Munc18c Interaction with Syntaxin 4 Monomers and SNARE Complex Intermediates in GLUT4 Vesicle Trafficking*

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In the process of insulin-stimulated GLUT4 vesicle exocytosis, Munc18c has been proposed to control SNARE complex formation by inactivating syntaxin 4 in a self-associated conformation. Using in vivo fluorescence resonance energy transfer in 3T3L1 adipocytes, co-immunoprecipitation, and in vitro binding assays, we provide data to indicate that Munc18c also associates with nearly equal affinity to a mutant of syntaxin 4 in a constitutively open (unfolded) state (L173A/E174A; LE). To bind to the open conformation of syntaxin 4, we found that Munc18c requires an interaction with the N terminus of syntaxin 4, which resembles Sly1 interaction with the N terminus of ER/Golgi syntaxins. However, both N and C termini of syntaxin 4 are required for Munc18c binding, since a mutation in the syntaxin 4 SNARE domain (I241A) reduces the interaction, irrespective of syntaxin 4 conformation. Using an optical reporter for syntaxin 4-SNARE pairings in vivo, we demonstrate that Munc18c blocks recruitment of SNAP23 to wild type syntaxin 4 yet associates with syntaxin 4LE-SNAP23 Q-SNARE complexes. Fluorescent imaging of GLUT4 vesicles in 3T3L1 adipocytes revealed that syntaxin 4LE expressed with Munc18c bypasses the requirement of insulin for GLUT4 vesicle plasma membrane docking. This effect was attenuated by reducing the Munc18c-syntaxin 4LE interaction with the I241A mutation, indicating that Munc18c facilitates vesicle docking. Therefore, in contradiction to previous models, our data indicates that the conformational “opening” of syntaxin 4 rather than the dissociation of Munc18c is the critical event required for GLUT4 vesicle docking.

Soluble N-ethylmaleimide-sensitive factor (NSF)3 attachment protein receptors (SNAREs) are central to vesicle fusion

1 The abbreviations used are: NSF, N-ethylmaleimide-sensitive factor; SNARE, soluble NSF attachment protein receptor; SM, Sec1/Munc18; FRET, fluorescence resonance energy transfer; FRAP, fluorescence recovery after photobleaching; GST, glutathione S-transferase; GFP, green fluorescent protein; YFP, yellow fluorescent protein; RFP, red fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; CFP, cyan fluorescent protein.

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allow syntaxin 1A to rapidly fluctuate between a folded (closed) conformation and an unfolded (open) conformation (13). The open conformation facilitates SNARE-SNARE pairing resulting from accessibility of the SNARE motif. The closed (SNARE pairing inactive) conformation of syntaxin 1A is stabilized by interaction with Munc18a (15), precluding interactions with other SNAREs (16, 17). Thus, the association of Munc18 suggests a mechanism for the regulation of the syntaxin SNARE motif through the control of syntaxin conformation. However, there is a lack of general correlation between SM function and a particular conformation of syntaxin. The situation is further complicated, because not all syntaxins assume folded conformations. For instance, the SM proteins Sly1 and Vps45, which regulate trafficking of intracellular transport vesicles, bind to a conserved N-terminal domain found upstream of the Habc domain in their cognate syntaxins (Sly1 to syntaxins 5 and 18, and Vps45 to syntaxin 16) (18–20). This subset of SM proteins does not hinder the kinetics of SNARE pairings (21). Comparatively, the yeast Munc18 homologue Sec1p stimulates fusion through its interaction with the ternary SNARE complex, only binding weakly to monomeric Sso1p/syntaxin (22, 23).

By analogy to the Munc18a-syntaxin 1A interaction shown in the crystal structure (14), the most prevalent model for Munc18c regulation of SNARE complex formation equates the association of Munc18c with a closed syntaxin 4 conformation to the inhibition of GLUT4 vesicle externalization. However, recent evidence suggests that exocytotic SM-syntaxin interactions may possess multiple binding and functional states. For example, Munc18a has been shown to bind to SNARE complexes and facilitate SNARE-mediated fusion in vitro (24), the basis of which is probably a binding mode for the SM protein utilizing both the N terminus of syntaxin 1A and interactions with the SNARE bundle (25). Comparatively, in vitro Munc18c associates with the ternary SNARE complex (26, 27). In addition, Munc18c possesses an interaction site for the N-terminal amino acids of syntaxin 4 resembling the syntaxin binding sites on Sly1 and Vps45 (27). However, genetic studies suggest that the mechanism of Munc18c action may be different from Munc18a. For example, the insulin-induced fusion of GLUT4 vesicles was enhanced in adipocytes prepared from Munc18c-null mice (11), whereas Munc18a deficiency was demonstrated to eliminate both evoked and spontaneous synaptic exocytosis (28). Additionally, insulin (6) as well as N-ethylmaleimide (26), which inhibits NSF-mediated SNARE core complex disassembly, have been reported to dissociate Munc18c from syntaxin 4. Thus, it is not yet evident how the Munc18c-syntaxin 4 interaction fits with other models for SM-syntaxin interactions.

The purpose of this study was to define the molecular properties of the Munc18c interaction with syntaxin 4, specifically how the Munc18c-syntaxin 4 complex influences SNARE core complex formation in the pathway of GLUT4 cycling. Our investigations utilized quantitative optical techniques, including fluorescence resonance energy transfer (FRET) and biochemical approaches, to directly report the state of the Munc18c-syntaxin 4 interaction in vivo. Using mutational analyses, we address the mode of Munc18c binding to syntaxin 4, including where and when the interaction occurs and which structural motifs are important for function. Our findings indicate that syntaxin 4 exists in a closed conformation that is stabilized by Munc18c, thus rendering the syntaxin 4 SNARE domain unavailable for SNARE complex formation. Using the system of insulin-stimulated GLUT4 exocytosis, we furthermore demonstrate that the binding of Munc18c to an open conformation of syntaxin 4 facilitates GLUT4 vesicle docking. Therefore, the change in the Munc18c-syntaxin 4 binding state precipitated by conformational opening of syntaxin is probably the critical regulatory point in the temporal sequence for the initiation of vesicle docking and fusion events.

EXPERIMENTAL PROCEDURES

Chemicals and Expression Constructs—The following antibodies were used at a 1:1000 dilution for Western blotting: anti-syntaxin 4 rabbit polyclonal (Sigma), anti-syntaxin 1A mouse monoclonal HPC-1 (Sigma), anti-SNAP23 polyclonal (Synaptic Systems), anti-FLAG polyclonal (Sigma), and anti-c-Myc mouse monoclonal 9B11 (Cell Signaling). To generate N-terminal fluoroprotein-labeled constructs, the following cDNAs were subcloned into the Sall-XbaI sites of pDNR-dual for use with the Cre recombinase-mediated Creator System (Clontech): rat Munc18a (pGex-KG-Munc18a), rat syntaxin 1A (pGEX-syntaxin 1A11), rat Munc18c (pcDNA3-FLAG-Munc18c), human syntaxin 4 (pcDNA4/TO/syntaxin 4-Myc-His), and human SNAP23 (pcDNA3-SNAP23) (gifts from J. Pevsner, R. Scheller, J. Pessin, and T. Weinberg). The recipient vectors pLoxP-ECFP-C1 and pLoxP-EcYFP-C1 (Q39M mutant of pEYFP-C1; citrine) were generated and mutated to their monomeric forms (A206K) from pLoxP-EGFP-C1 (Clontech) as described previously (17). To prepare pLoxP-mRFPP1-C1, the cYFP sequence from pLoxP-EcYFP-C1 was replaced with mRFPP1 (monomeric form) from pRSETB-mRFPP1 (a gift from R. Tsien) by PCR cloning. cDNAs in pDNR-dual were subcloned into the indicated recipient vectors using Cre recombinase according to the manufacturer's instructions. To prepare recombinant fusion proteins in Escherichia coli, syntaxin 4-(1–275) and Munc18c were PCR-amplified and cloned downstream of glutathione S-transferase (GST) cDNA contained in the pGEX-KG plasmid; pGEX-syntaxin 1A11 (syntaxin 1A1-(1–267)) was a gift from R. Scheller. pGex-KG-FLAG-loxP-Munc18c was created using a pGex-KG construct modified to include a loxP site suitable for use with the Clontech Creator system and an N-terminal FLAG epitope. The PCR-based QuickChange site-directed mutagenesis kit (Stratagene) was used to construct the following mutants: EGFP-SNAP23C/A, a cytosolic mutant lacking cysteine palmitoylation sites at 80, 83, 85, and 87, which were mutated to alanine; Munc18c (R240L); syntaxin 1A (L165A/E174A, LE; I209A; and I233A), and the homologous mutations in syntaxin 4 (L173A/E174A, LE; I217A, I241A). Syntaxin 4-(1–188) (ΔH3/TM) and syntaxin 1A-(1–181) were created by inserting an XbaI site with the TAG stop codon in frame at Leu189 and Ile182, respectively. The same XbaI site along with a SalI site upstream of the start codon were subcloned into the Sall site upstream of the start codon was used to facilitate construction of the following syntaxin chimeras: syntaxin 41 (syntaxin 4-(1–188) + syntaxin 1A-(182–288)) and syntaxin 14 (syntaxin 1A-(1–181) + syntaxin 4-(189–297)). Before use, the
XbaI restriction sites were back-mutated in the syntaxin 14 and syntaxin 41 plasmids to eliminate the stop codon and restore the Leu189 in the syntaxin 4 fragments and the Ile182 in the syntaxin 1A fragments. The sequence fidelity of all expression constructs was confirmed by DNA sequencing (University of Michigan DNA Sequencing Core).

