Glucose-stimulated insulin secretion is mediated by syntaxin 4-based SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein complexes and the Sec1/Munc18 protein Munc18c. Our laboratory recently reported that Munc18c-syntaxin 4 complexes are further regulated by the competitive binding of the double C2 domain protein Doc2β to Munc18c, although the underlying mechanism for this is unknown. Because the Doc2β binding region of Munc18c contained residue Tyr-219 and this residue becomes phosphorylated in response to glucose stimulation, we hypothesized that the mechanism would involve Munc18c phosphorylation. Coimmunoprecipitation analyses using detergent lysates prepared from pervanadate-treated MIN6 beta cells revealed that the tyrosine phosphorylation of Munc18c corresponded to a 60% decrease in Munc18c-Doc2β association with a coordinate 2-fold decrease in Munc18c-syntaxin 4 association with 18 U.S.C. Section 1734 solely to indicate this fact. This work was supported, in whole or in part, by National Institutes of Health Grants DK067912 and DK076614 (to D. C. T.). This work was also supported by American Heart Association Postdoctoral Fellowship 0720042Z (to E. O.) and a DeVault Diabetes predoctoral award (to J. L. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Evidence in favor of Munc18c as a negative regulator of exocytosis is principally based upon over-expression methodologies but was supported by crystallographic and NMR data showing that the neuronal Munc18c protein (nSec1/Munc18c-1/Munc18a) held its syntaxin 1 binding partner in a closed conformation in a 1:1 stoichiometric complex (22, 23) and in vitro protein-protein interaction studies stating the absence of Munc18c from the 7 S and 20 S SNARE complexes (24, 25). The role of Munc18c as a possible positive regulator in exocytosis is based upon aberrant glucose uptake and insulin secretion from Munc18c heterozygous knock-out mice and peptide interference of endogenous Munc18c-syntaxin 4 association in 3T3L1 adipocytes (13, 20). In addition, recent in vitro data have detected Sec1/Munc18c proteins associated with SNARE core complexes (26–29). Notably, these recent in vitro studies were conducted using milder detergent conditions than earlier studies that showed Sec1/Munc18c protein binding to monomeric syntaxin in cell lysates (30–33). These differing buffer conditions combined with in vitro versus cellular approaches have confounded our ability to designate a clear role for Munc18c proteins.

In addition to issues of detergent conditions and methodologies, it remains to be resolved just what contacts of the syntaxin molecule are sufficient to confer its interaction with Munc18. There is a general consensus that the N-terminal 20–30 residues of syntaxins are important for interaction with Munc18 proteins (34–36), and it is suggested that it is through this region that Munc18 proteins remain associated with the SNARE core complex (37). However, it has been disconnected protein; MKRBB, modified Krebs-Ringer bicarbonate buffer; PM, plasma membrane; GST, glutathione S-transferase; TAP, tandem affinity purification; VAMP, vesicle-associated SNARE protein; CBP, calmodulin-binding peptide; pV, pervanadate; GFP, green fluorescent protein.
puted that this N-terminal region of syntaxin 4 can independently confer binding specificity to the Munc18c isoform, as opposed to binding Munc18a or Munc18b (Munc18a and Munc18b specifically bind syntaxin isoforms 1–3). Thus, it has been suggested there are likely alternate or additional residues required to support and specify the association (37, 38).

Consistent with the dissociation model put forth by Zilly et al. (39), Munc18c-syntaxin 4 association can be significantly reduced upon the stimulus-induced phosphorylation of residue Tyr-219 of Munc18c (16). We have since determined that a region of Munc18c containing this particular residue is sufficient to confer binding to the C2 domain-containing protein Doc2β, which is localized at the plasma membrane in islet beta cells and adipocytes (14). Syntaxin 4 was excluded from the Munc18c-Doc2β complex, suggesting that some type of “switch mechanism” occurs for transitions between Munc18c-Doc2β and Munc18c-syntaxin 4 complex formation.

In the present study we have examined the protein-protein interactions among Munc18c and its binding partners syntaxin 4 and Doc2β to test the validity of such a “switch mechanism” using cell lysates under membrane protein-solubilizing conditions. Our experimental results show that, indeed, Doc2β preferentially bound to tyrosine-phosphorylated Munc18c and may function as an “acceptor” of Munc18c displaced from binding to syntaxin 4. Moreover, we have delineated the region containing the Hc-Linker region (residues 118–194) of syntaxin 4 capable of conferring stable interaction with Munc18c. Molecular modeling of these new contacts in syntaxin 4 with phosphorylation and Doc2β binding sites present in Munc18c provide new insights into the conformational changes incurred in these proteins.

EXPERIMENTAL PROCEDURES

Materials—Rabbit anti-Munc18c antibody was generated as previously described (17). Rabbit polyclonal anti-syntaxin 4 and N-terminal syntaxin 4 peptide (residues 2–23) were obtained from Chemicon (Temecula, CA). Antibodies raised against SNAP23, VAMP2, and phosphotyrosine (4G10) were obtained from Affinity BioReagents (Golden, CO), Synaptic Systems (Göttingen, Germany), and Upstate Biotechnology (Lake Placid, NY), respectively. Rabbit polyclonal anti-Doc2β antibody was a generous gift from Dr. Dr. Mattijs Verhage (Vrije Universiteit, The Netherlands). Rabbit and mouse anti-GFP antibodies were acquired from Abcam (Cambridge, MA) and Clontech Laboratories (Mountain View, CA), respectively. Protein G plus-agarose was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The MIN6 beta cells were a kind gift from Dr. John Hutton (University of Colorado Health Sciences Center). Goat anti-mouse and anti-rabbit horseradish peroxidase secondary antibodies and Transfectin lipid reagent were acquired from Bio-Rad. Monoclonal FLAG antibody, radioimmunoassay grade bovine serum albumin, and N-glucose were purchased from Sigma. Enhanced chemiluminescence (ECL) and SuperSignal West Femto reagents were obtained from Amersham Biosciences and Pierce, respectively. The human C-peptide radioimmunoassay kit was purchased from Linco Research Inc (St. Charles, MO).

