pipettes were prepared from borosilicate filaments (World Precision Instruments) that were pulled with a Sachs-Flaming micropipette puller (Sutter model P-97). Differentiated 3T3L1 adipocytes were impaled with an Eppendorf model 5171 micromanipulator and given injections of approximately 0.1 pl of GST fusion proteins (2 mg/ml in PBS) directly into the cell cytoplasm with an Eppendorf model 5246 transjector. Following microinjection of 100 to 200 cells per dish, the medium

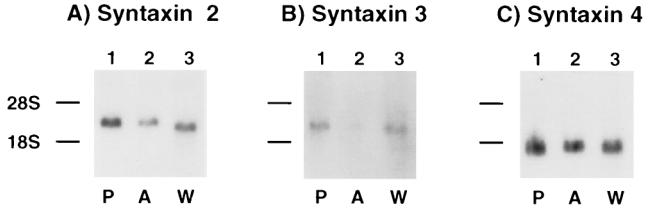

FIG. 1. 3T3L1 and rat adipose tissue express syntaxin 2, 3, and 4 mRNA. Total RNA was obtained from rat epididymal white adipocytes (W) and predifferentiated (P) and fully differentiated (A) 3T3L1 adipocytes. Portions (10 μg) were separated by agarose gel electrophoresis and subjected to Northern blotting with a syntaxin 2 (A), syntaxin 3 (B), or syntaxin 4 (C) ^{32}P-labeled probe as described in Materials and Methods. The syntaxin 2 and 4 Northern blots were developed after an overnight exposure, whereas the syntaxin 3 Northern blot required 4 days of exposure. These results are representative of two independently performed experiments.

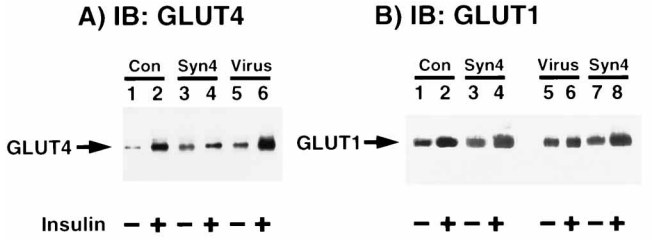

FIG. 2. Vaccinia virus expression of the syntaxin 4 cytoplasmic domain inhibits insulin-stimulated GLUT4 translocation. Fully differentiated 3T3L1 adipocytes were either uninfected (Con; lanes 1 and 2) or infected with the β-galactosidase-expressing recombinant vaccinia virus containing the cytoplasmic domain of syntaxin 4 (Syn4; lanes 3 and 4) or the empty β-galactosidase recombinant vaccinia virus (Virus; lanes 5 and 6) as described in Materials and Methods. After 4 h of infection, the cells were either unstimulated (lanes 1, 3, and 5) or stimulated with 100 nM insulin for 15 min at 37°C (lanes 2, 4, and 6). The cells were rapidly cooled to 4°C, and plasma membrane sheets were prepared as described in Materials and Methods. The isolated plasma membranes were then subjected to Western blotting with a GLUT4 antibody (A) or a GLUT1 antibody (B). These results are representative of three to seven independently performed experiments depending on the specific conditions and immunoblot (see the legend to Fig. 3).

was changed to Leibovitz's L-15 medium containing 0.2% bovine serum albumin, and the cells were incubated for an additional 2 h at 37°C.

Immunofluorescence and confocal microscopy. Sonicated plasma membrane sheets were fixed for 20 min in a solution containing 2% paraformaldehyde, 70 mM KCl, 30 mM HEPES (pH 7.5), 5 mM $MgCl_2$, and 3 mM EGTA. The fixed plasma membrane sheets were quenched for 15 min at 25°C in 100 mM glycine–PBS (pH 7.5). After three rinses in PBS, the sheets were blocked overnight at 4°C in PBS containing 5% donkey serum (Sigma). The blocked sheets were incubated at 25°C for 1 h with a 1:100 dilution of polyclonal rabbit anti-GLUT4 antiserum (East Acres Biologicals) in combination with a 1:5,000 dilution of polyclonal sheep anti-maltose binding protein antiserum (generously provided by Morris Birnbaum, University of Pennsylvania). The plasma membrane sheets were then washed three times with PBS and incubated for 1 h at 25°C with a 1:50 dilution of fluorescein isothiocyanate-conjugated donkey anti-sheep immunoglobulin G (IgG) in combination with a 1:50 dilution of lissamine-rhodamine-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, Inc.). Following incubation with the secondary antibodies, the membrane sheets were rinsed three more times in PBS and mounted for microscopic analysis with Vectashield mounting medium (Vector Laboratories Inc.). Confocal images were obtained on a Bio-Rad MRC 600 laser confocal microscope (University of Iowa Central Microscopy Research Facility).

RESULTS

Primary rat and 3T3L1 murine adipocyte expression of syntaxins 2, 3, and 4. Previous studies have identified the presence of the v-SNARE proteins (VAMP2 and VAMP3/cellubrevin) as components of GLUT4 vesicles (6, 57). Since the VAMP proteins direct the association of intracellular vesicles with t-SNARE proteins on the target membrane, we initially determined the relative expression of syntaxin 2, 3, and 4 mRNAs in isolated primary rat adipocytes and in cultured murine 3T3L1 adipocytes (Fig. 1). We did not assess the levels of syntaxin 1 or 5 mRNA, since syntaxin 1 is neuron specific and syntaxin 5, due to its intracellular localization, appears to function as a t-SNARE for endoplasmic reticulum-to-Golgi transport (18).

Northern blotting demonstrated that primary rat and cultured murine 3T3L1 adipocytes expressed predominantly syntaxin 2 and 4 mRNA (Fig. 1A and C). Although a small amount of syntaxin 3 mRNA was detectable, it was substantially smaller than the transcripts for syntaxin 2 and 4; detection required 4 days of exposure compared to 16 h for both syntaxin 2 and syntaxin 4 (Fig. 1B). Interestingly, the 3T3L1 preadipocytes expressed relatively higher levels of syntaxin 2 and 3 mRNA compared to the fully differentiated 3T3L1 adipocytes (Fig. 1A and B, compare lane 1 with lane 2). In contrast, syntaxin 4 mRNA remained unaffected by the differentiation of the 3T3L1 cells from the preadipocyte to the adipocyte state and was present in amounts comparable to those detected in primary isolated rat adipocytes (Fig. 1C). The slower migration of the syntaxin 2 and 3 mRNAs from the murine 3T3L1 cells than from the primary rat adipocytes (lanes 3) reflects species differences in the size of these transcripts. These data are consistent with recent studies demonstrating the presence of the syntaxin 2, 3, and 4 protein isoforms in adipocytes (55, 58).