Cell Culture and Transfection—3T3L1 fibroblasts (American Type Culture Collection, Manassas, VA) were maintained in DMEM containing 10% bovine serum (Invitrogen). Following 2 days of confluence, differentiation was induced by the addition of DMEM with 10% fetal bovine serum (FBS) containing 167 nM insulin, 0.25 µM dexamethasone, and 0.25 mM isobutylmethyl-xanthine for 3 days, followed by DMEM/FBS containing insulin for 2 days and subsequent removal of insulin for 2 days. Adipocytes were transfected by electroporation (0.16 kV, 950 microfarads) in phosphate-buffered saline, pH 7.4 (Invitrogen), with 50–200 µg of each plasmid. After electroporation, cells were allowed to adhere to coverglass for 18–24 h in DMEM/FBS prior to imaging. In selected experiments, cells were exposed to 100 nM insulin after 3 h of serum starvation in DMEM low glucose (Invitrogen) containing 0.5% FBS. HEK293-S3 cells, which contain a stably transfected N-type calcium channel (17), were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS (Invitrogen), 0.4 mg/ml hygromycin (Invitrogen), 0.4 mg/ml Geneticin (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). Cells were plated on coverglass affixed to the bottom of 35-mm plates 12–24 h prior to transfection using Lipo- mg/ml Geneticin (Invitrogen), and 1% penicillin/streptomycin for 3 days, followed by DMEM/FBS containing insulin for 2 days and subsequent removal of insulin for 2 days. Adipo- cytes were transfected by electroporation (0.16 kV, 950 micro- farads) in phosphate-buffered saline, pH 7.4 (Invitrogen), with 50–200 µg of each plasmid. After electroporation, cells were allowed to adhere to coverglass for 18–24 h in DMEM/FBS prior to imaging. In selected experiments, cells were exposed to 100 nM insulin after 3 h of serum starvation in DMEM low glucose (Invitrogen) containing 0.5% FBS. HEK293-S3 cells, which contain a stably transfected N-type calcium channel (17), were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS (Invitrogen), 0.4 mg/ml hygromycin (Invitrogen), 0.4 mg/ml Genetecin (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). Cells were plated on coverglass affixed to the bottom of 35-mm plates 12–24 h prior to transfection using Lipo- fectamine2000 (Invitrogen). Before live cell imaging, cells were transferred to physiologic saline solution containing 140 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM MgCl2, 2.2 mM CaCl2, 5 mM NaHCO3, and 10 mM HEPES (pH 7.4).

Homology Modeling of Munc18c-Syntaxin 4 Complex—Rat Munc18c (Stxbp3; GenBank™ accession number NP_446089) and syntaxin 4 (STX4; GenBank™ accession number NP_112387) sequences were threaded into the Munc18a-syntaxin 1A structure (Protein Data Bank code 1DN1) using O, based on a ClustalW alignment. Helical register, assessed using PredictProtein, was comparable; sequence gaps and insertions were not modeled. Figures were made with MOL- SCRIPT, RASTER 3D version 2.1.2, and GIMP.

GST Fusion Proteins—Chemically competent BL21DE3(RIPL) E. coli (Invitrogen) containing pGex-KG-syntxin 1-(1–275), pGex-KG-syntaxin 4-(1–275), or pGEX-KG-Munc18c were cultured and induced with 100 µM isopropyl 1-thio-β-D-galac- topyranoside for 5–6 h at 27 °C. Bacteria were lysed by French press (15,000 p.s.i. pressure differential), and the eluent was solubilized in phosphate-buffered saline containing 1% Triton X-100 for 1 h on ice. Proteins were subsequently purified using glutathione-Sepharose (Amersham Biosciences). Cleavage of the GST moiety from the fusion protein was accomplished by treatment with 1.4 NIH units of human thrombin (Amersham Biosciences) for 16 h at 4 °C. Purity of all isolated fusion pro- teins was confirmed by SDS-PAGE fractionation and subse- quent visualization with Coomassie Blue or Western blotting. Protein concentration was measured using the DC protein assay (Bio-Rad) against a bovine serum albumin standard (Sigma).

Trypsin Proteolysis Assay—Trypsin digest of recombinant GST-syntaxin 4-(1–275) and GST-syntaxin 4-(1–275)LE was modeled after Graham et al. (29). Proteins were incubated at 5 µM concentration in a total volume of 50 µl containing 60 nM trypsin (Type IX-S; Sigma) in phosphate-buffered saline (pH 7.4) for the times indicated. During trypsin incubation, GST fusion proteins remained bound to glutathione-Sepharose beads. The reactions were terminated by the addition of SDS sample buffer and immediate boiling. After separation by 12% SDS-PAGE, the samples were transferred to nitrocellulose and probed for syntaxin 1A or syntaxin 4. Proteins were visualized with a horseradish peroxidase-conjugated secondary antibody and ECL substrate (Amersham Biosciences).

Co-immunoprecipitation—For each treatment, a 6-well plate of confluent HEK293-S3 cells was transiently transfected with the indicated constructs. After a 2-day expression period, cells were rinsed twice in physiologic saline solution and lysed in a Dounce homogenizer in a buffer containing 2% sucrose, 1 µM EDTA, and 20 mM Tris (pH 7.5). After the homogenate was centrifuged (300 g for 3 min) to pellet the nuclei, the supernatant was diluted 1:1 in immunoprecipitation buffer (150 Tris, pH 7.4, 1 mM MgCl2, 0.1 mM EGTA, 2% Triton X-100). Samples were normalized for lysate volume and concentration (2–3 µg/µl) and incubated with 10 µg of anti-Myc 9B11 monoclonal antibody (Cell Signaling) for 4 h at 4 °C. The samples were then incubated with Protein G-Sepharose beads (Pierce) for 1 h, and washed in immunoprecipitation buffer. Finally the pellet was resuspended in SDS sample buffer and subject to fractionation by SDS-PAGE and Western blotting.

In Vitro Binding Assay—For all binding reactions in vitro, the GST moiety was cleaved from purified GST-syntaxin 4-(1–275), GST-syntaxin 4-(1–275)LE, and GST-Munc18c. Reactions used 0.03–30 pmol of Munc18c in a dilution series spotted in quadruplicate on nitrocellulose (BA-83; Schleicher and Schuell). After blocking in 2% milk, the blots were incubated with 500 nM syntaxin 4-(1–275) or syntaxin 4-(1–275)LE for 12 h at 4 °C. Syntaxin 4 proteins were visualized using an anti-syntaxin 4 polyclonal antibody and horseradish peroxi- dase-conjugated secondary antibody as described above. Integrated intensity (area/intensity) was quantified using Meta- morph (version 6.3r5; Universal Imaging, Inc., Malvern, PA).