Plasmids—The generation of pcDNA3.1-FLAG-Munc18c, pET28a(+)-His-Munc18c, and pGEX-Munc18c plasmids has been previously described (14, 15, 17). The TAP-Munc18c DNA constructs were generated by subcloning a PCR-generated Munc18c fragment into the XhoI and Clal sites of the pNTAP and pCTAP shuttle vectors (Stratagene, La Jolla CA) for adenosiviral particle production by Virasert (North Liberty, IA). The pGEX-4T1-syntaxin 4 (–193, –112), and (1–70) DNA constructs were generated by subcloning PCR-generated rat syntaxin 4 fragments into the Sall and XhoI restriction sites of the pGEX-4T1 expression vector (GE Healthcare). The pGEX-4T1-syntaxin 4 (–123, –93, –71–273, and –113–273) DNA constructs were made by subcloning PCR-generated rat syntaxin 4 fragments into the EcoR1 and XhoI sites of the pGEX-4T1. The pEGFP-C2-syntaxin 4 (–193, –112) DNA construct was generated by digesting the fragment from pGEX-4T1-syntaxin 4 (–113–193) with EcoR1 and XhoI and subcloning into the EcoR1 and SalI sites of the pEGFP-C2 vector (BD Biosciences Clontech).

Cell Culture, Transient Transfection, and Secretion Assays—MIN6 beta cells were cultured in Dulbecco’s modified Eagle’s medium (Dulbecco’s modified Eagle’s medium with 25 mM glucose) supplemented with 15% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 292 µg/ml L-glutamine, and 50 µM β-mercaptoethanol as described previously (14). MIN6 beta cells at 50–60% confluence were transfected with 40 µg of plasmid DNA per 10-cm² dish using Transfectin (Bio-Rad) to obtain ~50% transfection efficiency. After 48 h of incubation cells were washed twice with and incubated for 2 h in freshly prepared modified Krebs-Ringer bicarbonate buffer (MKRBB; 5 mM KCl, 120 mM NaCl, 15 mM HEPES pH 7.4, 2.4 mM NaHCO3, 1 mM MgCl2, 2 mM CaCl2, and 1 mg/ml radiomunnoassay grade bovine serum albumin). Cells were stimulated with 20 mM glucose or 0.1 mM pervanadate for the times indicated in the figures. Cells were subsequently lysed in Nonidet P-40 lysis buffer (25 mM Tris, pH 7.4, 1% Nonidet, 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, and 5 µg/ml leupeptin), and lysates were cleared by microcentrifugation for 10 min at 4 °C for subsequent use in co-immunoprecipitation experiments. For measurement of human C-peptide release, MIN6 beta cells were transiently co-transfected with each plasmid plus human proinsulin cDNA (a gift from Dr. Chris Newgard, Duke University) using Transfectin with 2 µg of each DNA per 35-mm dish of cells. Forty-eight hours after transfection, cells were preincubated for 2 h in MKRBB buffer and stimulated with 20 mM glucose for 1 h, and buffer was collected for quantitation of human C-peptide released.

CHO-K1 cell stocks were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 292 µg/ml L-glutamine. At 80–90% confluence, cells were electroporated with 40 µg of DNA as previously described (17) and incubated for 48 h. Detergent cell lysates were prepared by harvesting in 1% Nonidet P-40 lysis buffer, and lysates were cleared by cen-
trifugation at 14,000 × g for 10 min at 4 °C for use in co-immunoprecipitation experiments.

**Tandem Affinity Purification (TAP)***—The InterPlay Adenoviral TAP System (Stratagene) was used for purification of Munc18c-associated proteins from MIN6 beta cells. NTAP-Munc18c contained two affinity tags, a streptavidin-binding peptide and a calmodulin-binding peptide (CBP). MIN6 beta cells were transduced using pNTAP-Munc18c or pCTAP-Munc18c CsCl-purified adenoviral particles as described previously (40). Cells were harvested in the manufacturer's lysis buffer containing 0.1% Triton X-100 or in this buffer containing additional detergent (1% Triton X-100 final concentration). The cells were subjected to 3 successive rounds of freeze-thawing and centrifuged at 16,000 × g for 10 min for collection of supernatant. Supernatants were incubated with streptavidin resin followed by washes with lysis buffer (0.1% Triton X-100 and elution. Eluates were then subjected to a second purification over calmodulin resin. Column flow-through was collected. Purified TAP-Munc18c and associated proteins were eluted and resolved by 12% SDS-PAGE for immunoblotting.

**Subcellular Fractionation**—Subcellular fractions of beta cells were isolated as described previously (41). Briefly, MIN6 beta cells at 80–90% confluence were harvested into 1 ml of homogenization buffer (20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, and 1 mM sodium orthovanadate containing the protease inhibitors leupeptin (10 μg/ml), aprotinin (4 μg/ml), pepstatin (2 μg/ml), and phenylmethylsulfonyl fluoride (100 μM). Cells were disrupted by 10 strokes through a 27-gauge needle, and homogenates were centrifuged at 900 × g for 10 min. Plasma membrane fractions were obtained by mixing the postnuclear pellet with 1 ml of buffer A (0.25 M sucrose, 1 mM MgCl₂, and 10 mM Tris-HCl, pH 7.4) and 2 volumes of buffer B (2 M sucrose, 1 mM MgCl₂, and 10 mM Tris-HCl, pH 7.4). The mixture was overlaid with buffer A and centrifuged at 113,000 g for 1 h to obtain an interface containing the plasma membrane fraction. Interface was collected and diluted to 2 ml with homogenization buffer for centrifugation at 6000 × g for 10 min, and the resulting pellet was collected as the plasma membrane fraction. Pellets were resuspended in 1% Nonidet P-40 lysis buffer to solubilize membrane proteins.