Insulin-dependent GLUT4 translocation is inhibited in 3T3L1 adipocytes expressing the syntaxin 4 cytoplasmic domain. Standard transfection protocols such as Lipofectin, electroporation, and calcium phosphate treatment yield relatively low levels of exogenous gene expression in 3T3L1 adipocytes. To overcome this deficiency for biochemical analysis, we prepared recombinant vaccinia viruses that are expressed with a 100% efficiency in differentiated 3T3L1 adipocytes as assessed by in situ β-galactosidase staining (data not shown). As a control for potential cytotoxic effects following vaccinia virus infection, we compared the extent of insulin-stimulated GLUT4 translocation between uninfected cells and cells infected with the control β-galactosidase-expressing virus (Fig. 2A). GLUT4 immunoblotting of isolated plasma membrane sheets demonstrated the typical insulin-stimulated increase in GLUT4 protein levels due to its translocation from an intracellular vesicular pool to the plasma membrane (Fig. 2A, lanes 1 and 2). Following 4 h of vaccinia virus expression, there was no significant effect on the amount of plasma membrane GLUT4 protein in unstimulated cells (lane 5). In addition, insulin stimulation resulted in a comparable amount of GLUT4 translocation to the plasma membrane as observed in the uninfected cells (lane 6). In contrast, infection of 3T3L1 adipocytes with a recombinant vaccinia virus directing the expression of the syntaxin 4 cytoplasmic domain (amino acids 1 to 273) inhibited the insulin-stimulated appearance of GLUT4 in the plasma membrane (lanes 3 and 4).

Quantitation of multiple GLUT4 immunoblots by laser scanning densitometry indicated that insulin induced an approximately fourfold increase in plasma membrane sheet GLUT4 protein in both uninfected and vaccinia virus-infected cells (Fig. 3A). In contrast, vaccinia virus expression of the syntaxin 4 cytoplasmic domain resulted in only a 1.4-fold insulin stimulation of GLUT4 translocation. The apparent lower extent of GLUT4 translocation detected by immunoblotting of plasma membrane sheets compared to subcellular fractionation and/or surface labeling reflects the incomplete lysis of cells that occurs at the rim of the tissue culture dish (data not shown).

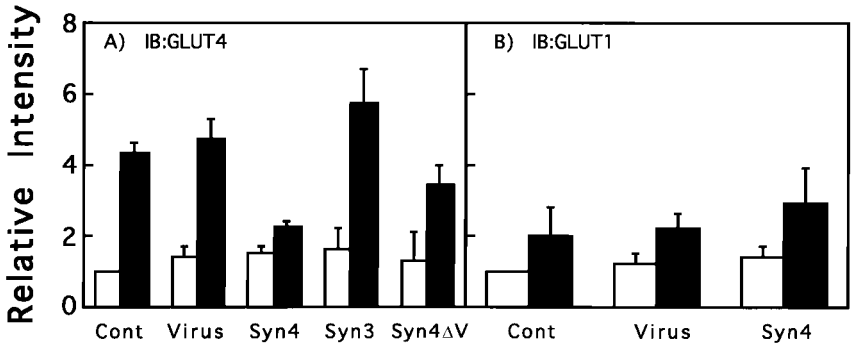

FIG. 3. Quantitation of multiple GLUT4 and GLUT1 immunoblots demonstrating the inhibition of insulin-stimulated GLUT4 but not GLUT1 translocation by vaccinia virus expression of the syntaxin 4 cytoplasmic domain. (A) 3T3L1 adipocytes were either uninfected (Cont, $n = 7$), infected with a vaccinia virus that expresses the LacZ gene (Virus, $n = 4$), the cytoplasmic domain of syntaxin 4 (Syn4, $n = 7$), the cytoplasmic domain of syntaxin 4 in which the VAMP binding site was deleted (Syn4ΔV, $n = 2$) and the cytoplasmic domain of syntaxin 3 (Syn3, $n = 3$). The cells were then treated without (open boxes) or stimulated with (solid boxes) 100 nM insulin for 15 min, and plasma membrane sheets were isolated and immunoblotted for GLUT4 as described in Materials and Methods. (B) 3T3L1 adipocytes were either uninfected (Cont, $n = 3$) or infected with a vaccina virus that expresses the LacZ gene (Virus, $n = 3$) or the cytoplasmic domain of syntaxin 4 (Syn4, $n = 4$). The cells were then treated without (open boxes) or stimulated with (solid boxes) 100 nM insulin for 15 min, and plasma membrane sheets were isolated and immunoblotted for GLUT1.

3T3L1 adipocytes also express the GLUT1 isoform, which is localized to both the plasma membrane and intracellular vesicular storage sites in the unstimulated state (61). However, GLUT1 displays a lesser degree of insulin-stimulated translocation, being part of a different constitutively recycling vesicle population (36, 37). As expected, insulin treatment of uninfected cells resulted in a relatively small increase in the amount of plasma membrane associated GLUT1 protein (Fig. 2B, lanes 1 and 2). Similarly, vaccinia virus infection per se did not have any significant effect on GLUT1 translocation (lanes 5 and 6). In contrast to GLUT4, the insulin-stimulated translocation of GLUT1 was independent of the presence of the syntaxin 4 cytoplasmic domain (lanes 3, 4, 7, and 8). The inability of the syntaxin 4 cytoplasmic domain to inhibit insulin-stimulated GLUT1 translocation was confirmed by several independent experiments, which are quantitated in Fig. 3B. Together, these data demonstrate that vaccinia virus infection itself does not alter the insulin-stimulated translocation of either GLUT4 or GLUT1. In addition, vaccinia virus infection did not alter the total cellular pool of the GLUT4 or GLUT1 proteins (data not shown). Thus, the expression of the syntaxin 4 cytoplasmic domain does not effect GLUT1 trafficking but functions in a dominant-interfering manner by inhibiting the insulin-stimulated translocation of GLUT4 to the plasma membrane.