Measurement of FRET Stoichiometry by Sensitized Emission—Live cell imaging of FRET was performed on transfected 3T3L1 adipocytes and HEK293 cells 24 h after transfection. Since false positive FRET signals have been observed when cytoplasmic donors come into contact with membrane-compartmentalized acceptors (30), syntaxin 4 was purposely tagged with CFP. However, performing the experiments using CFP-Munc18c and cYFP-syntaxin 4 resulted in similar FRET efficiency values (data not shown). Measurement of sensitized emission FRET was carried out as previously described (17, 31). The method- ology employed an inverted fluorescence microscope (Olym- pus, IX71) equipped with the following components: a TILL- Photonics Polychrome IV xenon lamp-based monochrometer (TILL-Photonics, Grafelfing, Germany), a polychromic mirror that allowed excitation of multiple fluorophores (436–500 nm; Chroma Technology Corp., Brattleboro, VT), a Planapo ×60 water immersion objective (1.2 numerical aperture), a multi-
spec microimager (Optical Insights, Santa Fe, NM) containing dichroic splitter (505dxcr) and emission filters (D465/30 and HQ535/30) to allow simultaneous two-channel monitoring of emission fluorescence, and a cooled digital CCD camera (TILL IMAGO QE). The multispec microimager hardware was calibrated to allow pixel-by-pixel alignment of images, and offline adjustments were made using the TILL-Vision software. For analyses of the acquired images were performed using Meta-morph image-processing software as previously described (17, 31). The apparent efficiency of acceptor (monomeric cYFP) in complex (Eₐ), the apparent efficiency of donor (monomeric ECFP) in complex (Eₐ), and the mole fraction of acceptor to donor (RATIO) values were determined using the following equations: $E_A = \frac{\gamma((DA - \beta/DD)/(\alpha AA) - 1)(1/E_C)}{1 - (DD/(DA - \alpha AA - \beta/DD)((\alpha \gamma) + DD))}((1/E_C); E_D = \frac{1 - (DD/(DA - \alpha AA - \beta/DD)((\alpha \gamma) + DD))}{(1/E_C); and RATIO = \frac{(\alpha \gamma)/((DA - \alpha AA - \beta/DD)((\alpha \gamma) + DD))}{(1/E_C)}$. Donor and acceptor excitations were 436 and 480 nm, respectively. Empirically determined constants were established in HEK293 cells: α, 0.017; β, 0.9406; γ, 0.0658; ξ, 0.0147. For all measurements, $E_A$ values were determined in regions of the cell where the mole fraction between cYFP-Munc18c and CFP-syntaxin 4 (RATIO) was between 0.9 and 1.1; $E_D$ is comparable with $E_A$ over this RATIO range.

Resolution of SNAP23/C/A-SNARE Interactions by Cytosolic Photobleach and Membrane FRAP—GFP-SNAP23/C/A bound to syntaxin 4 at the plasma membrane was observed by expression of the proteins when viewed with the FV500 Olympus Fluoview laser-scanning confocal microscope. In order to resolve the relative amount of plasmalemmal GFP-SNAP23/C/A, images were taken before and after 1 min of cytosolic photobleach (>90% bleach) using the 488-nm laser line of the argon laser. The localization of GFP-SNAP23/C/A fluorescence intensity remaining after photobleach was quantified using linescans averaged over a 10-pixel width (~½ cell diameter) normalized to peak intensity for each cell. For FRAP experiments, cytosolic photobleach was followed by a 10-s photobleach (>50%) of the cell membrane using simultaneous excitation with the 488-nm line of the Argon laser and 543-nm line of the helium neon laser to facilitate simultaneous bleach of GFP and RFP, respectively. FRAP images were taken following a 1.5-min recovery period.

Quantification of Insulin-induced GLUT4 Plasma Membrane Insertion—3T3L1 adipocytes stably expressing Myc-GLUT4-GFP were prepared as previously described (32) using a retroviral vector pMX/GLUT4mec7-EGFP (a gift from H. Lodish) (33). Cells were electroporated as described above with pcDNA-FLAG-Munc18c and pLoxP-ECFP-syntaxin 4 or mutants of syntaxin 4 as described in the figure legends. As a control experiment to verify the functionality of N-terminally tagged syntaxin 4 (supplemental Fig. S2), adipocytes were electroporated with pcDNA-syntaxin 4L4F and pLoxP-mCerulean-Munc18c. 24 h after electroporation, adipocytes were serum-starved for 3 h in DMEM containing 0.5% FBS. Subsequently, cells were treated with 100 nm insulin for the times indicated, fixed in phosphate-buffered saline containing 4% paraformaldehyde, and quenched with 50 mm glycine for 5 min. Externalized Myc-GLUT4-GFP was visualized by exposing the cells to an anti-Myc monoclonal antibody 9E10 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) without permeabilization, followed by Alexa594-conjugated goat anti-mouse secondary antibody (Molecular Probes, Inc., Eugene, OR) as discussed (32).

For cell counting experiments, ≥75 cells from each treatment possessing peripheral CFP-syntaxin 4 fluorescence were blindly examined for peripheral GFP fluorescence and/or surface Alexa594 fluorescence using an Olympus IX71 microscope equipped with a dual pass GFP/mRFP1 filter set (Chroma Technology). For quantification of GLUT4 localization, cells with equivalent CFP (syntaxin) and GFP (GLUT4) were chosen based on equivalent integrated density (cell area/intensity) from a 500-ms image taken at 436 and 480 nm, respectively. To quantify CFP and GFP localization, line scans (10-pixel width averages) drawn from the outside of the cell to the interior were normalized to fluorescence intensity at the plasma membrane region and plotted as a function of distance from the plasma membrane.

RESULTS

Modeling of Syntaxin 4 Structure and Generation of a Constitutively Open Syntaxin 4 Mutant—To facilitate design of syntaxin 4 mutations that would aid in characterization of the conformational states of syntaxin 4 for Munc18c binding, we constructed a homology model of syntaxin 4 based on the reported crystal structure of syntaxin 1A in complex with Munc18a (nSec1/Munc18-1) (14). The Munc18c and syntaxin 4 sequences (identity: 50%, Munc18; 46%, syntaxin) were threaded directly on the crystal structure of the Munc18a-syntaxin 1A complex (Protein Data Bank code 1DN1). No modeling predictions were made in areas with sequence gaps or insertions. Fig. 1A shows the generated Munc18c-syntaxin 4 homology model. An analysis of the model over highly conserved regions between syntaxin 1A and syntaxin 4 yielded two important observations. First, the outer surfaces of the syntaxin 4 Habc helices contain most of the nonconserved residues, whereas the regions of intramolecular interactions between the Habc domain and the H3 domain were found to be highly conserved (Fig. 1B, circled). This structural similarity suggested that the helical packing that allows syntaxin 1A to adopt a closed conformation would also allow syntaxin 4 to adopt a closed conformation. Second, the Habc-H3 linker region is stabilized against the Habc and H3 domains by a highly conserved hydrophobic pocket. A mutation in the syntaxin 1A linker helix (L165A/E166A; LE) has been reported to destabilize the N-terminal helices from interaction with the SNARE motif and result in a constitutively open syntaxin 1A conformation (15). This open conformation impairs Munc18a binding to syntaxin 1A, strongly facilitating its interaction with other SNAREs (3, 17, 29, 34). From the structural prediction (Fig. 1C), the homologous residues in syntaxin 4, Leu172, and Glu174 lie in a conserved pocket of the linker helix. The hydrophobic contacts of Leu173 are maintained with Ile156, Leu160, and the semeconserved Val168 (Ile149, Leu153, and Thr160 in syntaxin 1A). The hydrophobic regions of the nonconserved residues, Asp170 and Arg157 (Ser162 and Gln150 in syntaxin 1A), may form van der Waals interactions contributing to the hydrophobic pocket for...
Leu173, whereas their charged portions are solvent-exposed. Glu174 maintains one conserved salt bridge with Arg149, although a second with Val153 (Arg142 and Lys146 in syntaxin 1A) is lost. Taken together, the model predicts that significant Leu173 and Glu174 interactions are likely to be disrupted by dual mutation to alanine (L173A/E174A), hereafter termed syntaxin 4LE, and to confer a constitutively open conformation on the syntaxin 4 mutant.