**Co-immunoprecipitation and Immunoblotting**—MIN6 beta cells were preincubated in MKRBB for 2 h followed by glucose stimulation or treatment with 0.1 mM pervanadate. Pervanadate was made immediately before use by combining 1 mM sodium orthovanadate with 3 mM hydrogen peroxide for 15 min. Cells were subsequently lysed in 1% Nonidet P-40 lysis buffer. MIN6 beta cell-clearal detergent lysates (2–4 mg) were combined with primary antibody for 2 h at 4 °C followed by a second incubation with protein G-agarose for 2 h. The resultant immunoprecipitates were subjected to 10 or 12% SDS-PAGE followed by transfer to polyvinylidene difluoride membranes for immunoblotting. Munc18c and VAMP2 antibodies were used at 1:5000; syntaxin 4, Doc2 β, SNAP23, and 4G10 antibodies were used at 1:1000; FLAG and EGFP antibodies were used at 1:2000 and 1:800, respectively. Secondary antibodies conjugated to horseradish peroxidase were diluted at 1:5000 for all except for phosphotyrosine, which was used at 1:2000 for visualization by ECL for detection using a Chemi-Doc documentation imaging system (Bio-Rad).

**Recombinant Proteins and Interaction Assays**—The pGEX fusion constructs were transformed into Escherichia coli for expression of all GST fusion proteins and purified by glutathione-agarose affinity chromatography as described previously (42). Recombinant fragments of syntaxin 4 were obtained after thrombin cleavage and capture (Novagen). Recombinant His-tagged Munc18c was expressed in E. coli and purified by nickel-nitrilotriacetic acid chelating resin (Invitrogen) under native conditions (50 mM NaH₂PO₄, 0.5 mM NaCl). Eluted protein was further dialyzed overnight in 50 mM Tris, pH 8.0, supplemented with 1 mM dithiothreitol. *In vitro* GST interaction assays were conducted by incubating GST fusion proteins linked to Sepharose beads with recombinant purified proteins for 2 h at 4 °C in Nonidet P-40 lysis buffer followed by three stringent washes with lysis buffer and interacting proteins eluted from the beads and resolved on 10% or 12% SDS-PAGE followed by transfer to polyvinylidene difluoride membrane for immunoblotting. The syntaxin accessibility assay using GST-VAMP2 was performed similarly but using cleared detergent cell lysates instead of recombinant protein, as recently described (43).

**Surface Plasmon Resonance**—The binding kinetics for the interaction between syntaxin 4-(1–273) and syntaxin 4-(118–194) with Munc18c was determined by surface plasmon resonance using a BIAcore3000 instrument (Biacore AB, Uppsala, Sweden). Syntaxin 4-(1–273) and syntaxin 4-(118–194) were diluted in acetate buffer at pH 4.5 and immobilized on a CM5 sensor chip with amine coupling. The surface was activated by injecting a solution containing 0.4 M N-ethyl-N’-dimethylaminopropylcarbodiimide (EDC) and 50 mM N-hydroxysuccinimide (NHS) for a 7-min flow rate of 5 μl/min (44). Syntaxin 4 was then injected, and the surface was subsequently blocked with 1 M ethanolamine at pH 8.5 for 7 min. The target for final immobilization levels was 1000 resonance units, corresponding to ~1 ng of syntaxin 4/mm² (45). Munc18c was injected over the flow cell at 30 μl/min for 2 min and allowed to dissociate for 3 min. To determine the association (kₐ) and dissociation (kₐ) rate constants, Munc18c was injected at seven concentrations ranging from 0 to 53.3 nM. Between cycles, the surface was regenerated with 10 μl of 0.15 M acetic acid, 0.5 M NaCl. The data were then fitted to a 1:1 (Langmuir) binding model using the BiaEvaluation software. It was found that mass transfer was not a factor in Munc18c binding to syntaxin 4. All experiments were conducted in HBS-EP running buffer (Biacore, Piscataway, NJ) with Munc18c storage buffer added to a 1:50 dilution at 25 °C.

**Computer Modeling**—The three-dimensional structure of the syntaxin 4-Munc18c complex was guided by the crystal structure of the syntaxin 1A/Munc18 complex (PDB code 1DN1) and the crystal structure of Munc18c (PDB code 2PJX). A homology model of syntaxin 4 was constructed using Swiss-Model based on the crystal structure of syntaxin 1a. The syntaxin 4-Munc18c complex was constructed by superimposing these proteins onto their homologs in the syntaxin 1A-Munc18 complex. This was carried out with the computer program PyMol. All representations of three-dimensional structures in the manuscript were generated with PyMol.
Doc2β Preferentially Binds Tyrosine-phosphorylated Munc18c

RESULTS

Munc18c-Syntaxin 4 Binary Complexes Predominate in Detergent Lysates—Although recent studies show association of Munc18 proteins with ternary SNARE core complexes in vitro, this association of endogenous proteins within cells has yet to be demonstrated. To investigate the binding mode(s) of Munc18c in cells known to require Munc18c for regulated exocytosis, MIN6 beta cells were harvested in 1% Nonidet P-40 detergent. MIN6 cells were transduced with NTAP-Munc18c adenovirus (multiplicity of infection = 100). Lysates were subsequently prepared in buffer containing 0.1% Triton X-100 (lanes 1–3) or 1% Triton X-100 (lanes 4–6). Streptavidin-binding peptide-CBP column eluates were subjected to 12% SDS-PAGE for immunoblotting (IB). TrueBlot anti-rabbit Ig immunoprecipitation beads were substituted in for protein G plus agarose to facilitate detection of SNAP23. B, MIN6 cells were transduced with NTAP-Munc18c adenovirus. In addition, this system was further utilized to test the impact of detergent levels in lysis buffers upon Munc18c binding to syntaxin 4, SNAP23, and VAMP2. TAP purification under either detergent condition resulted in similar yields of TAP-tagged Munc18c as seen by immunoblotting for the CBP tag (Fig. 1B, lanes 3 and 6). Syntaxin 4 was precipitated by TAP-Munc18c, detected by relative differences in input and flow-through and its strong presence in eluates. However, TAP-tagged Munc18c precipitated ~65% less syntaxin 4 protein from lysates prepared with 0.1 versus 1% Nonidet P-40 detergent. Furthermore, neither SNAP23 nor VAMP2 protein was found to co-elute with this complex from lysates prepared under either detergent conditions. The levels of SNAP23 and VAMP2 in input lysates were comparable with that in flow-through, consistent with the lack of co-precipitation with TAP-Munc18c–syntaxin 4 complexes (Fig. 1B, lanes 1 and 2 and lanes 4 and 5). In addition, elution using high salt conditions (450 mM NaCl) failed to yield SNAP23 or VAMP2; C-terminal TAP-tagged Munc18c results were identical to N-terminal TAP-Munc18c (data not shown). These data suggest that the Munc18c–SNARE complex is labile under conditions required to adequately solubilize endogenous SNARE proteins.