Inhibition of insulin-stimulated GLUT4 translocation is specific for syntaxin 4. Previous in vitro binding studies have demonstrated that syntaxins 1 and 4 but not syntaxin 2 or 3 can specifically bind to the VAMPs (7, 35). To examine the specificity of the syntaxin 4 cytoplasmic domain to inhibit GLUT4 translocation, we expressed a cytoplasmic syntaxin 4 protein in which the putative VAMP binding domain (amino acids 205 to 273) was deleted based upon the identified VAMP binding site (amino acids 194 to 267) of syntaxin 1 (Fig. 4). As observed in Fig. 2, GLUT4 immunoblots of isolated plasma membrane sheets from cells infected with control vaccinia virus indicated the expected insulin-stimulated translocation of the GLUT4 protein (Fig. 4A, lanes 1 and 2). Similarly, vaccinia virus-mediated expression of the syntaxin 4 cytoplasmic domain with the VAMP binding site deletion resulted in only a partial reduction of insulin-stimulated GLUT4 translocation (lanes 3 and 4). This was in marked contrast to the full-length cytoplasmic domain of syntaxin 4 (amino acids 1 to 273), which almost completely inhibited the insulin-stimulated translocation of the GLUT4 protein to the plasma membrane (lanes 5 and 6). Immunoblotting of whole-cell extracts with the *myc* epitope-specific monoclonal antibody (9E10) demonstrated identical expression levels of the syntaxin 4 cytoplasmic domain and the syntaxin 4 cytoplasmic domain containing the VAMP binding-site deletion (data not shown).

In contrast to syntaxin 4, neither syntaxin 2 nor syntaxin 3 displays appreciable affinity for either VAMP1 or VAMP2 (7, 35). Thus, to further document the specificity of the syntaxin 4 cytoplasmic domain on GLUT4 translocation, we next examined the effect of the expressed syntaxin 3 cytoplasmic domain. 3T3L1 adipocytes infected with the β-galactosidase recombinant vaccinia virus displayed the expected insulin-stimulated

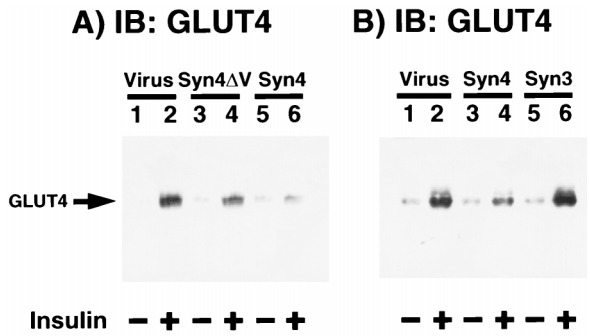

FIG. 4. The vaccinia virus-expressed cytoplasmic domain of syntaxin 4 but not syntaxin 3 inhibits insulin-stimulated GLUT4 translocation. (A) Differentiated 3T3L1 adipocytes were infected with the β-galactosidase-expressing recombinant vaccinia virus (Virus; lanes 1 and 2), the recombinant vaccinia virus containing the cytoplasmic domain of syntaxin 4 in which the VAMP binding domain was deleted (Syn4ΔV; lanes 3 and 4), or the recombinant vaccinia virus containing the cytoplasmic domain of syntaxin 4 (Syn4; lanes 5 and 6). (B) The 3T3L1 adipocytes were infected with the β-galactosidase-expressing recombinant vaccinia virus (Virus; lanes 1 and 2), the recombinant vaccinia virus containing the cytoplasmic domain of syntaxin 4 (Syn4; lanes 3 and 4), or the recombinant vaccinia virus containing the cytoplasmic domain of syntaxin 3 (Syn3; lanes 5 and 6). Following 4 h of infection, the cells were either unstimulated (lanes 1, 3, and 5) or stimulated with 100 nM insulin for 15 min at 37°C (lanes 2, 4, and 6). The cells were rapidly cooled to 4°C, and plasma membrane sheets were prepared and subjected to Western blotting with a GLUT4 antibody. These results are representative of two to seven independently performed experiments depending on the specific condition and immunoblot (see the legend to Fig. 3).

