Regulation of Insulin-stimulated GLUT4 Translocation by Munc18c in 3T3L1 Adipocytes*

(Received for publication, August 11, 1998, and in revised form, September 28, 1998)

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Insulin stimulates glucose transporter (GLUT) 4 vesicle translocation from intracellular storage sites to the plasma membrane in 3T3L1 adipocytes through a VAMP2- and syntaxin 4-dependent mechanism. We have observed that Munc18c, a mammalian homolog of the yeast syntaxin-binding protein n-Sec1p, competed for the binding of VAMP2 to syntaxin 4. Consistent with an inhibitory function for Munc18c, expression of Munc18c, but not the related Munc18b isoform, prevented the insulin stimulation of GLUT4 and IRAP/vp165 translocation to the plasma membrane without any significant effect on GLUT1 trafficking. As expected, overexpressed Munc18c was found to co-immunoprecipitate with syntaxin 4 in the basal state. However, these complexes were found to dissociate upon insulin stimulation. Furthermore, endogenous Munc18c was predominantly localized to the plasma membrane and its distribution was not altered by insulin stimulation. Although expression of enhanced green fluorescent protein-Munc18c primarily resulted in a dispersed cytosolic distribution, co-expression with syntaxin 4 resulted in increased localization to the plasma membrane. Together, these data suggest that Munc18c inhibits the docking/fusion of GLUT4-containing vesicles by blocking the binding of VAMP2 to syntaxin 4. Insulin relieves this inhibition by inducing the dissociation of Munc18c from syntaxin 4 and by sequestering Munc18c to an alternative plasma membrane binding site.

The binding of insulin to its heterotetrameric integral-membrane receptor activates its intracellular tyrosine kinase domain and thereby triggers a signaling cascade resulting in the translocation and fusion of intracellular GLUT41 isoform-containing vesicles to the plasma membrane (1–3). Although most cell types also constitutively express the GLUT1 isoform at the cell surface, the insulin-stimulated increase in plasma membrane-associated GLUT4 protein accounts for the majority of post-prandial glucose disposal in both muscle and adipose tissue (4).

The insulin-stimulated translocation of these GLUT4-containing vesicles has several features in common with the regulated exocytosis pathway of synaptic vesicle trafficking in neurotransmitter release (5). The machinery involved in the regulation of synaptic vesicle priming/docking/fusion entails the pairing of protein complexes in the vesicle compartment (v-SNAREs, for vesicle SNAP receptors) with their cognate receptor complexes at the target membrane (t-SNAREs, for target membrane SNAP receptors). Recently, several of the v- and t-SNARE proteins have been identified that specifically participate in the insulin-regulated docking and fusion of GLUT4 vesicles with the adipocyte plasma membrane. GLUT4 vesicles co-purify with both the VAMP2 and VAMP3 v-SNARE isoforms and specific proteolytic cleavage of VAMP2, expression of a dominant-interfering VAMP2 mutant or inhibitory peptides impairs insulin-stimulated GLUT4 translocation (6–11). In addition, transferrin-horseradish peroxidase ablation of recycling endosomes resulted in a selective loss of GLUT4 vesicles containing VAMP3 but did not prevent the insulin stimulation of GLUT4 to the plasma membrane (11). Although there are no specific endoproteases that cleave either syntaxin 4 or SNAP23/Syndet plasma membrane t-SNARE proteins, studies using blocking antibodies, dominant-interfering mutants, and/or peptide inhibitors have strongly implicated syntaxin 4 and SNAP23/Syndet as the required t-SNARE proteins for insulin-stimulated GLUT4 translocation (10, 12–14). Thus, these data provide compelling evidence that VAMP2 functions by directing the association of the GLUT4-containing vesicles with syntaxin 4 and SNAP23/Syndet.

In the case of synaptic transmission, there are also several modulators of v- and t-SNARE interactions (15–18). In particular, the mammalian Munc18 proteins are homologous to the Saccharomyces cerevisiae n-Sec1/rbSec1, Caenorhabditis elegans unc18, and Drosophila melanogaster Rop proteins (19–23). These proteins bind with high affinity to their cognate plasma membrane syntaxins, and null mutations in these genes cause dramatic reduction in vesicle exocytosis, suggesting that these proteins are essential for normal v- and t-SNARE function (24, 25). In mammals, the Munc18a isoform is predominantly expressed in neurons, where it inhibits the association of VAMP1 and SNAP25 with syntaxin 1 (21, 26). In contrast to Munc18a, Munc18b and Munc18c are expressed ubiquitously but only Munc18c binds syntaxin 4 with high affinity (27, 28).