Doc2β Preferentially Associates with Tyrosine-phosphorylated Munc18c—We have previously shown that treatment of MIN6 cells with the protein-tyrosine phosphatase inhibitor pervanadate (pV) results in accumulation of tyrosine-phosphorylated Munc18c, which corresponds to a decrease in Munc18c-syntaxin 4 interaction (16). The region of Munc18c bound by Doc2β contains a key stimulus-induced phosphorylation site, Tyr-219 (14). To determine whether the binding of Doc2β was related to the phosphorylation state of Munc18c, cells were treated with 0.1 mM freshly prepared pervanadate and detergent (1% Nonidet P-40 unless otherwise specified) cell lysates prepared for use in co-immunoprecipitation experiments. Pervanadate treatment resulted in a ~1.5 ± 0.06-fold increase in phospho-Munc18c (p < 0.01) as detected by anti-

![Image](226x26 to 254x38)
Doc2β Preferentially Binds Tyrosine-phosphorylated Munc18c

A) IP: Munc18c

| pV (mM): | Lysate | PM |
|---------|--------|----|
| 0       | 0.1    | 0  |
| 0.1     | 0.1    |    |

IB: Munc18c

IB: 4G10

IB: Syn4

IB: Doc2β

B) IP: Flag (Munc18c)

| pV (mM): | 0 | 0.1 |
|---------|---|-----|
| 0       |   |     |
| 0.1     |   |     |

IB: Flag

IB: 4G10

IB: Syn4

IB: Doc2β

Figure 2. An inverse correlation between syntaxin 4 and Doc2β association with tyrosine-phosphorylated Munc18c. A, MIN6 beta cells were incubated in MKRBB for 2 h before 5-min treatment with or without freshly prepared 0.1 mM pervanadate. Detergent-solubilized (1% Nonidet P-40) cleared lysates were prepared 0.1 mM pervanadate. Detergent-solubilized (1% Nonidet P-40) cleared lysates were prepared from MIN6 cells transfected with pcDNA3-FLAG-Munc18c and incubated 48 h before pervanadate treatment. Anti-FLAG antibody was used for immunoprecipitation, and proteins were resolved on 12% SDS-PAGE for immunoblotting. Band intensities were quantified using the Bio-Rad ChemiDoc system and normalized to untreated (no pV) bands in either syntaxin 4 (Syn4) or Doc2β immunoblots; data represent the average of three experiments.

Munc18c immunoprecipitation from detergent lyse followed by 4G10 antibody immunoblotting (Fig. 2A, first and second lanes), with a 60 ± 3% decrease in syntaxin 4 co-precipitation (p < 0.01), consistent with previous results (16). Interestingly, pV treatment resulted in a coordinate 1.8 ± 0.09-fold increase in co-precipitation of Doc2β compared with untreated cell lysates (p < 0.01). This pattern of binding with phospho-Munc18c was fully recapitulated using detergent-solubilized PM fractions (Fig. 2A, third and fourth lanes), indicating the PM as the principal cellular locale of these events (1.5 ± 0.06-fold increase in phospho-Munc18c, p < 0.01; 60 ± 5% decrease in syntaxin 4 co-precipitation, p < 0.01; 2.2 ± 0.2-fold increase in co-precipitation of Doc2β, p < 0.01). As an additional approach, detergent lysates prepared from MIN6 cells over-expressing recombinant FLAG-Munc18c treated with or without pV were immunoprecipitated using anti-FLAG antibody for subsequent immunodetection of tyrosine-phosphorylated Munc18c and co-precipitation of syntaxin 4 and Doc2β (Fig. 2B). Nearly identical results were obtained (1.5 ± 0.04-fold increase in phospho-Munc18c, p < 0.01; 60 ± 8% decrease in syntaxin 4 co-precipitation, p < 0.01; 1.8 ± 0.09-fold increase in co-precipitation of Doc2β, p < 0.01), suggesting that the selective binding of Doc2β to the region of Munc18c containing the pivotal Tyr-219 residue was enhanced by phosphorylation at this site.

The Hc-Linker Region of Syntaxin 4 (Amino Acids 118–194) Confers Binding to Munc18c—Recent crystallographic data has shown that the N-terminal 19 amino acids of syntaxin 4 constitute an important contact region for Munc18c binding that other contacts must contribute to its specificity of binding to Munc18c (37). To investigate additional contact regions that were both necessary and sufficient to confer binding to Munc18c, truncated forms of syntaxin 4 were used in GST interaction assays in vitro. First, GST-Munc18c and GST-syntaxin 4 C-terminal truncation proteins were expressed in E. coli and purified by glutathione-Sepharose precipitation. GST-syntaxin 4 truncation mutants were subsequently thrombin-cleaved from GST, and pure syntaxin 4 mutant proteins were captured for use in binding assays with GST-Munc18c linked to beads. As shown in Fig. 3A, four C-terminal truncation mutants of syntaxin 4 were generated, with sequential deletion of the H3 domain (containing residues 1–194), up to the Ha domain (containing residues 1–112), and up to the Ha domain (containing residues 1–70). Removal of the H3 domain failed to eliminate syntaxin 4 binding to GST-Munc18c compared with binding of the full-length soluble syntaxin 4 (1–273; no transmembrane domain) (Fig. 3A, lanes 1 and 2). However, further deletion of the Hc-linker and Ha domains eliminated all binding to GST-Munc18c (Fig. 3A, lanes 3 and 4). This lack of binding was not resultant from insufficient syntaxin 4 protein input (Fig. 3A, lanes 7 and 8). Further increases in input of the 1–70 protein failed to yield detection of association with GST-Munc18c (data not shown).