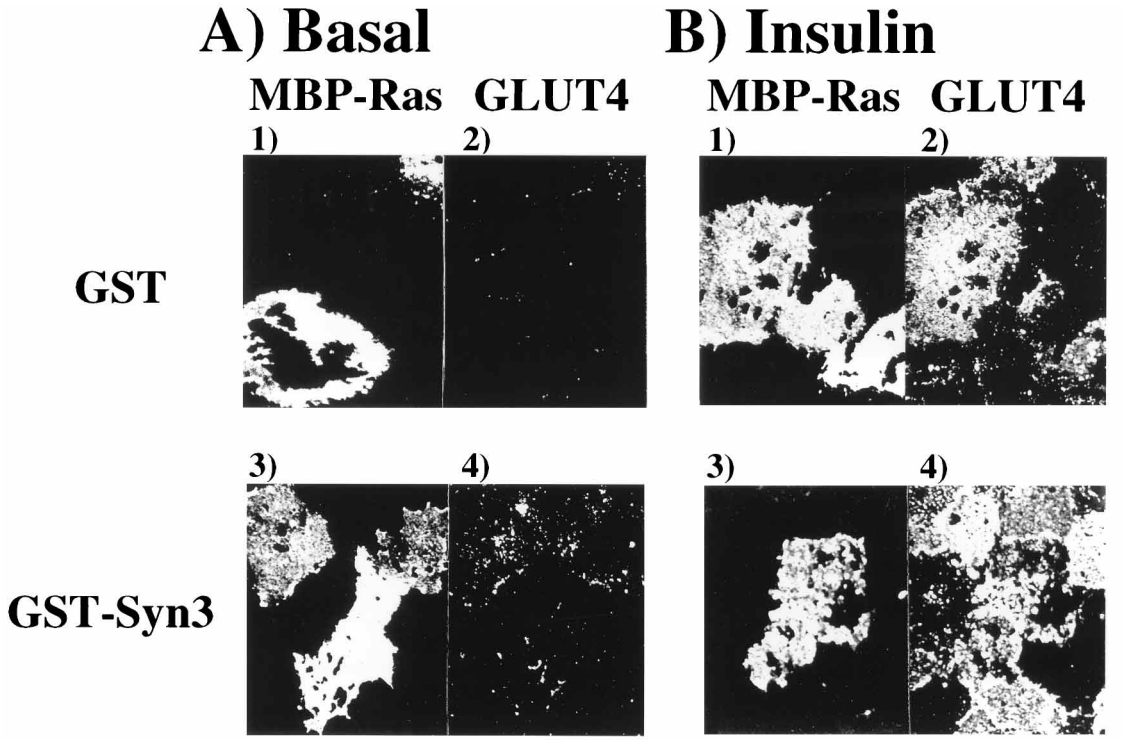

FIG. 5. Single-cell microinjection of GST or GST-syntaxin 3 fusion proteins (GST-Syn3) does not affect insulin-stimulated GLUT4 translocation. Fully differentiated 3T3L1 adipocytes were microinjected with 2 mg of MBP-Ras per ml mixed with 2 mg of GST per ml (panels 1 and 2) or 2 mg of GST-Syn3 fusion protein per ml (panels 3 and 4) as described in Materials and Methods. Following microinjection, the cells were either untreated (A) or stimulated with 100 nM insulin for 15 min at 37°C (B). Plasma membrane sheets were then prepared and subjected to confocal immunofluorescence microscopy with an MBP-specific antibody (panels 1 and 3) or a GLUT4-specific antibody (panels 2 and 4). These are representative fields from three independent experiments in which a total of 47 and 82 cells were scored for GLUT4 translocation in GST and GST-Syn3 microinjected cells, respectively. A total of 96% of the GST and 92% of the GST-Syn3 plasma membrane sheets displayed typical insulin-stimulated GLUT4 immunofluorescence.

translocation of GLUT4 to the isolated plasma membrane sheets (Fig. 4B, lanes 1 and 2). Again as observed in Fig. 2, 3, and 4A, expression of the syntaxin 4 cytoplasmic domain inhibited insulin-stimulated GLUT4 translocation (Fig. 4B, lanes 3 and 4). In parallel, vaccinia virus expression of the syntaxin 3 cytoplasmic domain had no significant effect on either the basal or insulin-stimulated translocation of the GLUT4 protein (lanes 5 and 6). Quantitation of these data are also presented in Fig. 3A, which confirm the inability of the syntaxin 3 cytoplasmic domain to inhibit insulin-stimulated GLUT4 translocation. These data demonstrate that the syntaxin 4 cytoplasmic domain, but not syntaxin 3, functions in a dominant-interfering manner for insulin-stimulated GLUT4 translocation and that the VAMP binding domain within syntaxin 4 is required for this effect.

Microinjection of GST-syntaxin fusion proteins into 3T3L1 adipocytes recapitulates the effects on GLUT4 translocation observed by recombinant vaccinia virus expression. The successful use of vaccinia virus infection as a means to examine insulin-stimulated GLUT4 translocation was based upon the broad host range of the virus in addition to the rapid induction of virus-encoded proteins under the control of immediate-early promoters. However, vaccinia virus is also a cytotoxic virus and, following infection, strongly inhibits protein synthesis from host mRNAs (34). Thus, even though infection of 3T3L1 adipocytes with vaccinia virus did not appear to alter the insulin regulation of GLUT4 trafficking, it remained possible, although unlikely, that the cytoplasmic domain of syntaxin 4 in concert with vaccinia virus infection was responsible for the inhibition of insulin-stimulated GLUT4 translocation. To address this issue, we used a completely independent approach consisting of single-cell microinjection of various GST fusion proteins followed by determination of GLUT4 translocation by confocal immunofluorescence analysis (Fig. 5).

To identify the specific 3T3L1 adipocytes that were microinjected following preparation of the plasma membrane sheets, the syntaxin GST fusion proteins were coinjected with the carboxyl-terminal domain of Ras fused to the maltose binding protein (MBP) (22). The ability to identify the plasma membrane sheets isolated from microinjected cells is demonstrated in Fig. 5. Following comicroinjection with GST and MBP-Ras, the isolated plasma membrane sheets demonstrated a specific MBP immunofluorescence signal from the single injected cell in this particular visualization field (Fig. 5A, panel 1). However, since the cells were unstimulated, no specific GLUT4 signal was detected (panel 2). Similarly, cells comicroinjected with GST and MBP-Ras and subjected to insulin stimulation resulted in the specific incorporation of the MBP-Ras marker into the plasma membrane sheets (Fig. 5B, panel 1). However, since these cells were stimulated with insulin prior to isolation of the plasma membrane sheets, there was a strong GLUT4-specific immunofluorescence signal in essentially all the cells visualized including the microinjected cells (panel 2). As an additional control, microinjection of the syntaxin 3 cytoplasmic domain as a GST fusion protein (GST-Syn3) had no significant effect on the basal levels of plasma membrane-associated GLUT4 in the microinjected cells (Fig. 5A, panels 3 and 4). Furthermore, the introduction of the GST-Syn3 fusion protein