Based upon these data, we have now investigated the functional role of Munc18c in modulating the insulin stimulation of GLUT4 vesicle translocation. In this report we demonstrate that Munc18c specifically competes for VAMP2 binding to syn-
taxin 4 and increased expression of Munc18c in 3T3L1 adipocytes inhibits GLUT4 translocation to the plasma membrane. The Munc18c inhibition was specific for GLUT4-containing vesicles, as there was no effect upon GLUT1 vesicle trafficking. Furthermore, insulin stimulation results in an uncoupling of the Munc18c-syntxin 4 complex. These data are consistent with a model in which Munc18c inhibits GLUT4 translocation in the basal state and with the hypothesis that insulin stimulation results in a de-repression of Munc18c function.

EXPERIMENTAL PROCEDURES

Materials—The GLUT4 and syntaxin 4 antibodies were isolated as described previously (10, 29). The Munc18c polyclonal antibody was obtained by immunization of a carboxyl-terminal synthetic peptide as described previously (13). VAMP2 and VAMP3 antibodies were prepared by immunization of sheep with glutathione-S-transferase fusion proteins containing the cytoplasmic domains of VAMP2 (amino acids 1–94) and VAMP3 (amino acids 1–81). Monoclonal antibody to the Flag epitope (M2) was purchased from Eastman Kodak Corp. Lissamine rhodamine-conjugated donkey anti-rabbit IgG was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Hypermune-conjugated donkey anti-mouse IgG was obtained from Amersham Pharmacia Biotech. Supersignal Ultra ECL reagent was purchased from Pierce. Yeast dropout media were obtained from Amresco. HEPES buffer was purchased from the American Type Tissue Culture repository and cultured in DMEM containing 25 mM glucose and 10% calf serum at 37 °C and 5% CO2. At confluence cells were differentiated by incubation in medium containing 25 mM glucose, 10% fetal bovine serum 1 μg/ml insulin, 1 mM dexamethasone, and 0.5 mM isobutyl-1-methylxanthine. After four days the medium was changed to DMEM, 25 mM glucose, and 10% fetal bovine serum. Differentiated adipocytes were used for the experiments 12 days after initiation of differentiation and were placed in serum-depleted medium for 3 h prior to insulin stimulation. Chinese hamster ovary cells expressing the human insulin receptor (CHO/IR) were obtained as described previously (30). CHO/IR cells were incubated in minimal Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO2. Fully confluent CHO/IR cells were transiently transfected by electroporation (0.34 kV and 960 microfarads) with 40 μg of plasmid DNA/cuvette as described previously (31). Following electroporation, the cells were allowed to adhere to 10-cm tissue culture dishes for 40–48 h and were then serum-starved for 6 h prior to stimulation with 100 nM insulin at 37 °C. Differentiated adipocytes were transfected by a modification of the electroporation method. Briefly, fully differentiated adipocytes were electroporated (0.16 kV and 960 microfarads) with 50 μg of the eGFP-tagged plasmids plus 200 μg of yeast dominant-negative (SNAP) repressor plasmid (32). Cells were treated with 0.55 mg/ml poly-L-lysine followed by three washes with hypotonic buffer (23 mM KCl, 10 mM HEPES, pH 7.5, 1 mM MgCl2, 1 mM EGTA). The cells were placed in sonication buffer (3× hypotonic buffer plus 100 μM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol) and sonicated with a Fisher probe membrane disrupter. Plasma membrane sheets were washed three times with sonication buffer and fixed in 2% paraformaldehyde for 20 min on ice. The fixed plasma membrane sheets were quenched for 15 min at 25 °C in 100 mM glycine. The isolated plasma membrane sheets were washed and blocked with 5% donkey serum for 45 min at 37 °C, followed by incubation with GLUT4 antibody (1:100 dilution of antisera) or GLUT1 antibody (1:50 dilution) for 15 h at 4 °C. Plasma membrane sheets were washed three times with PBS and then incubated in anti-rabbit (1:50 dilution) or anti-mouse (1:20 dilution) secondary antibody and Lissamine rhodamine-conjugated donkey anti-rabbit secondary antibody for 4 h in the dark. Washed three times with PBS, overlaid with a drop of Vectashield, and visualized by confocal fluorescence microscopy.