To determine whether the syntaxin 4LE conformation is different from the wild type protein in a manner consistent with a constitutively “open” syntaxin 4, we examined the effect of partial trypsin proteolysis on GST-syntaxin 4-(1–275) and GST-syntaxin 4-(1–275)LE. Fig. 2A shows that proteolytic treatment of wild type GST-syntaxin 4-(1–275) resulted in two dominant cleavage products recognized by the anti-syntaxin 4 polyclonal antibody, which fractionated at 15 and 30 kDa. After equivalent incubation, these products were dramatically reduced or absent in the GST-syntaxin 4-(1–275)LE digest. The increased resistance of wild type syntaxin 4 to trypsin relative to the LE mutant suggests that the wild type protein is more compact in structure, thereby limiting proteolysis, whereas the LE mutation promotes a more accessible, or open, structure for the proteolytic activity of the enzyme. As a control experiment, we also tested the trypsin sensitivity of GST-syntaxin 1A-(1–267)LE.

FIGURE 1. Homology modeling of the Munc18c-syntaxin 4 complex based on the Munc18a-syntaxin 1A crystal structure. A, Munc18c and syntaxin 4 were threaded directly into the Munc18a-syntaxin 1A crystal structure based on a ClustalW alignment. Munc18c is colored gray, and the syntaxin 4 helical domains are colored as follows: Ha in pale blue, Hb in gold, Hc in cyan, H3 in green, and the Habc-H3 linker region in orange. The syntaxin 4 point mutants used in this study (red) are mapped onto the Munc18c-syntaxin 4 structural model: L173A/E174A, I1217A, I1241A, and L1217A; I1241A. B, structural map of nonconserved syntaxin 4 residues viewed down the axis of the syntaxin 4 H3 domain (green) as it packs against the Habc helices. Side chains of nonconserved residues are shown. A region of highly conserved packing between the SNARE (H3) domain and the N-terminal helices is circled (rust, Ha; blue, Hb; magenta, Hc; red, H3; indigo, Habc-H3 linker). C, all contacts proposed to interact with the syntaxin 4 linker residues Leu173 and Glu174 are colored as conserved (yellow) and nonconserved (magenta); differing syntaxin 1A residues are shown after a slash. Residue numbers correspond to the rat syntaxin 4 sequence (GenBank™, NP_112387).
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(L165A/E166A) relative to wild type GST-syntaxin 1A-(1–267). Fig. 2B shows that the wild type syntaxin 1A was more resistant to trypsin than that of syntaxin 1A carrying the L165A/E166A mutation, confirming the efficacy of the approach initially published by Graham et al. (29). These parallel results suggest that syntaxin 4, like syntaxin 1A, forms a self-associated structure folded about the linker region. The syntaxin 4 LE mutation allowed investigation of the effects of a less restricted syntaxin 4 conformation on Munc18c binding.

Munc18c Interaction with the Closed and Open Conformations of Syntaxin 4 in Vivo—Syntaxin 4 has been reported to facilitate insulin-regulated GLUT4 vesicle externalization in 3T3L1 adipocytes. By comparison, Munc18c overexpression exerts an inhibitory effect on the same pathway through its interaction with syntaxin 4 (5–7), signifying that the level of complexation of Munc18c with syntaxin 4 is an important determinant of SNARE complex formation. To measure the structural parameters important for the direct Munc18c-syntaxin 4 protein interaction in living 3T3L1 adipocytes, we employed expression of cYFP-Munc18c and CFP-syntaxin 4 as a FRET pair. Sensitized emission FRET stoichiometry yields a spatial map of the apparent FRET efficiency of CFP donor ($E_d$) and cYFP acceptor ($E_a$) in complex as well as the mole fraction of acceptor to donor (RATIO) (31). Fig. 3A (top) shows an image series of a representative adipocyte coexpressing cYFP-Munc18c and CFP-syntaxin 4. Both the CFP and cYFP fluorescence is enriched at the plasma membrane region relative to the cytosol, as expected for the plasma membrane localization of the Munc18c-syntaxin 4 complex. Moreover, the corresponding FRET images $E_a$ and $E_d$ for this cell indicated that substantial FRET occurred between the coexpressed cYFP-Munc18c and CFP-syntaxin 4 proteins, thereby indicating direct protein-protein interactions. The absolute values of FRET ($E_a$ and $E_d$) are sensitive to the relative levels of expression of the FRET donor to acceptor (RATIO). Therefore, Fig. 3B compares $E_a$ values averaged from many cells where the range over which the FRET values are averaged was restricted to pixels where the molar ratio of cYFP to CFP expression is within the range of 0.9–1.1. This strategy allowed us to compare $E_a$ values between cells and across treatments. Importantly, as a control to confirm that the FRET signal resulted from a specific and direct interaction between Munc18c and syntaxin 4, we tested the effect of CFP-syntaxin 4 coexpression with a single point mutant of Munc18c (cYFP-Munc18cR240L). This temperature-insensitive mutant was based on the previously characterized temperature-sensitive SM mutants, Munc18cR264F and the Saccharomyces cerevisiae homolog Sly1pR266K, which demonstrated a strongly reduced affinity of interaction with syntaxin 4 and Sed5p, respectively (35, 36). In the following, the measured FRET efficiency was reduced by ~7-fold with respect to control upon coexpression of cYFP-Munc18cR240L with CFP-syntaxin 4. These results establish that FRET between cYFP-Munc18c and CFP-syntaxin 4 is specific to the bimolecular interaction in 3T3L1 adipocytes and changes in Munc18c-syntaxin 4 binding affinity. Notably, CFP-syntaxin 4 localized to the plasma membrane when coexpressed with cYFP-Munc18cR240L, suggesting that syntaxin 4 does not require a direct interaction with Munc18c for plasma membrane targeting. We next used the FRET assay to determine if the constitutively open mutant of syntaxin 4 (CFP-syntaxin 4LE) was capable of direct interaction with Munc18c. Remarkably, we observed only a slight reduction (~10%) in cYFP-Munc18c energy transfer with CFP-syntaxin 4 as compared with the wild type control, indicating the strong association of Munc18c with both closed and open conformations of syntaxin 4.

Our FRET experiments in adipocytes described above demonstrated a significant interaction of Munc18c with syntaxin 4LE, which contrasts with the strongly reduced interaction reported for the LE mutant of syntaxin 1A with Munc18a in vitro and in vivo (17, 29). This indicates that divergence occurs among vertebrate SM-syntaxin protein interactions. To directly compare the interaction of Munc18c and Munc18a with their cognate syntaxins in vivo, we measured FRET between the protein pairs when expressed in a common reporter cell line, HEK293 cells. Fig. 4A indicates that the apparent efficiency ($E_d$) of cYFP-Munc18c in complex with CFP-syntaxin 4LE was mildly reduced from that measured with CFP-syntaxin 4. By contrast, the apparent efficiency of energy transfer between Munc18a and syntaxin 1A1LE was ~50% lower than that measured for syntaxin 1A, a result consistent with the strong reduction in affinity conferred by the syntaxin 1A1LE mutation reported in other studies (17, 29). To further
The number of observations is indicated for treatments. The interaction between Munc18c and CFP-syntaxin 4 (LE) could be attributed to a change in the characteristic efficiency of the interaction ($E_C$) associated with conformational differences between syntaxin 4 and syntaxin 4(LE) or to changes in the affinity of the interaction. To obtain an estimate of $E_C$ for the interactions in live cells, we varied the expression ratio of cYFP-Munc18c to CFP-sytaxin 4 such that it encompassed an extended RATIO range, thus driving the apparent efficiency to asymptote to $E_C$. Fig. 4C shows the combined data for the imaged cells, plotting the calculated $E_A$ values of each image pixel versus the corresponding inverse RATIO values. A statistically significant difference in apparent $E_C$ was found between the LE mutant ($E_C = 26.92 \pm 0.05$, n = 67) and the wild type syntaxins ($E_C = 29.59 \pm 0.04$, n = 61). This difference may account for the reduced FRET between cYFP-Munc18c and CFP-sytaxin 4(LE) when compared with wild type CFP-sytaxin 4. However, the fit rate constants also differed slightly between conditions, indicating that small differences may also occur in the affinity of the protein-protein interactions. To test for differences in affinity, we measured the in vitro binding of bacterially expressed and purified sympathetic 4–1(275) or syntaxin 4–1(275)LE to Munc18c spotted on nitrocellulose and the wild type syntaxins (Fig. 5). To verify that equal amounts of each syntaxin 4 protein were used in the binding reaction, small standardized amounts of syntaxin 4–1(275) or syntaxin 4–1(275)LE were spotted alongside Munc18c. No apparent differences were observed between the wild type or syntaxin 4(LE) binding to Munc18c as measured over a 3 log concentration range, further confirming the promiscuous interaction of Munc18c with the closed and open conformation of syntaxin 4.