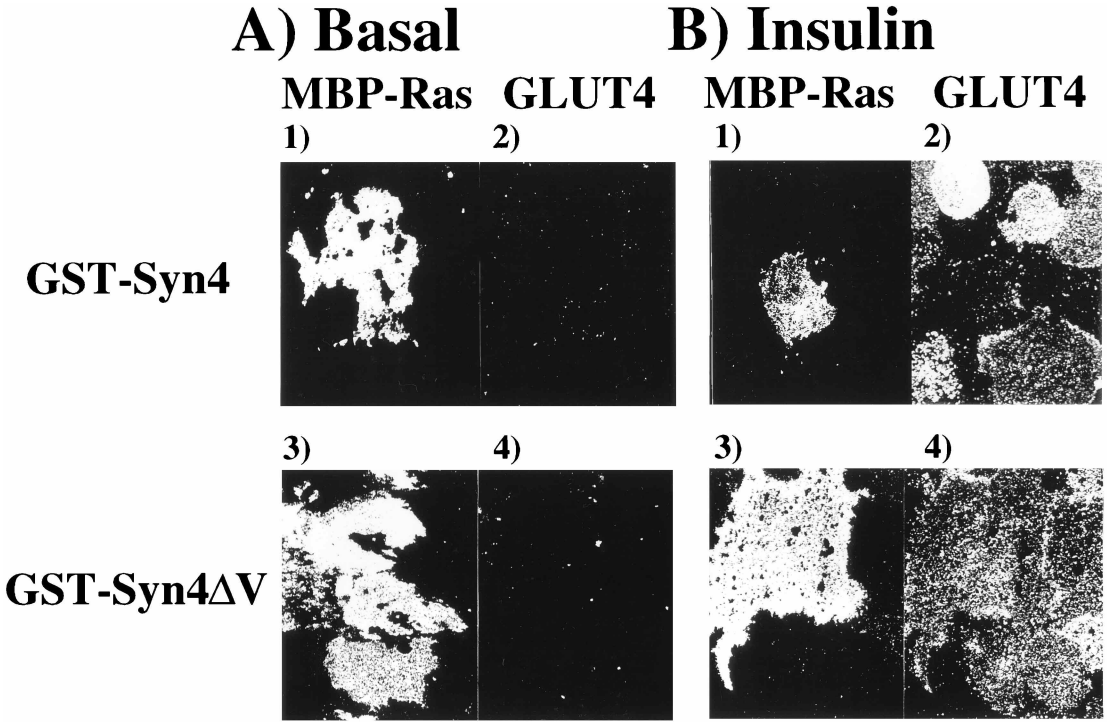

FIG. 6. Single-cell microinjection of GST-syntaxin 4 requires an intact VAMP binding site to inhibit GLUT4 translocation. Differentiated 3T3L1 adipocytes were microinjected with 2 mg of MBP-Ras per ml mixed with 2 mg of GST-Syn4 per ml (panels 1 and 2) or 2 mg of GST-Syn4ΔV fusion protein per ml (panels 3 and 4) as described in Materials and Methods. Following microinjection, the cells were either untreated (A) or stimulated with 100 nM insulin for 15 min at 37°C (B). Plasma membrane sheets were then prepared and subjected to confocal immunofluorescence microscopy with an MBP-specific antibody (panels 1 and 3) or a GLUT4 specific antibody (panels 2 and 4). These are representative fields from four GST-Syn4 and three (GST-Syn4ΔV) independent experiments in which a total of 155 and 63 cells were scored for GLUT4 translocation. Microinjection of GST-Syn4 inhibited GLUT4 translocation in 86% of the cells examined, whereas microinjection of GST-Syn4ΔV inhibited GLUT4 translocation in only 2% of the cells.

had no effect on the insulin-stimulation of GLUT4 translocation to the plasma membrane (Fig. 5B, panels 3 and 4). These data demonstrate that the single-cell microinjection method successfully recapitulated insulin-stimulated GLUT4 translocation and that introduction of the GST protein or GST fused to the cytoplasmic domain of syntaxin 3 did not affect the insulin stimulation of GLUT4 translocation.

We next examined the effect of microinjecting the cytoplasmic domain of syntaxin 4 on insulin-stimulated GLUT4 translocation in the 3T3L1 adipocytes (Fig. 6). As expected, microinjection of the cytoplasmic domain of syntaxin 4 fused to GST (GST-Syn4) did not affect the basal level of GLUT4 on the plasma membrane (Fig. 6A, panels 1 and 2). Consistent with vaccinia virus expression, introduction of the cytoplasmic domain of syntaxin 4 inhibited insulin-stimulated GLUT4 translocation in the microinjected cells but not in the surrounding noninjected cells (Fig. 6B, panels 1 and 2). Again in complete agreement with the vaccinia virus expression system, deletion of the VAMP binding domain (GST-Syn4ΔV) did not affect either the basal state (Fig. 6A, panels 3 and 4) or insulin stimulation of GLUT4 translocation to the plasma membrane (Fig. 6B, panels 3 and 4).

Coimmunoprecipitation of syntaxin 4 with GLUT4-containing vesicles. The functional requirement of syntaxin 4 in GLUT4 translocation implies that at some point during this process, the GLUT4-containing vesicles should interact with syntaxin 4. To assess this interaction, we again used the vaccinia virus system to express the *myc* epitope-tagged cytoplasmic domain of syntaxin 4 (Fig. 7). Immunoprecipitation of the *myc* epitope tag (9E10) from 3T3L1 adipocytes expressing the syntaxin 4 cytoplasmic domain (Syn4) demonstrated the coimmunoprecipitation of GLUT4 protein (Fig. 7A, lane 3). However, insulin treatment resulted in a greater extent of GLUT4 protein that was specifically coimmunoprecipitated with the cytoplasmic domain of syntaxin 4 (lane 4). To determine nonspecific absorption of the GLUT4 vesicles, the same samples were also subjected to immunoprecipitation with a control mouse monoclonal IgG (lanes 1 and 2). Although a small amount of GLUT4 vesicles bound nonspecifically, it was substantially less than that immunoprecipitated with the *myc* epitope-specific mouse monoclonal antibody (compare lanes 1 and 2 with lanes 3 and 4).

The specificity of the insulin-stimulated association between the GLUT4 vesicles and the syntaxin 4 cytoplasmic domain was also assessed in comparison to that of syntaxin 3 (Fig. 7B). Total-cell membrane vesicles were prepared from 3T3L1 adipocytes infected with vaccinia virus encoding either the *myc* epitope-tagged syntaxin 3 or syntaxin 4 cytoplasmic domains. Expression of these cytoplasmic domains had no effect on the cellular content of GLUT4 protein as detected by immunoblotting of the total-cell lysate (Fig. 7B, lanes 1 to 4). As observed in Fig. 7A, immunoprecipitation with the *myc* epitope-specific antibody (9E10) from the syntaxin 4 cytoplasmic domain-expressing cells resulted in an increased extent of GLUT4 coimmunoprecipitation from extracts of insulin-stimulated cells (Fig. 7B, lanes 7 and 8). However, there was no significant effect of insulin treatment on the amount of GLUT4 coimmunoprecipitated with the 9E10 antibody from cells expressing the *myc* epitope-tagged cytoplasmic domain of syntaxin 3 (lanes 5 and 6). These data further demonstrate that

