The Stimulus-induced Tyrosine Phosphorylation of Munc18c Facilitates Vesicle Exocytosis*

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Stimulus-induced tyrosine phosphorylation of Munc18c was investigated as a potential regulatory mechanism by which the Munc18c-Syntaxin 4 complex can be dissociated in response to divergent stimuli in multiple cell types. Use of 32P-orthophosphate incorporation, pervanadate treatment, and phosphotyrosine-specific antibodies demonstrated that Munc18c underwent tyrosine phosphorylation. Phosphorylation was apparent under basal conditions, but levels were significantly increased within 5 min of glucose stimulation in MIN6 beta cells. Tyrosine phosphorylation of Munc18c was also detected in 3T3L1 adipocytes and increased with insulin stimulation, suggesting that this may be a conserved mechanism. Syntaxin 4 binding to Munc18c decreased as Munc18c phosphorylation levels increased in pervanadate-treated cells, suggesting that phosphorylation dissociates the Munc18c-Syntaxin 4 complex. Munc18c phosphorylation was localized to the N-terminal 255 residues. Mutagenesis of one residue in this region, Y219F, significantly increased the affinity of Munc18c for Syntaxin 4, whereas mutation of three other candidate sites was without effect. Moreover, Munc18c-Y219F expression in MIN6 cells functionally inhibited glucose-stimulated SNARE complex formation and insulin granule exocytosis. These data support a novel and conserved mechanism for the dissociation of Munc18c-Syntaxin 4 complexes in a stimulus-dependent manner to facilitate the increase in Syntaxin 4-VAMP2 association and to promote vesicle/granule fusion.

In the early 1990s, the discovery of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)2 proteins elucidated the molecular basis for synaptic vesicle exocytosis (1, 2). Vesicle exocytosis entails the pairing of a vesicle-associated membrane protein (VAMP) SNARE with a binary cognate receptor complex at the target membrane composed of SNAP-25/23 and syntaxin in a stimulus-dependent manner to facilitate the increase in Syntaxin 4-VAMP2 association and to promote vesicle/granule fusion.

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2The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; VAMP, vesicle-associated membrane protein; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; SM, Sec1/Munc18; WT, wild-type; EGFP, enhanced green fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; MKRBB, modified Krebs-Ringer bicarbonate buffer; PVDF, polyvinylidene difluoride; PTPs, protein-tyrosine phosphatases.

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insulin-stimulated GLUT4 vesicle translocation rapidly and coordinately maintain whole body euglycemia, we propose that commonalities in post-translational modifications may provide the basis for a conserved mechanism by which the Munc18c-Syntaxin 4 complex regulates exocytosis.

The post-translational modification of Munc18 proteins by serine/threonine phosphorylation has been suggested as a mechanism to regulate exocytosis (34). For example, the phosphorylation of Munc18a by protein kinase C and Cdk5 (cyclin-dependent kinase 5) reduces the amount of Munc18 that binds to syntaxin and promotes secretory granule exocytosis (35–40). Munc18c was also recently shown to be phosphorylated by protein kinase C in rat parotid acinar cells and endothelial cells (41, 42). Furthermore, the platelet Munc18c homolog PSP (platelet Sec1 protein) becomes phosphorylated in vitro by protein kinase C, and phosphorylated PSP fails to bind to Syntaxin 4 in vitro (43) as well as in thrombin-stimulated platelets (44). However, this serine/threonine phosphorylation of Munc18 proteins has failed to fully account for the stimulus-induced alterations in the Munc18-syntaxin complex.

In this study, we present evidence demonstrating that Munc18c is regulated by tyrosine phosphorylation and that increased tyrosine phosphorylation of Munc18c can dissociate it from Syntaxin 4. Munc18c phosphorylation was mapped to multiple tyrosine residues present in the N-terminal 255 amino acids, and mutation of Tyr219 increased its binding to Syntaxin 4. Conversely, enhancement of Munc18c phosphorylation using the protein-tyrosine phosphatase inhibitor pervanadate decreased its association with Syntaxin 4. Glucose stimulation rapidly enhanced the tyrosine phosphorylation of endogenous Munc18c in MIN6 pancreatic beta cells, and Tyr219 was found to confer this effect. Moreover, mutation of Munc18c Tyr219 resulted in inhibited glucose-stimulated SNARE complex formation and insulin secretion. Because we also found that Munc18c became rapidly tyrosine-phosphorylated in response to insulin in 3T3L1 adipocytes, this stimulus-induced Munc18c phosphorylation may be part of a conserved mechanism in vesicle/granule fusion.