Subcellular Fractionation—Adipocyte subcellular membrane fractions were obtained using the differential centrifugation method described by Piper et al. (33). Briefly, control and insulin-stimulated 3T3L1 adipocytes were washed twice with PBS, and plasma membrane sheets were prepared as described by Robinson et al. (32). Cells were treated with 0.55 mg/ml poly-L-lysine followed by three washes with hypotonic buffer (23 mM KCl, 10 mM HEPES, pH 7.4, 1 mM EDTA, and 255 mM sucrose containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 5 μg/ml leupeptin). Cell lysates were prepared by shearing the cells through a 22-gauge needle 10 times. Lysates were then centrifuged at 19,000 × g for 20 min at 4 °C. The low speed pellet (LSP) was obtained by centrifugation of the resulting supernatant at 41,000 × g for 20 min at 4 °C. Supernatant was removed and centrifuged at 180,000 × g for 75 min at 4 °C to generate the high speed pellet and cytosol (supernatant) fractions. The crude plasma membrane pellet was obtained by resuspending the pellet from the initial 19,000 × g centrifugation in HES buffer followed by layering onto a 1.2 M sucrose cushion for centrifugation at 100,000 × g for 60 min. The plasma membrane layer was removed from the sucrose cushion and centrifuged at 40,000 × g for 20 min. All pelleted fractions were resuspended in HES buffer and assayed for soluble protein content (34). Fractions were subjected to electrophoresis on 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with antibodies indicated in the individual figure legends.

Immunoprecipitation and Immunoblotting—Whole cell detergent extracts were prepared by solubilization in an Nonidet P-40 lysis buffer (25 mM Tris, pH 7.4, 1% Nonidet P-40, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 137 mM sodium chloride, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 5 μg/ml leupeptin) for 10 min at 4 °C. Insoluble material was separated from the soluble extract by microcentrifugation for 10 min at 4 °C. Whole cell lysate (2–4 mg) was combined with 4.5 μg...
of affinity-purified sheep syntaxin 4 antibody for 2 h at 4°C, followed by a second incubation with protein A-Sepharose for 2 h. The resultant immunoprecipitates were subjected to electrophoresis on 10% SDS-PAGE, followed by transfer to polyvinylidene difluoride and immunoblotting.

**Yeast Two- and Three-hybrid Analyses**—The LexA two-hybrid system, originally described by Gyuris et al. (35), was used here to examine interactions between known proteins. This two-hybrid system employs the use of three vectors: pLexA or “bait,” pB42AD or “prey,” and the p8op-lacZ reporter plasmid. The bait vector is used to generate fusions of the DNA binding domain of LexA fused upstream of an in-frame cDN of interest. The prey vector generates fusions of a cassette consisting of the SV40 nuclear localization sequence, the 88-residue B42 acidic activator domain, and the hemagglutinin epitope tag. The reporter p8op-lacZ carries the lacZ reporter gene under the control of eight LexA operators and the minimal TATA region from the GAL1 promoter. p8op-lacZ exhibits no transcriptional activity in the absence of a LexA-fused activator. Syntaxin 4 was used as bait and co-transformed with the p8op-lacZ reporter vector into the pEGY48 strain of yeast cells. The bait vector and the reporter plasmid carry HIS3 and URA3 transformation markers, respectively. The LEU2 reporter gene is under absolute control of the LexA operators. The bait showed no intrinsic transcriptional activation in the absence of prey, and was shown to enter the yeast nucleus and bind the operators.

Yeast transformants carrying the bait reporter plasmids were made competent and transformed with various prey constructs as indicated, and plated with selective dropout media containing glucose, nitrogen source, and all amino acids minus uracil, histidine, and tryptophan (the prey vector carries a TRP1 transformation marker). After 2 days at 30°C, plates were replica plated onto four plates containing X-gal: glucose Ura-His-Trp-, glucose Ura-His-Trp-Leu-, gal/raf Ura-His-Trp-, and gal/raf Ura-His-Trp-Leu- and scored for interaction. Plates showing white colonies on glucose Ura-His-Trp-, no growth on glucose Ura-His-Trp-Leu-, and all blue colonies on the gal/raf media were given a score of +++++ as an indication of high level of interaction as measured by lacZ expression. No blue color was scored as −, while 25, 50, and 75% dark blue color were scored as +, ++, and ++++, respectively. lacZ expression as determined by liquid assay using o-nitrophenyl-β-D-galactopyranoside as substrate was consistent with our whole plate assay results shown above are representative of three independent sets of 3T3L1 adipocyte subcellular fractionations.

**RESULTS**

**Subcellular Distribution of v- and t-SNARE Proteins in 3T3L1 Adipocytes**—To examine the intracellular localization of Munc18c in comparison to GLUT4 and several SNARE proteins involved in insulin-stimulated GLUT4 translocation, we used differential centrifugation to isolate subcellular fractions from 3T3L1 adipocytes (Fig. 1). Immunoblot analysis demonstrated the presence of the GLUT4 protein in the whole cell extract, which was unaffected by insulin treatment (Fig. 1, lanes 1 and 2). GLUT4 protein was detected in the low speed pellet, high speed pellet, and plasma membrane fractions but was completely absent from the cytosol fraction (Fig. 1, lanes 3–10). Although insulin did not have a significant effect on the distribution of GLUT4 in the low speed pellet fraction (Fig. 1, lanes 3 and 4), insulin stimulation resulted in a loss of GLUT4 protein in the high speed pellet fraction with an increase in the amount of GLUT4 protein in the plasma membrane fraction (Fig. 1, lanes 7–10). The decrease of GLUT4 in the high speed pellet fraction concomitant with its recruitment to the plasma membrane is characteristic of insulin-stimulated GLUT4 translocation (2, 5, 36).