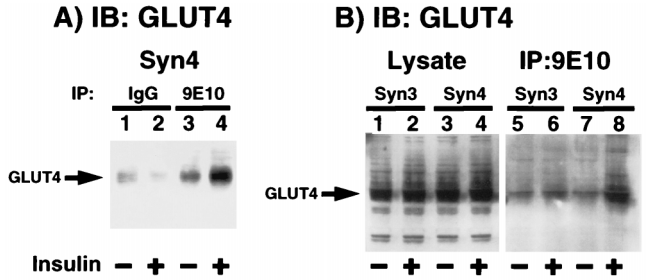

FIG. 7. Insulin stimulates the association of the syntaxin 4 cytoplasmic domain with the GLUT4-containing vesicles. (A) Differentiated 3T3L1 adipocytes were infected with the β-galactosidase-expressing recombinant vaccinia virus containing the cytoplasmic domain of syntaxin 4 (Syn4) fused to the *myc* epitope tag as described in Materials and Methods. Following 4 h of infection, the cells were either unstimulated (lanes 1 and 3) or stimulated with 100 nM insulin for 15 min at 37°C (lanes 2 and 4). Total-cell membranes were prepared and immunoprecipitated with either a control mouse monoclonal antibody (IgG; lanes 1 and 2) or the *myc* epitope-specific mouse monoclonal antibody (9E10; lanes 3 and 4). (B) Differentiated 3T3L1 adipocytes were infected with the β-galactosidase-expressing recombinant vaccinia virus containing either the cytoplasmic domain of syntaxin 3 (Syn3) or the cytoplasmic domain of syntaxin 4 (Syn4) fused to the *myc* epitope tag. Following 4 h of infection, the cells were either unstimulated (lanes 1, 3, 5, and 7) or stimulated with 100 nM insulin for 15 min at 37°C (lanes 2, 4, 6, and 8). The total-cell membranes were directly immunoblotted with a GLUT4 antibody (lanes 1 to 4) or initially immunoprecipitated with the *myc* epitope-specific mouse monoclonal antibody (9E10; lanes 5 to 8) followed by GLUT4 immunoblotting. These results are representative of three experiments for panel A and two experiments for panel B.

insulin induces an association of GLUT4 protein-containing vesicles with the expressed syntaxin 4 cytoplasmic domain but not with the syntaxin 3 cytoplasmic domain.

Insulin-dependent GLUT4 translocation is inhibited by expression of the VAMP cytoplasmic domain. In addition to the apparent colocalization of VAMPs in GLUT4-containing vesicles (6, 57), our data demonstrate that the VAMP binding domain of syntaxin 4 provides a necessary function for insulin-stimulated GLUT4 translocation. Based on these data, we next examined the effect of overexpression of both the VAMP2 and VAMP3/cellubrevin cytoplasmic domains (Fig. 8 and 9). Infection with the empty vaccinia virus had no significant effect on the basal level of GLUT4 protein detected on plasma membrane sheets by confocal immunofluorescence microscopy (Fig. 8A, panel 1). Consistent with the GLUT4 immunoblots (Fig. 2 to 4), there was a dramatic increase in plasma membrane-associated GLUT4 protein immunofluorescence following insulin stimulation (Fig. 8B, panel 1). As previously observed, under these conditions vaccinia virus infection did not alter the ability of the 3T3L1 adipocytes to translocate GLUT4 in response to insulin. Since there was little GLUT4 protein associated with the plasma membrane in the basal state, vaccinia virus expression of the cytoplasmic domain of VAMP2 had no effect (Fig. 8A, panel 2). In contrast, the cytoplasmic domain of VAMP2 greatly reduced the extent of insulin-stimulated GLUT4 translocation (Fig. 8B, panel 2). Similarly, expression of the VAMP3/cellubrevin cytoplasmic domain also reduced the extent of insulin-stimulated GLUT4 translocation (Fig. 9B, compare panels 1 and 2).

Quantitation of these results indicated that vaccinia virus expression of the VAMP2 and VAMP3 cytoplasmic domains increased the basal level of plasma membrane GLUT4 protein 1.4- and 2.0-fold, respectively (Fig. 10). Nevertheless, insulin induced a 5.5-fold increase in plasma membrane sheet GLUT4 immunofluorescence in vaccinia virus-infected cells but only 1.7- and 2.1-fold increases in 3T3L1 adipocytes expressing the VAMP2 and VAMP3 cytoplasmic domains, respectively. Thus, these data demonstrate that the cytoplasmic domains of either VAMP2 or VAMP3/cellubrevin also function in a dominant-

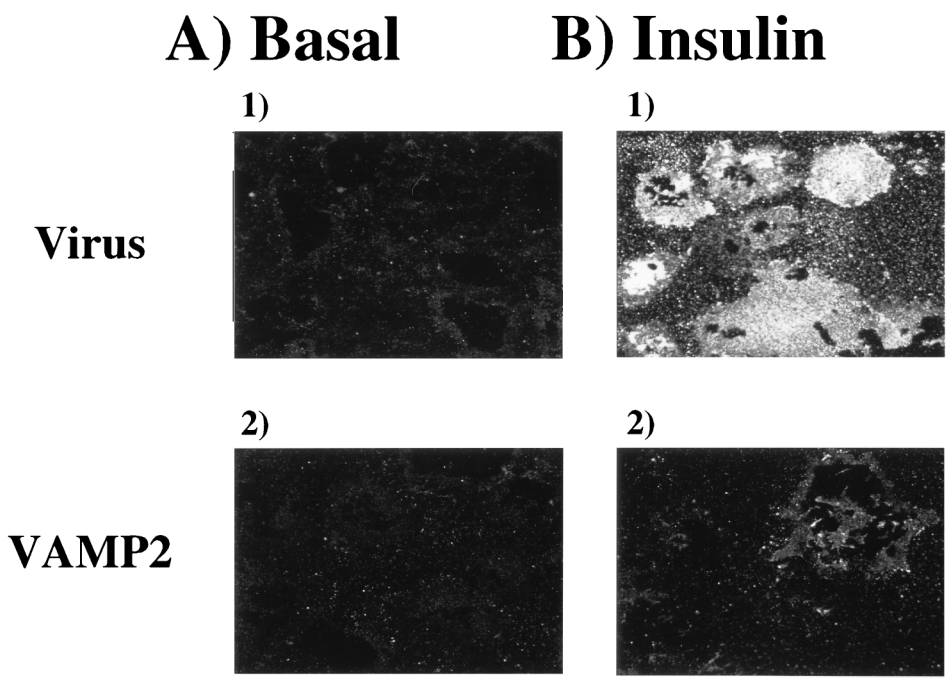

FIG. 8. Vaccinia virus-expressed cytoplasmic domain of VAMP2 inhibits insulin-stimulated GLUT4 translocation. Differentiated 3T3L1 adipocytes were infected with the β-galactosidase-expressing recombinant vaccinia virus (Virus; panels 1) or the recombinant vaccinia virus containing the cytoplasmic domain of VAMP2 (VAMP2; panels 2). Following 4 h of infection, the cells were either unstimulated (A) or stimulated with 100 nM insulin for 15 min at 37°C (B). Plasma membrane sheets were then prepared and subjected to confocal immunofluorescence microscopy with the GLUT4 specific antibody as described in Materials and Methods. This is a representative field from three independent experiments (see the legend to Fig. 10).