EXPERIMENTAL PROCEDURES

Materials—The rabbit anti-Munc18c antibodies were generated as described (16). The rabbit anti-Syntaxin 4 polyclonal and mouse anti-VAMP2 monoclonal antibodies were obtained from Chemicon International, Inc. (Temecula, CA), and Synaptic Systems (Göttingen, Germany), respectively. The rabbit anti-Myc polyclonal and mouse anti-Myc monoclonal (9E10) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. The anti-phosphotyrosine monoclonal antibodies were obtained from Upstate Biotechnology (4G10) and BD Transduction Laboratories (Pittsburgh, PA). The MIN6 cells were a gift from Dr. John C. Hutton (University of Colorado Health Sciences Center). TransFectin lipid reagent was purchased from Bio-Rad. Radioimmunoassay grade bovine serum albumin and d-glucose were purchased from Sigma. Enhanced chemiluminescence reagent and Hyperfilm-MP products were obtained from Amersham Biosciences. [32P]Orthophosphate (1 mCi/ml orthophosphoric acid in water) and X-Omat Blue XB-1 film were purchased from PerkinElmer Life Sciences.

Plasmids—Three N-terminal deletion constructs of Munc18c were generated by PCR amplification using different 5′- primers that initiated at internal Met sites with good Kozak sequences located at amino acid 172, 255, or 332: wild-type Munc18c (WT), 5′-GAATTTCCGGAATGGTGGCGGCCGCGG; Munc18c-Δ172, 5′-GAATTTCTGGTGTATGGGAGGCAATGG; Munc18c-Δ255, 5′-GAATTTCCAGGCAATGGGATGGCATATGATC; and Munc18c-Δ332, 5′-GAATTTCCAAAAGATGCGCCACCTTCC. The same 3′- primer was used for all deletion constructs: 5′-CGGATCCACTGCTCTTAAAGAAAC. PCR products were digested with EcoRI and BamHI and then subcloned into pcDNA3.1(−)/myc-His (Invitrogen). The Munc18c-(173–255)-WT-enhanced green fluorescent protein (EGFP) construct was generated by PCR amplification using the 5′- primer designed to make Munc18c-Δ172 and the 3′-primer 5′-CGGATCTCCGCTGAAAGTGCAGTTCC. The resulting PCR product was inserted into EcoRI and BamHI restriction sites of the pEGFP-N3 expression vector (BD Biosciences). Site-directed mutagenesis of individual Munc18c tyrosine residues to phenylalanine residues was carried out using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. pcDNA3-Syntaxin 4 was generated as described previously (24).

Cell Culture—MIN6 beta cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose and 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 (45, 46). 3T3L1 preadipocytes were purchased from the American Type Culture Collection and cultured in DMEM containing 25 mM glucose and 10% calf serum at 37 °C and 8% CO₂. At confluence, cells were differentiated by incubation in medium containing 25 mM glucose, 10% fetal bovine serum, 1 μg/ml insulin, 1 mM dexamethasone, and 0.5 mM isobutyl-1-methylxanthine. After 4 days the medium was changed to DMEM, 25 mM glucose, and 10% fetal bovine serum. Differentiated adipocytes were used for the experiments 12 days after the initiation of differentiation and were placed in serum-depleted medium for 2–3 h prior to insulin stimulation.

MIN6 Transient Transfection and C-peptide Assay—MIN6 beta cells plated on 10-cm² tissue culture dishes at 40–60% confluence were electroporated with 300 μg of plasmid DNA/plate (one 10-cm² dish/plate) to obtain ~50–70% transfection efficiency using the procedure described previously (32). After 48 h of incubation, cells were washed twice with and incubated for 2 h in 10 ml of modified Krebs-Ringer bicarbonate buffer (MKRBB; 5 mM KCl, 120 mM NaCl, 15 mM HEPES (pH 7.4), 24 mM NaHCO₃, 1 mM MgCl₂, 2 mM CaCl₂, and 1 mg/ml bovine serum albumin) (46) and stimulated with 20 mM glucose. Cells were subsequently lysed in Nonidet P-40 lysis buffer (25 mM Tris (pH 7.4), 1% Nonidet P-40, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 137 mM sodium chloride, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml 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 cotransfected with the human proinsulin expression vector pCB6/INS (a kind gift from Dr. Chris B. Newgard, Duke University) using TransFectin with 2 μg of each DNA/35-mm dish of cells at 50% confluence. Forty-eight hours following transfection, cells were starved for 2 h in MKRBB and stimulated with 20 mM glucose for 1 h. MKRBB was collected for quantitation of human C-peptide released using a human C-peptide immunoassay kit (Linco Research, Inc., St. Charles, MI).