Similar to GLUT4, VAMP2 and VAMP3 were localized to all the particulate fractions and were absent from cytosol fractions. Interestingly, the relative proportion of VAMP3 in both the low speed pellet and high speed pellet fractions (Fig. 1, lanes 3, 4, 7, and 8) compared with the plasma membrane fraction (Fig. 1, lanes 9 and 10) was significantly greater than that for VAMP2. This is consistent with VAMP3 being more localized to endosomes, which are enriched in the low speed pellet fraction (37). Furthermore, insulin stimulation resulted in a more marked translocation of VAMP2 from the high speed pellet to the plasma membrane fraction than VAMP3 (Fig. 1, lanes 7–10). These data are consistent with VAMP2 being predominantly localized to the insulin-responsive GLUT4 vesicle population distinct from the recycling endosome pool (11).

In contrast to these vesicular proteins, syntaxin 4 and Munc18c were primarily localized to the plasma membrane fraction, although a relatively small amount of Munc18c was found in the cytosol (Fig. 1, lanes 3 and 6). However, there was no significant redistribution of either syntaxin 4 or Munc18c following insulin stimulation (Fig. 1, lanes 7–10). Since Munc18c does not contain any domains directly responsible for membrane targeting, its localization to the plasma membrane probably reflects its interaction with other plasma membrane-bound proteins including syntaxin 4 (27, 38, 39).

**Munc18c Competes for VAMP2 and SNAP23 Binding to Syntaxin 4**—In vitro binding assays have indicated that Munc18c can selectively bind to syntaxin 2 and syntaxin 4 (28). To further investigate the interaction of syntaxin 4 with Munc18c, we used yeast two- and three-hybrid assay systems (Fig. 2). Initially, we examined the relative interaction of a syntaxin 4 LexA DNA binding domain fusion (bait) with several individual SNARE proteins. Syntaxin 4 specifically interacted with Munc18c activation domain fusion (prey) but not with the related Munc18b isoform (Fig. 2A). Similarly, VAMP2 displayed a specific positive interaction with syntaxin 4, whereas there was no detectable interaction between syntaxin 4 and VAMP3. In addition, both SNAP23 and αSNAP demonstrated strong interactions with syntaxin 4. The apparent relatively weak interaction of VAMP2 with syntaxin 4 is consistent with previous studies demonstrating that, for the neuronal isoforms, high affinity VAMP1 binding requires the bimolecular complex of syntaxin 1-SNAP25 (40).
Munc18c: A Repressor of GLUT4 Trafficking

Expression of Munc18c Inhibits Insulin-stimulated GLUT4 and IRAP/VP165 Translocation—Previous studies have indicated that the interaction between syntaxin 4 and Munc18c (Fig. 2B). As previously observed, syntaxin 4 strongly interacted with the Munc18c activation domain fusion protein, which was inhibited by expression of Munc18c itself but not Munc18b. In addition, VAMP2 but not VAMP3 effectively competed for the binding to syntaxin 4 by glutamate-inducible conditions. One hundred % of colonies as dark blue in color were scored as + + + , 75% as + + , 50% as + , 25% as +, and 0% as −. This scoring was shown to be representative, as determined by comparison of results with liquid assay using 3-nitrophenyl-β-d-galactopyranoside as substrate. B, the prey vector used above was modified to express both the Munc18c prey protein in addition to a non-fusion competitor protein as described under “Experimental Procedures.” The three-hybrid vector expressing only the Munc18c prey fusion protein (Vector) served as the negative control for competition, and 100% of all colonies were dark blue in color (+ + + ). Competition by Munc18c itself served as the positive control for competition, leaving 50% or fewer colonies expressing lacZ (−). Protein expression was verified by immunoblotting and was similar in all cases (data not shown). All experiments were tabulated based upon at least three independent transformations with 100–300 transformants/plate.

Since Munc18c and VAMP2 have been hypothesized to compete for the binding to syntaxin 4, we established a three-hybrid assay system to examine the ability of additional proteins to modulate the interaction between syntaxin 4 and Munc18c (Fig. 2B). As previously observed, syntaxin 4 strongly interacted with the Munc18c activation domain fusion protein, which was inhibited by expression of Munc18c itself but not Munc18b. In addition, VAMP2 but not VAMP3 effectively competed for syntaxin 4 binding to Munc18c. In contrast, αSNAP did not interfere with the interaction between syntaxin 4 and Munc18c, whereas expression of SNAP23 had only a modest effect. These data suggest that the interaction of syntaxin 4 with Munc18c and VAMP2 is mutually exclusive and that Munc18c functions as a competitor for VAMP2 binding to syntaxin 4.