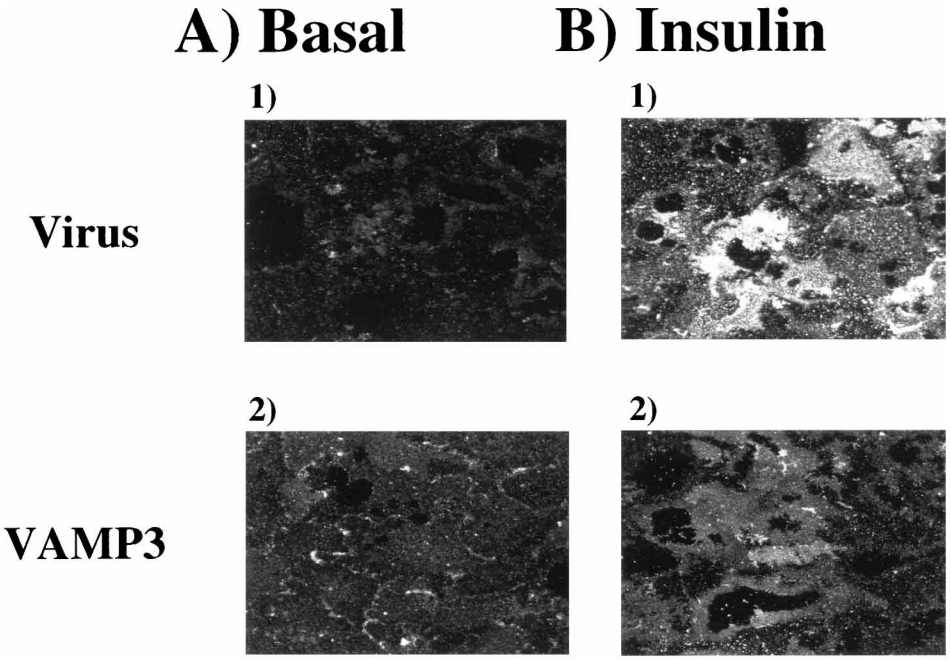

FIG. 9. Vaccinia virus-expressed cytoplasmic domain of cellubrevin inhibits insulin-stimulated GLUT4 translocation. Differentiated 3T3L1 adipocytes were infected with the β-galactosidase-expressing recombinant vaccinia virus (Virus; panels 1) or the recombinant vaccinia virus containing the cytoplasmic domain of VAMP3/cellubrevin (VAMP3; panels 2). Following 4 h of infection, the cells were either unstimulated (A) or stimulated with 100 nM insulin for 15 min at 37°C (B). Plasma membrane sheets were then prepared and subjected to confocal immunofluorescence microscopy with the GLUT4-specific antibody as described in Materials and Methods. This is a representative field from three independent experiments (see the legend to Fig. 10).

interfering manner by inhibiting insulin-stimulated GLUT4 translocation.

DISCUSSION

Intracellular vesicular trafficking pathways are essential processes for regulation of multiple events including the endocytosis of extracellular and cell surface molecules, movement of cargo from the endoplasmic reticulum to and through the Golgi stack, and release of prestored vesicular components by fusion with the plasma membrane. Although these appear to be a diverse set of signaling events, over the past few years several common mechanistic themes that are fundamental to all intracellular trafficking steps have emerged. In the case of neurotransmitter release from the active zone at the presynaptic membrane, specific proteins have been identified in both the cargo-containing vesicle (v-SNAREs) and plasma membrane (t-SNAREs) that are required for this docking/fusion mechanism (2, 4, 5, 24). The presence of these proteins or closely related isoforms in nonneuronal tissues strongly suggests a generalized role for these proteins in vesicle trafficking (8).

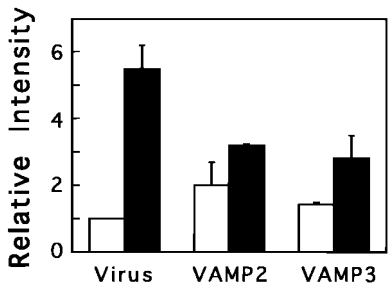

FIG. 10. Quantitation of the inhibition of GLUT4 translocation by expression of the VAMP2 and VAMP3 cytoplasmic domains. 3T3L1 adipocytes were either infected with a vaccinia virus that expresses the *lacZ* gene (Virus, $n = 3$), the cytoplasmic domain of VAMP2 ($n = 3$), or the cytoplasmic domain of VAMP3 ($n = 3$). The cells were then treated without (open boxes) or stimulated with (solid boxes) 100 nM insulin for 15 min, and plasma membrane sheets were isolated and subjected to GLUT4 immunofluorescence as described in Materials and Methods. The relative signal intensity per sheet surface area was determined with NIH 1.6 image analysis software.

It has been well established that the acute insulin stimulation of glucose transport in adipocytes results from a translocation of a specific glucose transporter isoform (GLUT4) from an intracellular vesicular storage to the plasma membrane (9, 16, 23, 53). Similar to synaptic vesicles, subcellular fraction and coimmunoprecipitation studies have demonstrated that GLUT4 vesicles colocalize with the VAMPs VAMP2 and VAMP3/cellubrevin (6, 57). In addition, both synaptic and GLUT4 vesicle trafficking require the functional association with Rab family members of the small GTP binding proteins, Rab3A and Rab4, respectively (14, 15, 20, 44). These features suggest that molecular events regulating synaptic vesicle docking and fusion may have some commonality with those of the insulin-stimulated GLUT4 vesicle translocation.