To determine the requirement for the Hc-linker region of syntaxin 4 in mediating contact with Munc18c, N-terminal deletions of syntaxin 4 were made. GST-syntaxin 4 N-terminal deletion mutants were expressed and purified as described above and retained on Sepharose beads for interaction studies with recombinant His-Munc18c protein. His-Munc18c protein was expressed in E. coli and purified for use as previously described (14). As depicted in Fig. 3B, GST-syntaxin 4 N-terminal deletion mutants were generated by removal of the first 38 residues (GST-(39–273)), deletion through the Ha domain (GST-(71–273)), deletion through the Hb domain (GST-(113–273)), or removal of all but the Hc-linker region (GST-(118–194)). All three syntaxin 4 N-terminal deletion mutants bound to His-Munc18c, as did the isolated Hc-linker region, whereas GST alone failed to precipitate the His-Munc18c protein (Fig. 3B, lanes 1–6). Thus, these data delineated the minimal region of
syntaxin 4 that was both necessary and sufficient to confer binding to Munc18c.

Alignment of this Hc-linker region of syntaxin 4 with other mammalian PM-localized syntaxin isoforms was performed using ClustalW. Syntaxin 4 and syntaxin 1A share 53 and 37% identity in their Hc and linker regions, respectively (Fig. 4).

Residues 155–163 at the C-terminal end of the Hc domain are highly conserved, suggesting against this region functioning in syntaxin binding specificity for its cognate Munc18 protein. However, the N-terminal 6 residues of the linker region (amino acids 164–169) of syntaxin 4 vary considerably from those of syntaxins 1A, 2, and 3. These data suggest that non-conserved residues present in the Hc-linker region of syntaxin 4 may participate in conferring its specificity for binding to Munc18c.

The Hc-Linker Region (118–194) of Syntaxin 4 Competitively Inhibits Munc18c-Syntaxin 4 Association—Next we sought to determine whether the Hc-linker region of syntaxin 4 was capable of competing with full-length soluble syntaxin 4 for binding to Munc18c. Either GST alone or GST-Munc18c proteins linked to Sepharose beads were pre-incubated with recombinant purified syntaxin 4 (residues 1–273) and centrifuged to eliminate unbound syntaxin 4 protein. Syntaxin 4 binding was dose-dependent and specific, as determined by lack of interaction with GST alone or GST-nSec1 (neuronal Sec1/Munc18-1) (Fig. 5A, lane 2, and B, lane 5). Subsequent addition of Hc-linker protein to reactions containing pre-bound GST-Munc18c-syntaxin 4 complexes decreased syntaxin 4 binding to the GST-Munc18c (Fig. 5B, lanes 2–4). Because of its small molecular mass (~8 kDa), the Hc-linker region of syntaxin 4 protein was difficult to visualize by Coomasie or Ponceau S staining and lacks the epitope for syntaxin 4 antibody recognition. Thus, the Hc-linker region of syntaxin 4 protein was fused to the C terminus of EGFP for confirmation of its binding to Munc18c and use in cell studies (see the schematic in Fig. 6A).

To determine whether the Hc-linker region bound to Munc18c in cells, pEGFP-(118–194) or pEGFP-C2 vector were co-electroporated with pcDNA3-FLAG-Munc18c into CHO-K1 cells, and detergent lysates were prepared for use in immunoprecipitation reactions. CHO-K1 cells transfected with very high efficiency, as evidenced by EGFP and FLAG immunoblotting of lysates in Fig. 6B, lanes 1 and 2, but fail to express detectable.

FIGURE 3. The region containing the HC and linker domains (118–194) of syntaxin 4 binds directly to Munc18c in vitro. A, bacterially expressed GST-Munc18c or GST proteins were purified and linked to beads for in vitro binding studies with bacterially expressed full-length or C-terminal truncations of soluble syntaxin 4. The syntaxin 4 fragments (1–70, 1–112, 1–194, and 1–273) were thrombin-cleaved to remove the GST tag and prepared at the concentration 1 mg/ml. Bound proteins were subjected to 18% SDS-PAGE for immunoblotting (IB) with anti-Munc18c and anti-syntaxin 4 antibodies. Asterisks denote bands of expected Mr. TM, transmembrane.

B, bacterially expressed N-terminal truncations GST-syntaxin 4 (1–273, 118–194, 71–273, 113–273, and 39–273) or GST proteins were purified and linked to beads for in vitro binding studies with bacterially expressed full-length His-Munc18c. Bound proteins were subjected to 12% SDS-PAGE for immunoblotting with anti-Munc18c antibody. Ponceau S staining shows input of GST-syntaxin 4 truncation proteins.
Doc2β Preferentially Binds Tyrosine-phosphorylated Munc18c

levels of endogenous Munc18c. Anti-FLAG immunoprecipitation specifically co-precipitated the EGFP-(118–194) and not EGFP alone (Fig. 6B, lanes 3 and 4). The pEGFP-(118–194) construct was then transfected into MIN6 cells to determine its ability to bind to endogenous Munc18c (Fig. 6C). Anti-Munc18c selectively co-immunoprecipitated EGFP-(118–194) with endogenous Munc18c, similar to results obtained in CHO-K1 cells. These data indicated that the interaction of EGFP-(118–194) with Munc18c was specific and that this region of syntaxin 4 was sufficient to confer binding in a relevant cell type.