**The Syntaxin 4 Binding Sites for Munc18c**—The interaction of Munc18c with multiple conformations of syntaxin 4 is in...
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**A.** SM binding partners for each syntaxin isoform are listed, although not all combinations exhibit equal affinity (20). In syntaxin 4 chimeric mutants, termed 14 and 41 (not pictured), the syntaxin 4 Habc or H3 domains were replaced with syntaxin 1A sequence upstream or downstream of Leu189, as detailed under “Experimental Procedures.” Syntaxin 4H3/TM contains a stop codon in place of Leu189. B. Identification of the syntaxin 4 domains required for Munc18c binding. A, diagram illustrating the relative position of syntaxin 4 mutants used to identify interacting sites with Munc18c. Alignments of the syntaxin N termini are shown for the four vertebrate exocytotic isoforms as well as syntaxins 5 and 18 (Sed5p and Ufe1p in yeast, respectively). The aspartic acid residue mutated in syntaxin 4D3R is highlighted in gray. The SM binding partners for each syntaxin isoform are listed, although not all combinations exhibit equal affinity (20). In syntaxin 4 chimeric mutants, termed 14 and 41 (not pictured), the syntaxin 4 Habc or H3 domains were replaced with syntaxin 1A sequence upstream or downstream of Leu189, as detailed under “Experimental Procedures.” Syntaxin 4H3/TM contains a stop codon in place of Leu189. 4A, averaged apparent energy transfer (Ea) between cYFP-Munc18c and the indicated mutants of CFP-syntaxin 4. Averaged Ea values determined for FRET RATIO values of 0.9–1.1 were selected. Significant differences are relative to syntaxin 4WT or syntaxin 4LE (white bars; controls redisplayed from Fig. 4A). Numbers of observations are indicated above bars. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**B.** ‘closed’ syntaxin

**C.** ‘open’ syntaxin

direct contrast to the belief that vertebrate exocytotic SM proteins bind solely to the closed form of syntaxin. As a possible explanation, Munc18c-syntaxin 4 interactions may resemble a subset of SM proteins that function in the ER, Golgi, TGN, and endosomal networks, Sly1 and Vps45, which are known to require only short N-terminal sequences of their cognate syntaxins (18–20). Since syntaxin 4 shares some sequence homology with the motif that allows rSly1 to bind the N terminus of syntaxins 5 and 18 (Fig. 6A), it remained possible that the N terminus of syntaxin 4 upstream of the Habc domain could be important for binding to Munc18c, as first suggested by in vitro studies (27). Yamaguchi et al. (20) previously demonstrated that an arginine substitution at Asp5 in Sed5p/syntaxin 5, a residue common to syntaxins 1–4 as Asp5, resulted in complete loss of Sly1p binding when mutated to arginine. Shown in Fig. 6B, CFP-syntaxin 4D3R results in a ~40% reduction in FRET with cYFP-Munc18c when compared with the wild-type proteins expressed in HEK293-S3 cells. Moreover, the D3R mutant completely eliminated Munc18c binding to the constitutively open mutant of syntaxin 4. However, the N terminus of syntaxin 4 is insufficient to bind Munc18c, since a mutant of CFP-syntaxin 4 lacking its H3 and transmembrane domains (ΔH3/TM) exhibited negligible energy transfer with cYFP-Munc18c. The N terminus of syntaxin 4 could therefore stabilize the association of Munc18c with both the closed and open syntaxin 4 conformations. Furthermore, these results point to a mode of Munc18c-syntaxin 4 binding that shares the characteristics of SM binding to both ER/Golgi syntaxins and the exocytotic syntaxins, which require both the N and C termini of syntaxin for SM binding.

We next assessed the extent of divergence between the Munc18c-syntaxin 4 binding interaction and the Munc18a-syntaxin 1A interaction, which facilitates neuro-exocytosis. To ascertain whether conserved C-terminal syntaxin 4 residues are important for Munc18c binding, we substituted the syntaxin 1A H3 domain into syntaxin 4 after residue 189 (syntaxin 41) (Fig. 6B). FRET between Munc18c and syntaxin 41 was not different from wild type syntaxin 4, indicating that Munc18c probably utilizes residues conserved between the two syntaxins to bind the SNARE domain. Mutation of syntaxin 1A at two conserved residues, Ile369 and Ile373, has been reported to reduce binding with SNAP25 and Munc18a, respectively (17, 29, 37, 38). These two amino acids lie within the group of hydrophobic residues that form the interior of the fully assembled SNARE complex. Therefore, we tested the homologous alanine mutations in syntaxin 4, I217A and I241A, on Munc18c binding. FRET between cYFP-Munc18c and CFP-syntaxin 4I217A was found to be reduced moderately (~16%) when compared with wild type syntaxin 4. Yet, I217A did not affect binding of Munc18c to constitutively open syntaxin 4 (Fig. 6C). These results are consistent with the prediction of the model of Fig. 1A: I217A is not expected to directly contact Munc18c but may disrupt the helical packing of syntaxin 4 in the closed conformation. Comparatively, Ile383 is predicted to be a critical residue for contacting the central cavity of Munc18c, and it reduced interaction with wild type syntaxin 4 by ~60%. To an equivalent extent, the I241A mutation impaired the interaction of Munc18c with the open mutant of syntaxin 4, revealing that the membrane-adjacent SNARE domain is critically important for Munc18c binding regardless of syntaxin 4 conformation. Given the effects of the D3R mutant and the I241A mutant on Munc18c binding, our data indicate that the Munc18c actions to regulate SNARE complex formation require association with both the N and C termini of syntaxin 4.

**Effects of Syntaxin 4 Conformation and Munc18c Binding on Q-SNARE Complex Formation**—The nucleation of syntaxin 4-mediated SNARE complex formation is believed to be negatively regulated by Munc18c (5–7, 11, 12, 36). Since Munc18c binds to both closed and open conformations of isolated syntaxin 4 (Figs. 3–6) as well as to the syntaxin 4-SNAP23-VAMP2 complex in vitro (26, 27), we next investigated whether the opening of syntaxin 4 is sufficient to relieve the Munc18c inh
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SNAP23 in vitro (12). Of specific importance, we next investigated if the association of Munc18c with the open form of syntaxin 4 inhibits SNAP23 binding. For this analysis, we coexpressed syntaxin 4LE with GFP-SNAP23C/A in the absence or presence of mRFP-Munc18c. After photobleach, GFP-SNAP23C/A remained bound to syntaxin 4LE despite the association of RFP-Munc18c at the membrane, signifying that the conformational transition of syntaxin 4 is sufficient to relieve the inhibition of Munc18c on SNAP23 binding. The localization of GFP-SNAP23C/A in both experiments was quantified using line scans drawn from the outside of the cell to its center (Fig. 7B). The sharp “ear” in the line scan is consistent with membrane localization of GFP-SNAP23C/A, whereas the GFP intensity plateaus in cells where photobleach of diffusible GFP-SNAP23C/A has occurred upon the addition of mRFP1-Munc18c. To confirm that a Munc18c-syntaxin 4LE-SNAP23C/A complex is formed on the plasma membrane, we performed FRAP experiments on the plasma membrane following the cytosolic photobleach. As anticipated, in the syntaxin 4WT/RFP-Munc18c/GFP-SNAP23C/A coexpression condition, no further bleaching of GFP was observed on the membrane, although membrane-associated RFP-Munc18c signal recovered after membrane photobleach, consistent with the Munc18c inhibition of Q-SNARE complex formation (supplemental Fig. S1). However, that both RFP-Munc18c and GFP-SNAP23C/A signals recover after a subsequent plasma membrane photobleach when coexpressed with syntaxin 4LE supports the interpretation that a Munc18c-syntaxin 4LE-SNAP23C/A complex is formed specifically at the plasma membrane.