Expression of Munc18c Inhibits Insulin-stimulated GLUT4 and IRAP/VP165 Translocation—Previous studies have indicated that the interaction between syntaxin 4 with VAMP2 is required for insulin-stimulated GLUT4 translocation to the plasma membrane (10–12, 28). Since Munc18c appears to compete for VAMP2 binding, we next assessed the effect of Munc18c expression on GLUT4 translocation using a GLUT4-eGFP fusion protein (Fig. 3). Fully differentiated 3T3L1 adipocytes were co-electroporated with the GLUT4-eGFP construct and the empty pcDNA3 vector (Fig. 3, panels a and b). In the absence of insulin, GLUT4-eGFP was present as punctate vesicular structures throughout the cell interior without any evidence for plasma membrane localization (Fig. 3, panel a). However, following insulin stimulation, there was a clear fluorescence signal around the cell surface membrane indicative of GLUT4 translocation (Fig. 3, panel b). Similarly, co-expression of Munc18c had no effect on the intracellular distribution of GLUT4-eGFP in the basal state or on the insulin-stimulated translocation to the plasma membrane (Fig. 3, panels c and d). Although co-expression of Munc18c did not alter the localization of GLUT4-eGFP in the absence of insulin, there was a marked reduction of insulin-stimulated plasma membrane fluorescence (Fig. 3, panels e and f). A comparison of GLUT4-eGFP localization in three independent experiments demonstrated that co-transfection with pcDNA3 or Munc18b resulted in 93% of the expressing adipocyte cell population displaying insulin-stimulated GLUT4 translocation, which was inhibited to 18% by co-transfection with Munc18c.

Previous studies have documented that GLUT4 translocation can be readily assessed by determining the appearance of GLUT4 immunoreactivity in isolated plasma membrane sheets (32, 41). To confirm the effects of Munc18c on the endogenous GLUT4 protein, we isolated plasma membrane sheets and determined GLUT4 translocation using an endofacial GLUT4 antibody (Fig. 4). In the basal state, there was no apparent GLUT4 immunofluorescence in plasma membrane sheets isolated from 3T3L1 adipocytes quantitatively transfected with pcDNA3, Munc18b, or Munc18c (Fig. 4, panels a, c, and e). As expected, insulin stimulation resulted in a marked increase in GLUT4 immunofluorescence in the plasma membrane sheets isolated from both pcDNA3- and Munc18b-transfected adipocytes (Fig. 4, panels b and d). However, expression of Munc18c dramatically inhibited the extent of endogenous GLUT4 immunofluorescence in the isolated plasma membrane sheets from insulin-stimulated cells (Fig. 4, panel f).

In addition to GLUT4, these translocating vesicles are also enriched with a 165-kDa aminopeptidase termed vp165 or IRAP (42–46). Thus, to determine if the inhibitory effect of Munc18c was recapitulated for other cargo specific to the insulin-responsive vesicle population, we examined the translocation of an eGFP-IRAP/vp165 fusion protein (Fig. 5). Similar to GLUT4-eGFP, co-expression of eGFP-IRAP/vp165 with the empty vector, Munc18b, or Munc18c demonstrated essentially...
Increased expression of Munc18c inhibits insulin-stimulated translocation of endogenous GLUT4 in 3T3L1 adipocytes.

Differentiated 3T3L1 adipocytes were electroporated with 600 µg of the empty vector (pcDNA3), pcDNA3-Flag-Munc18b (Munc18b), or pcDNA3-Flag-Munc18c (Munc18c) and allowed to recover for 48 h on collagen-coated tissue culture plates. The cells were then stimulated without (Control; panels a, c, and e) or with 100 nM insulin (Insulin; panels b, d, and f) for 30 min and plasma membrane sheets prepared as described under “Experimental Procedures.” The plasma membrane sheets were incubated with a GLUT4 antibody for 15 h at 4 °C, followed by addition of anti-rabbit lissamine rhodamine. Rhodamine staining was visualized by confocal microscopy (original magnification, ×60). These results are representative of three independent sets of electroporated adipocytes.

![IF: GLUT4](image)

**Fig. 4.** Increased expression of Munc18c inhibits insulin-stimulated translocation of endogenous GLUT4 in 3T3L1 adipocytes. Differentiated 3T3L1 adipocytes were electroporated with 600 µg of the empty vector (pcDNA3), pcDNA3-Flag-Munc18b (Munc18b), or pcDNA3-Flag-Munc18c (Munc18c) and allowed to recover for 48 h on collagen-coated tissue culture plates. The cells were then stimulated without (Control; panels a, c, and e) or with 100 nM insulin (Insulin; panels b, d, and f) for 30 min and plasma membrane sheets prepared as described under “Experimental Procedures.” The plasma membrane sheets were incubated with a GLUT4 antibody for 15 h at 4 °C, followed by addition of anti-rabbit lissamine rhodamine. Rhodamine staining was visualized by confocal microscopy (original magnification, ×60). These results are representative of three independent sets of electroporated adipocytes.