Functional Impact of Hc-Linker Region (Amino Acids 118–194) Expression upon Insulin Exocytosis—Two types of proteins are known to interact with the N termini of syntaxin proteins: Munc18 and Munc13 (33, 47). Of these, only Munc18c is known to bind to syntaxin 4. Munc13-1 binds to the second helix, known as Hb, of syntaxins 1–3, but it is not yet known whether it associates with syntaxin 4. Given this selectivity of binding, we predicted that the EGFP-(118–194) could be used in MIN6 cells to determine the functional impact of dissociating endogenous Munc18c-syntaxin 4 complexes. Transfection of MIN6 cells with the pEFGP-(118–194) or pEGFP constructs resulted in ~30–50% cells exhibiting EGFP fluorescence. Expression of the EGFP-(118–194) protein reduced coinmunoprecipitation of syntaxin 4 with Munc18c by 25 ± 5% (p < 0.01) compared with the impact of EGFP expression alone (Fig. 7A).

Using a second approach, recombinant peptide was added to anti-Munc18c immunoprecipitation reactions to evaluate the ability of the free peptide to disrupt endogenous Munc18c-syntaxin 4 protein complexes. The advantages of this approach were the ability to control peptide input level as well as obviate concern over EGFP-peptide cellular localization or differential susceptibility to proteasomal degradation. As seen in Fig. 7B, the Hc-linker region (residues 118–194) effectively disrupted endogenous Munc18c-syntaxin 4 complexes by 50 ± 1% (p < 0.005), whereas an identical quantity of the N-terminal syntaxin 4 peptide (residues 2–23) had no significant impact (97 ± 6% of control). Taken together, these assays indicated that the 118–194 residue region of syntaxin 4 had the capacity to function as a competitive inhibitor of Munc18c-syntaxin 4 association in MIN6 cells.

To evaluate its effect upon glucose-stimulated secretion, EGFP-(118–194) was co-transfected with human proinsulin cDNA into MIN6 cells. The human C-peptide derived from expression of human pro-insulin is immunologically distinct from the mouse C-peptide produced by MIN6 cells and, thus, serves as a reporter of secretion only from transfectable cells. In control EGFP-expressing MIN6 cells, glucose induced a 38% increase in human C-peptide secretion (Fig. 7C), similar to that in other reports (48, 49). However, expression of the EGFP-(118–194) significantly increased glucose-stimulated secretion to nearly 60%. As seen in Fig. 7D, this secretory increase paralleled a 67 ± 3% increase in the capacity of endogenous syntaxin 4 to bind to exogenous GST-VAMP2 compared with EGFP expression alone (p < 0.01). Over-expression of EGFP-(118–194) had no effect upon expression of endogenous cognate interacting proteins (Fig. 7E). These data are consistent with recent Doc2β over-expression studies (14) showing that partial disruption of Munc18c binding to syntaxin 4 results in
enhanced glucose-stimulated insulin release via enhancing syntaxin 4 accessibility for docking.

The 118–194 Region Does Not Associate with Phosphorylated Munc18c—Having identified the 118–194 region of syntaxin 4 as a functional binding site for Munc18c in a relevant cell system, we next focused upon determining the relationship between the complexation of Munc18c with syntaxin 4 and the tyrosine phosphorylation of Munc18c in MIN6 cells. Cells transiently expressing EGFP control protein or EGFP-(118–194) were briefly treated with pV, and cleared detergent lysates were prepared for immunoprecipitation of tyrosine-phosphorylated proteins using anti-4G10 antibody (Fig. 8). Although the EGFP-containing lysate showed typical phospho-Munc18c and Doc2β co-immunoprecipitation, the EGFP-(118–194)-containing lysates revealed 50% less phospho-Munc18c (p < 0.01) and 75% less Doc2β (p < 0.01). Importantly, EGFP-(118–194) binding to Munc18c was fully abolished by the tyrosine phosphorylation of Munc18c. This was not due to reduced expression of EGFP-(118–194) or increased expression of endogenous syntaxin 4, as expression levels of each were similar to that of EGFP (Fig. 8, lysate panels) and also to those present in non-pV-treated cells (data not shown). This suggests that the 118–194 fragment may trap Munc18c in a conformation that prevents Munc18c phosphorylation at Tyr-219 and, hence, abolishes its ability to co-precipitate Doc2β.

Modeling the Munc18c-Syntaxin 4 Complex Interaction Sites—Although it is assumed that Munc18c will bind to syntaxin 4 with high affinity, based upon similarity to Munc18-1 interactions, we next focused upon determining the relationship as a functional binding site for Munc18c in a relevant cell system.
tion with syntaxin 1A (30), kinetic measurements of the Munc18c-syntaxin 4 interaction are lacking. To address this, surface plasmon resonance data were collected using recombinantly expressed and purified His-Munc18c, syntaxin 4 (1–273), and syntaxin 4 (118–194) proteins (Fig. 9). Analysis of surface plasmon resonance data revealed an association rate constant ($k_a$) of $9.22 \times 10^5$ and a dissociation rate constant ($k_d$) of $2.96 \times 10^2$ with Munc18c-syntaxin 4 (1–273) as shown in Fig. 9A. From these data, the calculated equilibrium dissociation constant ($K_D = k_d / k_a$) was 32 nM. The fast association rate, which approaches the diffusion limit, is suggestive of a small free energy barrier for association of Munc18c with syntaxin 4. The slow dissociation rate suggests a steeper barrier for the reverse process indicating that the complex formation is exergonic. Munc18c binding to the 118–194 region of syntaxin 4 resulted in association and dissociation rate constants of $473 \text{s}^{-1}$ and $2.4 \times 10^{-3} \text{s}^{-1}$, respectively (Fig. 9B). These values led to a dissociation equilibrium constant of $5 \times 10^{-6} \text{M}^{-1}$, which is nearly 3 orders of magnitude lower than that of full-length soluble syntaxin 4. The lower affinity for the fragment is mostly attributed to its significantly slower association rate to Munc18c, suggesting a larger free energy barrier that the complex must overcome to bind. Although different from that of full-length soluble syntaxin 4, these data clearly demonstrate the ability of the 118–194 region to confer syntaxin binding to Munc18c.