As a final measure of whether the interaction of Munc18c affects Q-SNARE complex formation, we tested for co-immunoprecipitation of the proteins when coexpressed in HEK293-S3 cells. Since the HEK293 cell line contains endogenous syntaxin 4, an epitope tag was used to limit immunoprecipitation to proteins interacting with the open mutant of syntaxin 4 (syntaxin 4LE-Myc2), thereby facilitating a direct comparison with syntaxin 4WT-Myc2. As shown in Fig. 7C, immunoprecipitation with an anti-Myc antibody resulted in co-precipitation of endogenous SNAP23 only when syntaxin 4LE, not syntaxin 4WT, was used for pull-down. Moreover, the syntaxin 4LE-SNAP23 complex was not dissociated by the additional overexpression of FLAG-Munc18c, which also bound to

FIGURE 7. Munc18c effects on Q-SNARE complex nucleation. A, confocal images of HEK293-S3 cells expressing GFP-SNAP23C/A and syntaxin 4 in the absence or presence of RFP-Munc18c are shown after a 1-min photobleach (488 nm) of a region in the cytosol. The localization of diffusible GFP-SNAP23C/A after cytosolic photobleach was used as an optical reporter for SNARE pairings with syntaxin 4, 8, averaged GFP-SNAP23C/A fluorescence intensity remaining after photobleach was quantified by plotting the average GFP pixel intensity across the plasma membrane region for cells co-expressing syntaxin 4 (top) or syntaxin 4LE (bottom) in the absence (black squares) or presence (gray circles) of RFP-Munc18c. Line scans are normalized to peak GFP intensity (n = 8–12 cells analyzed per experiment; three independent experiments). B, immunoblots showing co-immunoprecipitation of endogenous SNAP23 from HEK293-S3 cell lysates containing expressed syntaxin 4WT/Myc2 or syntaxin 4LE-Myc2 in the presence or absence of expressed FLAG-Munc18c. Lysates were incubated with anti-Myc monoclonal antibody (9B11), pulled down with protein G-Sepharose, and subject to 12% SDS-PAGE fractionation and Western blot analysis. Blots were cut and separately exposed to rabbit polyclonal antibodies against syntaxin 4, SNAP23, and FLAG. Cell lysates (Lys) and supernatants (Sup) are shown for reference. No SNAP23 or FLAG-Munc18c pull down was observed when syntaxin 4-Myc2 was absent (right) (n = 4).

bition of SNARE complex nucleation in vivo. To address this question, we developed an optical reporter system to assess how Munc18c affects the interaction of closed or constitutively open syntaxin 4 with SNAP23 (Fig. 7A). We expressed limiting quantities of syntaxin 4 in living HEK293-S3 cells with an excess of GFP-SNAP23C/A, a cytosolic mutant of SNAP23 lacking its four-cysteine palmitylation sequence (39). After a 1-min photobleach of a delimited region within the cytosol, GFP-SNAP23C/A was resolved at the plasma membrane, consistent with the formation of a dimeric SNAP23-synactin 4 complex. The additional expression of mRFP-Munc18c resulted in the complete loss of GFP-SNAP23C/A signal after photobleach, signifying that GFP-SNAP23C/A was freely diffusible and not tethered at the plasma membrane. This result intimates that Munc18c binding to syntaxin 4 occludes SNAP23 binding to syntaxin 4. Indeed, the presence of membrane-associated mRFP-Munc18c was observed after the photobleach, indicative of its binding to syntaxin 4. The membrane association of Munc18c was not due to binding of endogenous proteins in the HEK293 cells, since expressed Munc18c was cytosolic in the absence of expressed syntaxin 4 (data not shown). In summary, the data are consistent with Munc18c occluding syntaxin 4 interactions with SNAP23C/A coexpression condition, no further bleaching of GFP was observed on the membrane, although membrane-associated RFP-Munc18c signal recovered after membrane photobleach, consistent with the Munc18c inhibition of Q-SNARE complex formation (supplemental Fig. S1). However, that both RFP-Munc18c and GFP-SNAP23C/A signals recover after a subsequent plasma membrane photobleach when coexpressed with syntaxin 4LE supports the interpretation that a Munc18c-syntactin 4LE-SNAP23C/A complex is formed specifically at the plasma membrane.
expressed syntaxin 4\textsuperscript{LE}. Comparatively, coexpression of FLAG-Munc18c inhibited the binding of syntaxin 4\textsuperscript{WT}-Myc\textsubscript{2} to SNAP23. Importantly, detectable levels of both SNAP23 and FLAG-Munc18c were present equivalently in the lysate and supernatant in cells lacking expressed syntaxin 4, which demonstrates that both proteins were specifically pulled down by the Myc-tagged syntaxins. Since coprecipitation of Munc18c and SNAP23 may be explained by immunoprecipitation of a mixture of syntaxin 4\textsuperscript{LE} in heterodimeric complex with Munc18c and SNAP23, we tested for a Munc18c-syntaxin 4\textsuperscript{LE}-SNAP23 tripartite complex, as suggested by the \textit{in vitro} binding of Munc18c to preformed syntaxin 4\textsuperscript{4TM}-SNAP23 binary complexes (27). Whereas expression of Munc18c inhibited co-precipitation of syntaxin 4\textsuperscript{WT} by Myc-SNAP23 (Fig. 8, left), immunoprecipitation of Myc-tagged SNAP23 resulted in co-precipitation of both expressed syntaxin 4\textsuperscript{LE} and FLAG-Munc18c (Fig. 8, middle). Notably, SNAP23 did not bind directly to Munc18c such that pull-down of Munc18c must have occurred through a trimeric complex (Fig. 8, right). Endogenous VAMP2 was not found associated with this complex, suggesting that either VAMP2 is prohibited from entering SNARE complexes when Munc18c is bound (5) or that ternary SNARE complex formation was transient and went undetected by our assay. However, it is possible to conclude from both the photobleach analysis and immunoprecipitation experiments that 1) Munc18c inhibition of Q-SNARE complex formation is relieved by a conformational change in syntaxin 4, and 2) Munc18c binding to Q-SNARE complexes suggests an additional regulatory capacity on downstream fusion events.

**Functional Effects of Munc18c-Syntaxin 4 Binding States on GLUT4 Trafficking**—The above results demonstrate two distinct binding states of the Munc18c-syntaxin 4 interaction: Munc18c binding to a conformation of syntaxin 4 that is inhibitory to SNARE complexes and furthermore to an open conformation, through binding to Q-SNARE complexes (Figs. 7 and 8). We next sought to determine the functional correlate of each Munc18c-syntaxin 4 binding state on GLUT4 vesicle plasma membrane recruitment and fusion under expression conditions favoring the interaction of Munc18c with either the closed or open forms of syntaxin 4. Our assay of SNARE complex formation was based on quantification of the insulin-stimulated plasma membrane insertion of GLUT4 storage vesicles in 3T3L1 adipocytes stably expressing Myc\textsubscript{7}-GLUT4-GFP (32, 33). In these cells, the resting distribution of GLUT4 vesicles is primarily intracellular, whereas insulin promotes a large net increase in the number of plasma membrane-bound transporters, catalyzed by syntaxin 4-SNARE complexes (8). The movement of GLUT4 was monitored by measuring GFP fluorescence at the cell periphery. Immunostaining of the exofacial Myc, epitope, contained in the first extracellular loop of GLUT4, was used to distinguish vesicle fusion from vesicle plasma membrane localization.

We examined the effect of Munc18c overexpressed with CFP-syntaxin 4\textsuperscript{WT}, CFP-syntaxin 4\textsuperscript{LE}, CFP-syntaxin 4\textsuperscript{I241A}, or CFP-syntaxin 4\textsuperscript{LE} + I241A on GLUT4 trafficking in 3T3L1 adipocytes. The cells were fixed before or after stimulation with 100 nM insulin, immunostained against the Myc epitope, and blindly scored for the presence of translocated (peripheral GFP) or fused (peripheral indirect Myc immunofluorescence) GLUT4 vesicles (summarized in Table 1). Representative confocal images for each treatment are shown in Fig. 9A. In the absence of insulin, the extent of GLUT4 movement to the cell periphery in cells expressing CFP-syntaxin 4\textsuperscript{WT} and Munc18c was limited to 18 ± 10% of cells (n = 75), with very little vesicle fusion (2.5 ± 1.5%). Insulin stimulated a 4.5-fold increase in vesicle recruitment (83 ± 4%, n = 114). These results are comparable with GLUT4 recruitment in untransfected adipocytes in the absence (12 ± 8%, n = 75) and presence (95 ± 3%, n = 75) of insulin. By comparison, 79 ± 3% (n = 100) of the cells expressing CFP-syntaxin 4\textsuperscript{LE} displayed unregulated recruitment of GLUT4 vesicles to the plasma membrane in the absence of insulin, despite the presence of coexpressed Munc18c (Table 1). To rigorously quantify the GLUT4 localization, we examined cells exhibiting equivalent expression of