Increased expression of Munc18c has no effect on insulin-stimulated translocation of endogenous GLUT1 in 3T3L1 adipocytes.

Differentiated 3T3L1 adipocytes were electroporated with 600 µg of the empty vector (pcDNA3), pcDNA3-Flag-Munc18b (Munc18b), or pcDNA3-Flag-Munc18c (Munc18c) and allowed to recover for 48 h on collagen-coated tissue culture plates. The cells were then stimulated without (Control; panels a, c, and e) or with 100 nM insulin (Insulin; panels b, d, and f) for 30 min and plasma membrane sheets prepared as described under “Experimental Procedures.” The plasma membrane sheets were incubated with a GLUT1 antibody for 15 h at 4 °C, followed by addition of anti-rabbit lissamine rhodamine. Rhodamine staining was visualized by confocal microscopy (original magnification, ×60). These results are representative of two independent sets of electroporated adipocytes.

![IF: GLUT1](image)

**Fig. 6.** Increased expression of Munc18c has no effect on insulin-stimulated translocation of endogenous GLUT1 in 3T3L1 adipocytes. Differentiated 3T3L1 adipocytes were electroporated with 600 µg of the empty vector (pcDNA3), pcDNA3-Flag-Munc18b (Munc18b), or pcDNA3-Flag-Munc18c (Munc18c) and allowed to recover for 48 h on collagen-coated tissue culture plates. The cells were then stimulated without (Control; panels a, c, and e) or with 100 nM insulin (Insulin; panels b, d, and f) for 30 min and plasma membrane sheets prepared as described under “Experimental Procedures.” The plasma membrane sheets were incubated with a GLUT1 antibody for 15 h at 4 °C, followed by addition of anti-rabbit lissamine rhodamine. Rhodamine staining was visualized by confocal microscopy (original magnification, ×60). These results are representative of two independent sets of electroporated adipocytes.

Expression of Munc18c Does Not Affect Insulin-stimulated GLUT1 Trafficking—In addition to GLUT4 and IRAP/vp165, 3T3L1 adipocytes express another glucose transporter isoform termed GLUT1. In the basal state, GLUT1 is primarily localized to the plasma membrane but does undergo a modest degree of insulin-stimulated translocation to the plasma membrane (5). To determine if the inhibitory effect of Munc18c was specific to the GLUT4 glucose transporter or acted as a general inhibitor of insulin-stimulated vesicular trafficking in adipocytes, we examined the translocation of GLUT1 using the plasma membrane sheet assay (Fig. 6). In contrast to GLUT4, plasma membrane sheets isolated from control cells displayed a significant amount of GLUT1 immunofluorescence indicating the presence of the GLUT1 protein at the plasma membrane in the basal state (Fig. 6, panels a, c, and e). Since the GLUT1 was already localized to the plasma membrane, insulin stimulation only resulted in a small increase in endogenous GLUT1 immunofluorescence (Fig. 6, panel b). Nevertheless, expression of neither Munc18b nor Munc18c had any significant effect on the basal or insulin-stimulated redistribution of endogenous GLUT1 protein (Fig. 6, panels c–f). These data were also confirmed by transfection of the adipocytes with an eGFP-GLUT1 construct (data not shown). Thus together, these data demonstrate that the Munc18c-dependent inhibition of vesicular trafficking in adipocytes is specific for the insulin-responsive GLUT4- and IRAP/vp165-containing vesicle population.

**Insulin Induces the Dissociation of Munc18c from Syntaxin 4—**Since Munc18c appears to function as an inhibitor of insulin-stimulated GLUT4 translocation, we next examined whether Munc18c could also function as a regulatory target of insulin signal transduction. Initially, we expressed a Munc18c construct containing an in-frame Flag epitope tag at the amino...
Munc18c from endogenous syntaxin 4 in CHO/IR cells. CHO/IR cells were electroporated with 40 μg of pcDNA3-Flag-Munc18c DNA and allowed to recover for 48 h. The cells were then incubated without (lanes 1) or with 100 nM insulin (lanes 2) for 30 min. Whole cell detergent extracts (Lysate) were prepared and immunoprecipitated (IP) with an affinity-purified syntaxin 4 antibody (Syn4) as described under “Experimental Procedures.” Whole cell lysates (A) and the syntaxin 4 immunoprecipitates (B) were immunoblotted with a Flag antibody. C, the syntaxin 4 immunoprecipitate was also immunoblotted with a syntaxin 4-digoxigenin-conjugated antibody. These results are representative of six independent sets of electroporated CHO/IR cells.