To model this high affinity interaction, we used the three-dimensional crystal structure of Munc18c-syntaxin 4 (1–19) and nSec1-syntaxin 1A complex (50) to construct a structure of the Munc18c-syntaxin 4 complex. More specifically, the crystal structure of Munc18c was used, and a homology model of syntaxin 4 was constructed by using the crystal structure of syntaxin 1A. The structure is likely highly accurate in light of the high (80%) sequence identity between syntaxin 4 and syntaxin 1A (50). We modeled the Munc18c-syntaxin 4 complex, illustrating how syntaxin 4 anchors it to the plasma membrane through a C-terminal transmembrane helix (Fig. 10A). Munc18c is shown in blue, and the Doc2β binding site on Munc18c, the N-terminal region residues 173–255 (14), is in orange. The short red region next to the Doc2β binding site corresponds to the first 19 amino acids of syntaxin 4 that have been crystallized in complex with Munc18c (37). Syntaxin 4 N-terminal domains Ha, Hb, Hc, and linker (residues 39–193) regions are shown in red (right), and syntaxin 4 C-terminal domain H3 (residues 194–273), also known as the SNARE binding domain, is shown in yellow. The absence of the region between residues 1–19 and 39–273 of syntaxin 4 suggests that this region is highly dynamic in solution. The N-terminal region of syntaxin 4 (1–19) is remarkably close to the Doc2β and the Tyr-219 residue of Munc18c known to undergo phos-
phorylation (16). More specifically, Fig. 10B shows that interactions between syntaxin 4 and Munc18c close to Tyr-219 are mediated by R2 located on syntaxin 4 and appears to be a water-mediated interaction with Munc18c. Another interaction occurs between Lys-17 and Glu-223 on Munc18c and a backbone nitrogen in syntaxin 4. Collectively, these interactions contribute to the introduction of sufficient stability to the Munc18c-syntaxin 4 complexes so as to maintain Munc18c in the conformation where Tyr-219 is buried and unable to be phosphorylated and may underlie the binding preference of syntaxin 4 for unphosphorylated Munc18c. Furthermore, upon dissociation of syntaxin 4 from Munc18c, a loop that holds the Tyr-219 residue might more easily flip open to expose Tyr-219 for phosphorylation and Doc2β/H9252 binding.

The Hc-linker region of syntaxin 4 (residues 118–194) shown in green interacts with Munc18c in two distinct areas as indicated by the arrows (Fig. 10C). One interaction (right arrow) includes possible hydrophobic interactions between syntaxin 4 residues Val-118, Met-122, Gln-126, Val-129, Gln-132, and Gln-133 and Munc18c. This is intriguing given that Met-122, Gln-132, and Gln-133 are found only in the syntaxin 4 isoform and not in syntaxin isoforms 1–3, whereas Gln-126 is conserved among all isoforms. The other hydrophobic interaction region of syntaxin 4 contains a conserved Arg-149. Future crystallographic analyses of these regions or the entire soluble syntaxin 4 protein will be required to validate these implicated contacts.

DISCUSSION

In an effort to reconcile discrepancies in models of Munc18c-syntaxin 4 complex dynamics derived primarily from in vitro data, we used the islet beta cell as model system of regulated exocytosis that is known to require Munc18c-syntaxin 4 interaction. Herein, we demonstrate that the induction of Munc18c tyrosine phosphorylation is an important trigger for Munc18c to switch its binding specificity from syntaxin 4 to Doc2β in the beta cell. Modeling studies showed a putative role for the N-terminal peptide of syntaxin 4 in this switch mechanism, given its close proximity to the Tyr-219 site lying within the Doc2β binding site on Munc18c. However, mutagenesis studies further revealed the necessity of the Hc-linker region of syntaxin 4, which was also found to be sufficient to confer binding to Munc18c. Binding of the novel Hc-linker binding site was confirmed by surface plasmon resonance, and its expression in MIN6 cells was found to effectively disrupt endogenous Munc18c-syntaxin 4 complexes. Functionally, this enhanced glucose-stimulated insulin exocytosis via increasing syntaxin 4 accessibility to VAMP2. Moreover, phosphorylation of Munc18c reduced its association with Hc-linker, suggesting that the Hc-linker region is involved in the switching mechanism. These novel data represent a new and important region of syntaxin 4 on which to focus upon in our efforts to delineate the dynamic conformational changes and associated functional

![FIGURE 10. Ribbon representation of the Munc18c-syntaxin 4 complex. A, overall illustration of syntaxin 4 interacting with Munc18c in close proximity to the PM. Munc18c is shown in blue, Doc2β binding site on Munc18c is in orange, and the syntaxin 4 N terminus (amino acids 1–19) to the left is in red. To the right in red is syntaxin 4 N-terminal domains Ha, Hb, and Hc. The syntaxin 4 C-terminal domain H3 (SNARE binding domain) is illustrated in yellow. B, a close-up view illustrating syntaxin 4 R2 interacting with full-length Munc18c and Munc18c Glu-223 interacting with a nitrogen in the backbone of syntaxin 4, which results in a strong hydrogen bond. C, a close-up illustration showing two contact sites between Munc18c (blue) and the Hc-Linker domain (green) of syntaxin 4. Arrows point to the two contact regions between Munc18c and syntaxin 4 (118–194).]
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modes of the Munc18c-syntaxin 4 complex as it pertains to regulated exocytosis.

In contrast to some recent reports of preferential Munc18 interaction with syntaxins engaged in SNARE core complex assembly using in vitro paradigms, our results were obtained using endogenous proteins present in MIN6 beta cell lysates and demonstrate the predominance of binary Munc18c-syntaxin 4 complexes in cells, with no detection of Munc18c-SNARE complexes. Although it is certainly possible that Munc18c-SNARE complexes might exist transiently, our data combined with the current literature suggest that in endocrine cells such as pancreatic beta cells and 3T3L1 adipocytes, Munc18c mostly associates with monomeric syntaxin 4 (37).