[32P]Orthophosphate Labeling of MIN6 Beta Cells—Metabolic labeling using [32P]orthophosphate was performed as described previously (47) with minor modification. Briefly, MIN6 beta cells (70% confluent on 10-cm² tissue culture dishes) were incubated in DMEM containing 2.8 mM glucose at 37 °C for 16 h. The cells were washed twice with phosphate-free DMEM and incubated for 4 h in 5 ml of the same medium containing 100 μCi/ml [32P]orthophosphate. The medium was then removed, and the cells were washed twice with ice-cold buffer containing 20 mM HEPES (pH 7.4) and 150 mM NaCl. One milliliter of
ice-cold Nonidet P-40 lysis buffer was added to the cells, and cleared cell lysates were prepared and used for immunoprecipitation with anti-Munc18c antibody. The Munc18c immunoprecipitates were subjected to 10% SDS-PAGE, and orthophosphate incorporation was detected by autoradiography.

Subcellular Fractionation—Subcellular fractions of beta cells were isolated as described previously (48, 49). Briefly, MIN6 beta cells at 80–90% confluence were harvested in 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)). Plasma membrane fractions were isolated as described previously (50). Cells were disrupted by 10 strokes through a 27-gauge needle, and homogenates were centrifuged at 900 × g for 10 min. The resulting pellets were mixed 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 mM 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 60 min, and the resulting pellet was collected as the plasma membrane fraction. Fractions were assayed for soluble protein content as described previously (51).

Plasma membrane fractions of 3T3L1 adipocytes were obtained using the differential centrifugation method as described previously (16, 52). Briefly, 3T3L1 adipocytes were washed with and resuspended in HES buffer (20 mM HEPES (pH 7.4), 1 mM EDTA, and 255 mM sucrose containing 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml pepstatin, 10 µg/ml aprotinin, and 5 µg/ml leupeptin). Lysates were sheared 10 times through a 22-gauge needle and centrifuged at 19,000 × g for 20 min at 4 °C. The resulting pellet was resuspended in HES buffer and layered onto a 1.12 M sucrose cushion for centrifugation at 100,000 × g for 60 min. The plasma membrane layer was removed from the cushion and centrifuged at 40,000 × g for 20 min. The plasma membrane pellet was resuspended in HES buffer and assayed for protein content as described above.

Co-immunoprecipitation and Immunoblotting—MIN6 beta cells were incubated with MKRBB for 2 h and preincubated with or without pervanadate for 5 min prior to glucose stimulation. Pervanadate was made immediately prior to use by combining 1 mM sodium orthovanadate with 3 mM hydrogen peroxide for 5 min (53). Cells were subsequently lysed in Nonidet P-40 lysis buffer. MIN6 beta cell cleared detergent homogenates (2–3 mg) were combined with rabbit anti-Munc18c antibody, rabbit anti-Syntaxin 4 antibody, or mouse anti-phosphotyrosine antibody for 2 h at 4 °C, followed by a second incubation with protein G Plus-agarose for 2 h. The resultant immunoprecipitates were subjected to 10% SDS-PAGE, followed by transfer to polyvinylidene difluoride (PVDF) membranes for immunoblotting. Anti-Munc18c, anti-Syntaxin 4, and anti-phosphotyrosine (PY20) antibodies were used at 1:5000, 1:500, and 1:1000 dilutions, respectively, and horseradish peroxidase-conjugated secondary antibodies were used at a dilution of 1:5000 for visualization by chemiluminescence. Immunoprecipitations using adipocyte cleared detergent cell lysates were performed similarly to those using MIN6 cell lysates.

RESULTS

Munc18c Undergoes Tyrosine Phosphorylation in MIN6 Beta Cells—To first determine whether Munc18c can be phosphorylated in MIN6 beta cells, metabolic labeling with [32P]orthophosphate and immunoprecipitation with anti-Munc18c antibody were performed (Fig. 1A). MIN6 cell lysates contained a high abundance of [32P]phosphate-labeled proteins. However, immunoprecipitation with anti-Munc18c antibody revealed incorporation into a single band at ~68 kDa, corresponding to a band detected by anti-Munc18c antibody in cell lysates. Immunoprecipitation of labeled lysates using anti-Syntaxin 4 antibody failed to show any incorporation of phosphate, indicating that Syntaxin 4 is not phosphorylated in beta cells (data not shown) and that phosphorylation is specific to Munc18c in this complex.

To determine whether Munc18c phosphorylation occurs in a tyrosine-specific manner, we treated MIN6 cells with the protein-tyrosine phosphatase inhibitor pervanadate. Treatment with pervanadate was predicted to increase the abundance of tyrosine-phosphorylated Munc18c, but not serine/threonine phosphorylation, if in fact Munc18c
undergoes tyrosine phosphorylation. Pervanadate was added to MIN6 cells for 5 min prior to preparation of lysates for immunoprecipitation. Pervanadate treatment resulted in 2.6 ± 0.5-fold increase (p < 0.02) in levels of phosphotyrosine-modified Munc18c as detected by immunoprecipitation with anti-phosphotyrosine antibodies (Fig. 1B). Munc18c abundance in cell lysates was unaffected by pervanadate treatment. In a reciprocal immunoprecipitation using anti-Munc18c antibody, pervanadate treatment induced a similar increase in phosphorylated Munc18c (Fig. 1C). Subsequent immunoblotting of the same membrane confirmed the migration of the phosphotyrosine band as identical to that of the Munc18c band at 68 kDa. This indicated that Munc18c and not some other tyrosine-phosphorylated protein with a molecular mass of ~68 kDa was indeed tyrosine-phosphorylated.