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**TABLE 1**

**Effects of syntaxin 4 mutants on GLUT4 translocation in 3T3L1 adipocytes**

3T3L1 adipocytes stably expressing Myc\textsubscript{7}-GLUT4-GFP were transfected with the indicated constructs, fixed before or after treatment with 100 nM insulin for 30 min at 37 °C, and stained with Myc antibody. The percentage of GFP-positive cells exhibiting peripheral GLUT4 (as detected by GFP fluorescence) or GLUT4 externalization (as detected by indirect Myc immunofluorescence) is displayed ± S.E. (data are averaged from three independent experiments).

| Transfection conditions | No insulin | Insulin |
|-------------------------|------------|---------|
|                         | GLUT4 (%), Myc (%), n | GLUT4 (%), Myc (%), n |
| Munc18c + CFP-syntaxin 4\textsuperscript{WT} | 18 ± 10, 2.5 ± 1.5, 75 | 83 ± 4, 46 ± 2, 114 |
| Munc18c + CFP-syntaxin 4\textsuperscript{LE} | 79 ± 3, 5 ± 3, 100 | 92 ± 6, 52 ± 2, 125 |
| Munc18c + CFP-syntaxin 4\textsuperscript{I241A} | 12 ± 4, 2.5 ± 1.5, 75 | 92 ± 2, 65 ± 1, 75 |
| Munc18c + CFP-syntaxin 4\textsuperscript{LE} + I241A | 40 ± 6, 4.5 ± 0.5, 60 | 88 ± 4, 45 ± 2, 60 |
both CFP-syntaxin 4 (or mutants) and Myc-GLUT4-GFP across treatments (Fig. 9B). As depicted in Fig. 9C, CFP and GFP fluorescence intensity was measured along a 10-pixel-wide line, and average fluorescence intensities were plotted as a function of distance from the plasma membrane. All four CFP-syntaxin 4 constructs tested exhibited proper membrane localization when expressed with Munc18c. However, cells expressing CFP-syntaxin 4LE exhibited significantly increased plasma membrane-localized Myc7-GLUT4-GFP. Thus, GLUT4 vesicle recruitment was strictly promoted by the conformational change in syntaxin 4. However, that only 5 ± 3% of the cells co-expressing syntaxin 4LE and Munc18c exhibited GLUT4 fusion (without insulin) (Table 1) suggests that a further mechanism exists to arrest GLUT4 vesicles at the plasma membrane without permitting vesicle fusion. To eliminate the possibility that the CFP tag used on the syntaxin 4 constructs inhibits fusion, we transfected adipocytes with unlabeled syntaxin 4WT or syntaxin 4LE together with Cerulean-Munc18c. Coexpression of syntaxin 4 was confirmed based on the plasma membrane targeting of Cerulean-Munc18c (supplemental Fig. S2), which does not occur to a significant extent in the absence of overexpressed syntaxin 4. As before, GLUT4 vesicles were found abnormally docked in the absence of insulin in cells expressing syntaxin 4LE (but not syntaxin 4WT) and Cerulean-Munc18c. These complementary results support the interpretation that a conformational change in syntaxin 4 is sufficient to
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tether GLUT4 vesicles to the plasma membrane without allowing their fusion.

Notably, our syntaxin 4 experiments were performed with overexpressed Munc18c, which leaves open the possibility that Munc18c is required for the conformational switch in syntaxin 4 at either the docking or fusion stages of GLUT4 trafficking. To address this issue, we overexpressed Munc18c with CFP-syntaxin 4L241A, which exhibits reduced binding with Munc18c regardless of syntaxin 4 conformation. GLUT4 movement to the plasma membrane was unaffected in unstimulated adipocytes (12 ± 4%, n = 75) (Fig. 9), indicating that Munc18c binding to syntaxin 4 is not strictly required to inhibit GLUT4 vesicle recruitment, consistent with results in Munc18c-null cells (11). To test for a role of Munc18c in docking GLUT4 vesicles to the plasma membrane, we expressed the double mutant CFP-syntaxin 4L4E + L241A with Munc18c. The number of cells exhibiting docked vesicles in the absence of insulin was reduced by ~50% (40 ± 6%, n = 60) when compared with the CFP-syntaxin 4LE and Munc18c co-expression condition (Table 1), concomitant with a quantifiable decrease in plasma membrane GLUT4 fluorescence (Fig. 9C). These results indicate that although conformational opening of syntaxin 4 is sufficient to promote GLUT4 vesicle docking, Munc18c facilitates the docking process.

DISCUSSION

SM proteins exert functional effects at different stages of SNARE complex assembly primarily through specific, nonconserved, interactions with cognate syntaxins. In this study, we have focused on identifying and characterizing the molecular underpinnings of the interactions between the ubiquitous SM protein Munc18c and its Q-SNARE cognate, syntaxin 4, an interaction of critical importance in the regulation of insulin-stimulated GLUT4 exocytosis in adipose tissue and muscle (8). Using a combination of optical techniques and biochemical approaches, we have demonstrated that Munc18c is capable of multiple binding interactions with syntaxin 4 in vivo, which probably occur during the stepwise assembly of SNARE complexes. These interactions include Munc18c binding to a closed conformation of syntaxin 4 that is inhibitory to SNARE complex nucleation as well as to an open conformation, which permits Munc18c binding to heteromeric Q-SNARE complexes. Point mutants were used to identify the syntaxin 4 SNARE domain as essential for mediating Munc18c interaction with both syntaxin 4 conformations, although the N terminus of syntaxin 4 was strictly required, but insufficient, for Munc18c binding to the open conformation. Last, we provide evidence that these multiple Munc18c binding states with Q-SNAREs are critical determinants of distinct functional effects on Glut4 vesicle translocation in differentiated 3T3L1 adipocytes.

Recent reports have indicated that Munc18c possesses an ability to directly interact with syntaxin 4 while it is bound to other SNARE proteins (26, 27). Our findings in living 3T3L1 adipocytes extend the analysis of direct Munc18c interactions to an in vivo situation, with a specific focus on its interactions with syntaxin 4. We report that Munc18c exhibits nearly equal FRET efficiency with either wild type syntaxin 4 or a mutant that maintains a constitutively open conformation (L173A/E174A; LE). Therefore, the affinity of Munc18c for syntaxin 4 appears to be largely unaffected by the conformational state of syntaxin 4. Our direct in vitro binding studies using bacterially expressed GST fusion proteins as well as co-immunoprecipitation results from cell lysates confirmed the in vivo findings. We and others have previously reported that Munc18a also exhibits limited binding to the constitutively open LE mutant of syntaxin 1A in vivo, (15, 17), a finding that has been recently extended to include Munc18a interaction with syntaxin 1A in heteromeric Q-SNARE complexes (40) and fully assembled SNARE complexes (24, 25). Comparatively, the yeast exocytotic SM protein Sec1p interacts weakly with a closed conformation of Sso1p, favoring binding to Sso1p-Snc2p Q-SNARE complexes (22, 23). Therefore, the interaction of exocytotic SM proteins with their cognate syntaxins appears to be converging on a general model where exocytotic Sec/Munc18 proteins demonstrate multiple binding states.

Our data establishing that Munc18c is able to interact with an open conformation of syntaxin 4 in vivo prompted experiments to map the primary structural sites that mediate the interaction. To date, the interacting sites between these proteins have been postulated to resemble the crystal structure of Munc18a in complex with syntaxin 1A (14). Indeed, our initial threading of the Munc18c and syntaxin 4 sequences directly into the Munc18a-syntaxin 1A crystal structure predicted similar interactions between Munc18c and the C terminus of syntaxin 4 when it is in the closed conformation. In confirmation of this prediction, we show that homologous I241A and I233A SNARE domain mutations in syntaxin 4 and syntaxin 1A, respectively, both dramatically reduced binding to their SM partners (17, 29). Furthermore, the entire SNARE domain of syntaxin 1A substituted for the syntaxin 4 SNARE domain exhibited no consequence for Munc18c binding. Notably, the complementary substitution (i.e. replacement of syntaxin 1A SNARE motif with syntaxin 4 sequence) resulted in a complete loss of Munc18a binding to the syntaxin 1A chimera (data not shown). These results correlate well with the binding of Munc18c to both syntaxin 4 and, with lesser affinity, syntaxin 1A, whereas Munc18a shows specificity for syntaxin 1A (41).