Insulin stimulation induces a dissociation of expressed Munc18c from endogenous syntaxin 4 in CHO/IR cells. CHO/IR cells were electroporated with 40 μg of pcDNA3-Flag-Munc18c DNA and allowed to recover for 48 h. The cells were then incubated without (lanes 1) or with 100 nM insulin (lanes 2) for 30 min. Whole cell detergent extracts (Lysate) were prepared and immunoprecipitated (IP) with an affinity-purified syntaxin 4 antibody (Syn4) as described under “Experimental Procedures.” Whole cell lysates (A) and the syntaxin 4 immunoprecipitates (B) were immunoblotted with a Flag antibody. C, the syntaxin 4 immunoprecipitate was also immunoblotted with a syntaxin 4-digoxigenin-conjugated antibody. These results are representative of six independent sets of electroporated CHO/IR cells.

Syntaxin 4 Localizes Munc18c to the Plasma Membrane—Munc18c does not contain any membrane targeting sequences but does associate with syntaxin 4 and possibly other plasma membrane-localized proteins, in an analogous fashion as Munc18a (28, 38, 39). Although insulin induced the dissociation of Munc18c from syntaxin 4 (Fig. 7A, lanes 1 and 2). Immunoprecipitation of endogenous syntaxin 4 demonstrated the co-immunoprecipitation of the expressed Flag-Munc18c protein (Fig. 7B, lane 1). However, insulin stimulation resulted in a decreased amount of Flag-Munc18c protein that was immunoprecipitated with syntaxin 4 (Fig. 7B, lane 2). This difference was not due to unequal expression or immunoprecipitation of endogenous syntaxin 4, as determined by syntaxin 4 immunoblotting of the syntaxin 4 immunoprecipitates (Fig. 7C, lanes 1 and 2). Thus, these data demonstrate that insulin can regulate the binding interaction between syntaxin 4 and Munc18c protein complexes.

FIG. 7. Insulin stimulation induces a dissociation of expressed Munc18c from endogenous syntaxin 4 in CHO/IR cells. CHO/IR cells were electroporated with 40 μg of pcDNA3-Flag-Munc18c DNA and allowed to recover for 48 h. The cells were then incubated without (lanes 1) or with 100 nM insulin (lanes 2) for 30 min. Whole cell detergent extracts (Lysate) were prepared and immunoprecipitated (IP) with an affinity-purified syntaxin 4 antibody (Syn4) as described under “Experimental Procedures.” Whole cell lysates (A) and the syntaxin 4 immunoprecipitates (B) were immunoblotted with a Flag antibody. C, the syntaxin 4 immunoprecipitate was also immunoblotted with a syntaxin 4-digoxigenin-conjugated antibody. These results are representative of six independent sets of electroporated CHO/IR cells.

DISCUSSION

It has been well established that insulin stimulates glucose uptake in both muscle and adipose tissue through the redistribution of the GLUT4 glucose transporter isoform from intracellular storage sites to the plasma membrane (1, 2, 36). This insulin-regulated translocation of GLUT4-containing vesicles is a critical process necessary for the maintenance of normal glucose homeostasis (4, 47, 48). However, the signaling pathways and protein components directly involved in GLUT4 vesicle translocation to the plasma membrane have only been poorly defined. Recent studies have demonstrated that adipocytes utilize several isoforms of the well established SNARE protein complexes in the trafficking of GLUT4-containing vesicles (reviewed in Ref. 5). These include the v-SNARE VAMP2 and the plasma membrane t-SNAREs, syntaxin 4, and SNAP23 (10–13). Various agents that have been used to disrupt these interactions have all resulted in an impairment of insulin-stimulated GLUT4 translocation underlying the necessity of these proteins in the budding, priming, docking, and/or fusion events. However, none of these core proteins appear to be targets of insulin action.

There are two general models that can account for the insulin-stimulated translocation of GLUT4 to the plasma membrane. The retention model predicts that specific sequences in the GLUT4 protein target it to vesicles that become sequestered away from the constitutively recycling endosome pool.
Insulin stimulation allows these vesicles to enter the constitutive recycling endosome pathway and thereby results in a default trafficking to the plasma membrane. This model is consistent with the predominant localization of GLUT4 predominantly in tubulo-vesicular elements beneath the plasma membrane, with the remaining protein found in the trans-Golgi network, clathrin-coated vesicles, and endosome structures (49, 50). In addition, the cytoplasmic domain of IRAP/vp165 and the carboxyl-terminal domain of GLUT4 appear to provide retention information and disruption of this signal results in plasma membrane translocation (42, 51, 52). Alternatively, a substantial proportion of the GLUT4-containing vesicles appear to be segregated away from recycling endosome markers, having characteristics analogous to small synaptic vesicles. This includes the co-localization of GLUT4 with VAMP2 and that a substantial portion of the GLUT4 remains distinct from the recycling of the transferrin receptor (11, 37). Furthermore, ablation of the recycling transferrin-containing endosome population does not prevent insulin-stimulated GLUT4 translocation (11). Although these two models are not mutually exclusive, several studies have also demonstrated that syntaxin 4, SNAP23, and VAMP2 play essential functions in the insulin-stimulated translocation of GLUT4 that is more consistent with a synaptic-like model of vesicle trafficking (10, 12, 13, 28).