Previous studies have reported that syntaxins 1a and 1b are neuron specific and are not present in tissues that display insulin-responsive glucose transport activity (2, 58). In addition, syntaxin 5 has been localized to the *cis*-Golgi, suggesting that it plays a role in the trafficking of vesicles from the endoplasmic reticulum (18). Furthermore, in vitro binding studies have demonstrated that VAMPs specifically bind to syntaxin 1a, syntaxin 1b, and syntaxin 4 but not to syntaxin 2 or syntaxin 3 (7, 35). Thus, based on a model in which specific v-SNARE–t-SNARE interactions would be necessary for insulin-stimulated GLUT4 translocation through the VAMPs, we reasoned that syntaxin 4 would be the most logical candidate as a func-

tional t-SNARE for GLUT4 translocation. Consistent with this hypothesis, Northern blot analysis demonstrated that the differentiated 3T3L1 adipocytes express predominantly syntaxins 2 and 4 with substantially lower levels of syntaxin 3. These findings are in agreement with those of recent immunoblot analyses indicating the presence of syntaxin 2, 3, and 4 proteins in adipocytes (55, 58).

Thus, to investigate the potential functional role of syntaxin 4 in insulin-stimulated GLUT4 translocation, we took advantage of the observation that expression of the cytoplasmic domain of syntaxin 1 can function as a dominant-interfering mutant for neurotransmitter release (2). This presumably results from the competition of the soluble syntaxin fragment with the plasma membrane-bound syntaxin for the vesicle-associated v-SNARE proteins (VAMP1 and VAMP2). This is also consistent with specific interactions between VAMP2 and syntaxin 4 demonstrated through use of the yeast two-hybrid system (25) and through in vitro binding studies with bacterially expressed proteins (7, 35).

In any case, we used two completely independent approaches (infection with recombinant vaccinia virus and single-cell microinjection) to introduce various syntaxin mutants in the 3T3L1 adipocyte cell line. In both expression systems, only the cytoplasmic domain of syntaxin 4 inhibited insulin-stimulated GLUT4 translocation. Control experiments confirmed the specificity for syntaxin 4, since syntaxin 3 had no effect. Furthermore, this inhibition was specific for the VAMP binding domain within syntaxin 4, since deletion of this region did not result in inhibition of insulin-stimulated GLUT4 translocation. In addition, the role of syntaxin 4 in the intracellular vesicle trafficking of adipocytes appears to be relatively specific for GLUT4 vesicles, since there was no inhibition of the plasma membrane translocation of GLUT1.

Unlike GLUT4, the GLUT1 isoform is part of a constitutively recycling pathway that is only moderately responsive to insulin (23). In primary rat adipocytes, the GLUT1 vesicle population is largely distinct from the GLUT4 compartment both functionally and morphologically (30, 63). However, in differentiated 3T3L1 adipocytes, these two compartments are not completely divergent, since a small fraction of the GLUT1 pool has been localized to GLUT4-containing vesicles, the extent of which appears to be dependent on cell culture conditions or clonal variability (36, 37). Thus, the difference in the susceptibility of GLUT1 and GLUT4 translocation to inhibition by syntaxin 4 in the present study was somewhat surprising. It is possible that different cell lines or degrees of differentiation affect the portion of insulin-regulatable GLUT4 protein that colocalizes to the same vesicle compartment containing GLUT1. Alternatively, the low degree of insulin-stimulated GLUT1 translocation may occur through a vesicular trafficking population distinct from that used by GLUT4. In either case, the data presented in this study clearly demonstrates that syntaxin 4, but not syntaxin 3, has the characteristics of a t-SNARE for insulin-stimulated GLUT4 translocation to the plasma membrane.

Having established that the syntaxin 4 cytoplasmic domain can inhibit insulin-stimulated GLUT4 translocation, we used a similar approach to identify potential v-SNAREs that are functionally important in GLUT4 translocation. Based on the known presence of both VAMP2 and VAMP3/cellubrevin in the GLUT4 vesicles, expression of the cytoplasmic domain of either VAMP2 or VAMP3/cellubrevin also inhibited insulin-stimulated GLUT4 translocation. These data are consistent with the requirement of an intact VAMP binding domain within the syntaxin 4 for it to function in a dominant-interfering manner. Furthermore, these findings are in excellent agreement with recent studies demonstrating that relatively specific proteolysis of VAMP2 or VAMP3 by *Clostridium botulinium* neurotoxins can abrogate insulin-stimulated GLUT4 translocation (11, 54). Together, these data strongly implicate a functional interaction between syntaxin 4 and the VAMPs in the regulation of GLUT4 trafficking.

In this regard, it has been reported that the addition of recombinant NSF to solubilized plasma membranes and GLUT4 vesicle proteins results in the formation of a complex containing NSF, syntaxin 4, VAMP2, and VAMP3 (55). Thus, it is reasonable to speculate that one important function of insulin is to promote the formation of a docking complex between these proteins in a manner analogous to that observed for the docking of t- and v-SNARE proteins associated with synaptic vesicle fusion. Importantly, we have observed an insulin-stimulated increase in the amount of syntaxin 4 cytoplasmic domain that associates with GLUT4-containing vesicles in vivo. We estimate that under these conditions, the syntaxin 4 cytoplasmic domain was coimmunoprecipitated with approximately 10% of the GLUT4 protein. Although we have not identified the membrane compartment containing the GLUT4 protein that is coimmunoprecipitated with the syntaxin 4 cytoplasmic domain, this interaction is indirect as detergent solubilization prevents the coimmunoprecipitation of GLUT4 with the syntaxin 4 cytoplasmic domain (data not shown).

Finally, we wish to emphasize that in these studies we have determined the relative insulin-stimulated increase of GLUT4 and GLUT1 proteins in isolated plasma membrane sheets either by immunofluorescence or by immunoblotting. In accordance with previous reports, we have operationally defined the appearance of immunoreactive GLUT4 or GLUT1 protein on the plasma membrane as translocation. However, at present it is unclear whether this assay system can distinguish between docking and/or fusion with the plasma membrane, where docking refers to specific membrane association and fusion indicates a physical incorporation into the plasma membrane. Further development of this assay system will be necessary to distinguish between these two events. Nevertheless, the data presented in this study provides direct functional analysis demonstrating the participation of syntaxin 4, VAMP2, and VAMP3/cellubrevin binding sites in the regulation of insulin-stimulated translocation of GLUT4 intracellular vesicles with the plasma membrane.

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A.L.O. and J.B.K. contributed equally to this study.

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