By comparison with the relatively well defined interactions of Munc18c with a closed state of syntaxin 4, the specific structural determinants that allow interaction of Munc18c with an open conformation of syntaxin 4 have been largely unstudied. In the current study, we show that the N terminus of syntaxin 4 is required for binding of Munc18c to the open mutant of syntaxin 4, but not to wild type syntaxin 4, via examination of an arginine substitution at syntaxin 4 Asp3 in FRET binding assays. This residue is conserved in both exocytotic and ER/Golgi syntaxins (syntaxins 1–4, 5, and 18), suggesting that SM protein interaction with the N terminus of syntaxin is a common mechanism for association with the open conformation in intracellular SNARE complexes. This is consistent with the N-terminal motif of Sed5p/syntaxin 5 and Ufe1p/syntaxin 18 providing a platform for their SM partner, Sly1p, to bind both monomeric syntaxin proteins and SNARE complexes (20, 21). Our findings complement a recent report that used an in vitro binding assay to elucidate the Munc18c binding pocket.
predicted to hold the N terminus of syntaxin 4 (27). However, a notable discrepancy between these collective findings is whether the syntaxin 4 N terminus is sufficient for Munc18c binding. Using an in vitro binding assay, Latham et al. (27) reported significant association between Munc18c and a mutant syntaxin 4 that lacked the SNARE motif (H3 domain) and transmembrane domain (syntaxin 4E341I). However, our in vivo analysis using a FRET-based assay displayed a loss of Munc18c interaction with syntaxin 4E341I, thereby suggesting that the N-terminal interaction is insufficient to mediate the Munc18c-syntaxin 4 interaction, in parallel to the in vivo findings of Ter Beest et al. (42). The necessity of the syntaxin 4 C terminus was additionally confirmed using the I241A mutation of syntaxin 4, a residue predicted to bind to the central cavity of Munc18c. The syntaxin 4I241A mutant eliminated Munc18c binding to constitutively open syntaxin 4 and severely reduced interaction with wild type syntaxin 4. Therefore, our data favor a model in which Munc18c interaction with either the open or closed conformation of syntaxin 4 depends upon SNARE motif interactions, with an additional requirement for an N-terminal interaction with syntaxin 4 in the open conformation. A similar requirement has been demonstrated for Munc18a binding to the open conformation of syntaxin 1A in SNARE complexes (25).

The SNARE assembly cycle begins with syntaxin nucleation of Qb- and Qc-SNARE complexes followed by the final SNARE motif contributed by vesicular R-SNAREs (43). It has remained unclear which steps are the relevant sites of action for the exocytotic SM proteins. In the present study, we demonstrate that Munc18c can interact with a SNARE pairing-competent conformation of syntaxin 4 in vivo. These findings extend in vitro binding studies, which showed Munc18c associated with preformed syntaxin 4-SNAP23 Q-SNARE complexes as well as ternary SNARE complexes composed of syntaxin 4, SNAP23, and VAMP2 (26, 27). It is difficult to directly demonstrate that Munc18c-containing SNARE complexes form in vivo within the regulated secretory pathway. However, as support that these complexes form, we show that constitutively open syntaxin 4E4LE recruits both Munc18c and diffusible GFP-SNAP23C/A to the plasma membrane, as resolved by photobleach/FRAP of GFP and RFP. Our SNAP23 immunoprecipitation data additionally confirm that a Munc18c-syntaxin 4E4LE-SNAP23 complex can be co-precipitated from lysates of HEK293-S3 cells overexpressing these proteins. This complex is prohibited from forming by Munc18c binding to syntaxin 4E341I. Thus, the stabilization of an open syntaxin 4 conformation relieves the Munc18c block on SNARE complex nucleation, although the functional significance of the Munc18c-Q-SNARE complexes remains unclear. That endogenous VAMP2 was not found associated with this complex in immunoprecipitation experiments is difficult to interpret. Either the Q-SNARE-Munc18c complex is prohibitive of VAMP2 binding despite the constitutive secretion in these cells, or ternary SNARE complex formation was transient and went undetected. We suggest the latter possibility in light of recent reports that both Munc18c and Munc18a facilitate SNARE assembly in vitro (24, 27).

Of fundamental importance to our study was the correlation of distinct Munc18c-syntaxin 4 binding states with specific functional effects on the GLUT4 translocation pathway. The accumulation of GLUT4 vesicles at the plasma membrane prior to detectable increases in glucose uptake or prior to the accessibility of exofacial GLUT4 epitopes (44, 45) has led to a trafficking model that identifies separate tethering, docking, and fusion steps as the critical sites of hormone action on exocytosis. To distinguish these separate kinetic steps, we performed functional assays in differentiated 3T3L1 adipocytes in which dual labeled Myc-C-GLUT4-GFP was used as a tool to differentiate between GLUT4 vesicle docking and GLUT4 vesicle fusion. Our results suggest that the specific conformation of syntaxin 4 together with its state of interaction with Munc18c defines whether GLUT4 vesicles are retained in recycling pathways or undergo fusion. GLUT4 vesicles were observed to accumulate at the plasma membrane regions following overexpression of the constitutively open LE mutant of syntaxin 4 with Munc18c, even in the absence of insulin stimulation. By comparison, no such accumulation was observed upon coexpression of wild type syntaxin 4 with Munc18c. Indeed, reports from other groups indicate that GLUT4 vesicles cycle to the plasma membrane in the absence of insulin, although studies using total internal reflection fluorescence microscopy indicate that these vesicles do not normally dock (46, 47). Our results indicate that the constitutively open conformation of syntaxin 4E4LE bypasses the requirement for insulin-regulated signaling pathways, uncoupling the normal checkpoint between vesicle recycling and vesicle docking. Interestingly, although SNARE proteins are not traditionally thought of as docking factors, particularly in neurons, it has been recently reported that syntaxin 1A is required for vesicle docking in adrenal chromaffin cells (48).

Several lines of evidence suggest that insulin ultimately reduces Munc18c-syntaxin 4 complexation through the activity of phosphatidylinositol 3-kinase and protein kinase C δ (10, 11). However, it appears that dissociation of Munc18c from syntaxin 4 may only be required at a site downstream of SNARE complex formation and committed vesicle docking. Two lines of evidence support this notion. First, overexpression of Munc18c with the syntaxin 4I241A mutant, which demonstrates greatly reduced Munc18c binding, did not recapitulate the docking phenotype exhibited by the syntaxin 4E4LE mutant. Moreover, GLUT4 vesicles dock normally in an insulin-regulated fashion in adipocytes lacking Munc18c altogether (11). Second, the same evidence indicates that the release of Munc18c from syntaxin4 is furthermore insufficient to render syntaxin 4 SNARE-competent, a role originally identified for priming factors, such as Munc13 (34). Rather, we have identified Munc18c as a facilitator of vesicle docking, since the syntaxin 4E4LE + I241A double mutant demonstrated decreased GLUT4-GFP at the plasma membrane relative to the syntaxin 4E4LE mutant. Munc18c, however, is not required for vesicle docking, since GLUT4 vesicles fused in response to insulin in both Munc18c-null adipocytes as well as those expressing syntaxin 4E4LE + I241A. By comparison, in chromaffin cells lacking Munc18a, the number of docked vesicles is reduced 10-fold, and calcium-dependent secretion is also reduced (49).

In summary, our data indicate that Munc18c probably regulates syntaxin 4-mediated SNARE complex formation via two
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discrete binding states: 1) Munc18c prevents monomeric syntaxin 4 from entering into Q-SNARE complexes with SNAP23, an inhibition that is probably overcome by the priming factors that open syntaxin 4; 2) through binding to an open conformation of syntaxin 4, Munc18c facilitates SNARE complex nucleation and vesicle docking. Downstream of SNARE complex formation, Munc18c may also serve as a scaffold for additional proteins to further regulate SNARE disassembly or recycling. An important focus of future experiments will be to elucidate the specific signaling pathways that alter the Munc18c-syntaxin 4 binding mode as well as determine the specific priming factors that control syntaxin 4 conformation in insulin-stimulated adipocytes.

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