The rationale behind Munc18c binding to and facilitating formation of syntaxin-SNARE complexes has been founded upon in vitro data. However, we show here that the N-terminal peptide is dispensable for conferring syntaxin 4 binding in cells and, rather, that the association is conferred by the Hc-linker region. Moreover, the N-terminal peptide failed to competitively inhibit endogenous Munc18c-syntaxin 4 association, whereas the Hc-linker was an effective competitor, and surface plasmon resonance data supported its ability to stably interact with Munc18c. Comconitant with these findings, other reports demonstrate that the N-terminal peptide of syntaxin 1A or syntaxin 4 is insufficient to confer the binding interaction (38, 51). Most recently, Fasshauer and co-workers (52) used isothermal titration calorimetry and fluorescence spectroscopy to confirm that the binary interaction between Munc18-1 and syntaxin 1A for the closed conformation did require contact sites which included the N-terminal peptide but show that removal of the first 24 residues of syntaxin 1A only reduces the $K_d$ from 1 to 8 nm, indicating that there is only a small contribution of the N-peptide.

Our modeling studies indeed support the concept that the N-terminal peptide of syntaxin 4 binds to Munc18c but also expand upon this by further speculating that this peptide might actively participate in the dissociation mechanism involving exposure of Tyr-219 of Munc18c along with participation of the Hc-linker region of syntaxin 4. Evidence supportive of Hc-linker function in this phosphorylation dissociation mechanism stems from the loss of Hc-linker binding to phosphorylated Munc18c, contrasting with its otherwise robust binding to Munc18c in vitro and in unstimulated MIN6 cells. One possible explanation for this is that the 118–194 fragment held Munc18c in a conformation that is unfavorable for Munc18c to become phosphorylated. Alternatively, Munc18c phosphorylation may have triggered dissociation of Hc-linker, as it does with full-length endogenous syntaxin 4. However, our data do not fully support this latter scenario given the 50% loss of Munc18c phosphorylation detected in EGF-118–194-expressing cells. Thus, although the first explanation seems more plausible, future conformational studies of the phosphorylated form of Munc18c will be required to confirm or dispute this concept.

A recent NMR study demonstrated that Munc18-1 binds to and prevents the opening of syntaxin 1A and that an additional factor helps to transition syntaxin into the open conformation (53). Doc2β would seem a suitable candidate for the “additional factor,” given our finding that it preferentially bound tyrosine-phosphorylated form of Munc18c, and that the increase in binding to Doc2β was proportional to the decrease in syntaxin 4 association. Our modeling studies revealed a close proximity of the syntaxin N-terminal peptide to a solvent-exposed Doc2β binding site on Munc18c that contains the Tyr-219 phosphorylation site. Incorporation of Doc2β into the binding model with Munc18c-syntaxin 4 provides new insight into the mechanism by which Munc18c dissociates, whether partially or fully, from syntaxin 4. However, it is predicted that this protein triad is further impacted by proteins such as Munc13 and Tomosyn, which like Doc2β have been shown to regulate and inhibit Munc18-syntxin complexes. Although Munc13-1 has not yet been shown to participate in syntaxin 4-based complexes, the UNC-13 homolog from Caenorhabditis elegans was shown to displace syntaxin from the Munc18-syntxin (UNC-18-syntxin) complex by competing for binding to Munc18 (54). In addition, Munc13-1 can bind directly to an N-terminal region of Doc2β and also to the Hb domain of syntaxin 1A, which was later shown to stabilize syntaxin in its open conformation (55).

Assigning a role to Munc18c as a positive SNARE complex interacting protein as opposed to a negative closed syntaxin 4 “clamping” protein has also varied as a function of detergent conditions used. For example, titration studies demonstrated the ability of Munc18 to displace SNAP25 and VAMP2 from syntaxin 1, coordinate with the ability to co-immunoprecipitate only syntaxin 1 with anti-Munc18 antibody but not SNAP25 or VAMP2 from detergent-solubilized (1% Triton X-100) synaptosomal membranes (24, 33, 46). However, more recent studies show that although the Munc18-syntxin binary complex does predominate, under in vitro conditions containing little (0.1% Triton X-100) or no detergent, SNARE complexes bound by Munc18 were detected (27, 28, 51, 57). Using a novel immunofluorescent approach, it was shown that syntaxin 1 bound to Munc18-1 could also bind SNAP25 but that the addition of VAMP2 displaced Munc18-1 (39). The current general consensus is, therefore, that Munc18-1-syntaxin 1 complexes predominate and likely function in regulating vesicle docking (58). The molecular mechanism of Munc18c function is equally controversial. For example, Munc18c reduced the binding of SNAP23 to syntaxin 4 in a concentration-dependent manner when evaluated in 1% Triton X-100-solubilized cell lysates (31), yet under low stringency detergent conditions Munc18c binding to syntaxin 4-bound SNARE core complexes was detected (27). Indeed the benefit of using low stringency buffers was the ability to detect the otherwise elusive transient docking complex. However, biochemical validation of the formation of this complex by endogenous proteins in cells awaits further investigation.

In summary, the model presented in this paper provides a new base/scaffold for building in these additional syntaxin4-
Munc18c-Doc2β-interacting proteins and serves to explain in part the mechanistic role for Munc18c in stimulus-induced syntaxin 4 engagement in exocytosis. In addition to tyrosine phosphorylation, other modifications such as glycosylation and serine/threonine phosphorylation of Munc18c still need to be incorporated into this model. Munc18c becomes O-linked-glycosylated in 3T3L1 adipocytes to inhibit insulin-stimulated GLUT4 vesicle translocation (59). Serine/threonine phosphorylation of Munc18c by protein kinase C has been suggested to dissociate it from syntaxin 4 and facilitate SNARE complex assembly in pancreatic acinar cells (60). Very recently, S-nitrosylation of syntaxin 1 was shown to alter affinity for Munc18-1 (61). Therefore, further studies of various post-translational Munc18c modifications will be important for fully understanding its impact upon syntaxin 4 conformation and role in exocytosis in physiologically relevant exocytosis events.

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