In this study, we have further explored the role of SNARE proteins in the insulin regulation of GLUT4 translocation by examining the functional properties of Munc18c in 3T3L1 adipocytes. Consistent with in vitro binding studies (13, 28), we utilized a yeast three-hybrid assay system to demonstrate that VAMP2 and Munc18c strongly compete with each other for binding to syntaxin 4. Since VAMP2 binding to syntaxin 4 is necessary for insulin-stimulated GLUT4 translocation, then any protein that interferes with this interaction will have an inhibitory function. Indeed, we have observed that increased expression of Munc18c, but not Munc18b, specifically inhibits the insulin-stimulated translocation of GLUT4 and vp165/IRAP-containing vesicles. Consistent with these data, it was also recently observed that adenovirus-mediated expression of Munc18c but not Munc18b inhibited insulin-stimulated GLUT4 translocation in 3T3L1 adipocytes (53).

The inhibition of GLUT4 vesicle translocation by Munc18c can be analogized with the role of the Drosophila and yeast Munc18 homologs Rop and n-Sec1. In these systems, both inactivating mutations and deletions prevent vesicle exocytosis suggesting a necessary positive signaling role (25, 54, 55). Nevertheless, increased Rop expression also prevents synaptic transmission consistent with an inhibitory function (21, 55, 56). This apparent dual function for Rop can be reconciled by the observation that Rop expression does not alter the number of docked vesicles at the synapse, implying at least some post-docking function for Rop (55). Similarly, in addition to vesicle docking, syntaxin has also been shown to play a post-docking role and, in sole combination with SNAP25 and VAMP, is sufficient to constitute membrane fusion (57, 58).

If syntaxin 4 is associated with Munc18c, which prevents VAMP2 binding, how can a productive syntaxin 4-VAMP2 vesicle complex assemble at the plasma membrane? One possibility is that there are multiple populations of syntaxin 4 docking/fusion sites, some of which are occupied with Munc18c and others which are not. In this situation, only the Munc18c-deficient syntaxin 4 complexes would be available for the docking/fusion of the GLUT4-containing vesicles. This would be consistent with recent studies suggesting the presence of two distinct GLUT4 vesicle populations in muscle and 3T3L1 adipocytes (37, 59, 60). Alternatively, our data indicate that insulin can directly regulate the interaction of Munc18c with syntaxin 4 and thereby de-repress its inhibitory function. This conclusion is consistent with the observation that protein kinase C phosphorylation of Munc18a prevents its association with syntaxin 1a, thereby de-repressing the inhibition of VAMP1 binding to syntaxin 1 (26). Although the data presented in this report do not directly distinguish between these models, both are consistent with an increased expression of Munc18c inhibiting insulin-stimulated GLUT4-containing vesicle translocation. Further studies will be necessary to determine the existence of multiple syntaxin 4 complexes in adipocytes as well as the role of Munc18c in the inhibition of GLUT4 vesicle translocation at either pre- and/or post-docking steps.

In summary, the data presented in this report directly demonstrate that Munc18c is a specific competitor of VAMP2 binding to syntaxin 4. Consistent with the reported requirement of a syntaxin 4-VAMP2 complex in GLUT4 translocation, increased expression of Munc18c specifically inhibited the insulin-stimulated translocation of GLUT4-containing vesicles. The inhibitory function of Munc18c also occurred for IRAP/vp165 translocation, as this cargo is also a constituent of GLUT4 vesicles. However, the trafficking of GLUT1 containing vesicles was unaffected, consistent with the distinct vesicular localization of GLUT1. Finally, insulin appears to de-repress the inhibitory function of Munc18c by stimulating the dissociation of Munc18c from syntaxin 4.

Acknowledgments—We thank Drs. Richard Scheller, Hideki Katagiri, Steven Waters, Sidney Whiteheart, and Paul Roche for providing the cDNAs for syntaxin 4, Munc18b, IRAP/vp165, αSNAP, and SNAP23, respectively. We also thank Dr. Michael Mueckler for providing the rabbit polyclonal antibody to GLUT1.

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J. Biol. Chem. 1998, 273:33876-33883.
doi: 10.1074/jbc.273.50.33876

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