**Tyrosine Phosphorylation of Munc18c Leads to Its Dissociation from Syntaxin 4: A Conserved Mechanism**—To determine whether the tyrosine phosphorylation of Munc18c alters its ability to interact with Syntaxin 4, we treated MIN6 cells with increasing concentrations of pervanadate and prepared lysates for immunoprecipitation with anti-Munc18c antibody. Similar levels of Munc18c were immunoprecipitated under all treatment conditions (Fig. 2). Munc18c became increasingly tyrosine-phosphorylated as the pervanadate concentration was increased. At 1 mM pervanadate (Fig. 2, lane 4), Munc18c tyrosine phosphorylation exceeded that of the basal level (lane 1) by 4 ± 1-fold (p < 0.05). By contrast, Syntaxin 4 co-precipitating with Munc18c decreased as pervanadate levels were increased. Syntaxin 4 protein expression levels were unaffected by pervanadate treatment. These data showed that pervanadate treatment specifically increased Munc18c phosphorylation at tyrosine residues, which correlated with its dissociation from Syntaxin 4.

We (16) and others (28) have proposed that stimulation of vesicle fusion must somehow trigger the dissociation of the Munc18c-Syntaxin 4 complex, enabling Syntaxin 4 to then form the heterotrimeric SNARE core complex with SNAP-25/23 and VAMP2. Moreover, this dissociation is predicted to occur at the plasma membrane, the location of the Syntaxin 4 and SNAP-25/23 proteins. Although Munc18c is a soluble protein, it localizes to the plasma membrane through its interaction with Syntaxin 4 (16). To investigate whether this particular pool of Munc18c can be tyrosine-phosphorylated in a stimulus-dependent manner, MIN6 cells were subfractionated according to previously described methods (48), and plasma membrane fractions were used in immunoprecipitation experiments. Munc18c immunoprecipitated from plasma membrane fractions prepared from unstimulated cells showed some phosphotyrosine immunoreactivity (Fig. 3A). However, stimulation for 5 min with glucose increased the amount of tyrosine-phosphorylated Munc18c by 2.8 ± 0.6-fold (p < 0.05). From the same fraction preparations, Syntaxin 4 co-immunoprecipitated phosphorylated Munc18c under unstimulated conditions (Fig. 3B); however, within 5 min of glucose stimulation, the amount of phosphorylated Munc18c immunoprecipitated decreased by ~50 ± 16% (p < 0.02). The phosphotyrosine band was identified as Munc18c based upon Munc18c immunoblotting and migration of the band at ~68 kDa. In addition, the identity of this band was confirmed not to be the tyrosine-phosphorylated forms of other Syntaxin 4-binding proteins such as N-ethylmaleimide-sensitive factor (NSF) or SNAP-25 because N-ethylmaleimide-sensitive factor migrated at ~75–80 kDa and SNAP-25 migrated at 25–30 kDa (data not shown) and were distinctly different from the 68-kDa band seen here. Moreover, the Syntaxin 4-interacting protein Synip (70 kDa) has also been suggested to be phosphorylated, but at serine residues and not tyrosine. These data suggested that, as Munc18c became increasingly tyrosine-phosphorylated in response to glucose stimulation, it dissociated from Syntaxin 4.

To determine whether Munc18c-Syntaxin 4 complex dissociation by Munc18c tyrosine phosphorylation in response to stimuli is a conserved mechanism, parallel experiments were performed in 3T3L1 adipocytes. In 3T3L1 adipocytes and skeletal muscle, Munc18c-Syntaxin 4 interactions are critical for mediating insulin-stimulated GLUT4 vesicle fusion (16, 54). Fully differentiated 3T3L1 adipocytes left unstimulated or stimulated with 100 μM insulin for 5 min were harvested, and plasma membrane fractions were prepared as described previously (25). Munc18c was equivalently immunoprecipitated from each fraction, and tyrosine-phosphorylated Munc18c was increased by ~1.5-fold in fractions prepared from cells stimulated with insulin (Fig. 3C). Immunoprecipitation of Syntaxin 4 resulted in the co-immunoprecipitation of 80% less...
tyrosine-phosphorylated Munc18c from insulin-stimulated lysates compared with unstimulated lysates (Fig. 3D). With 15 min of insulin stimulation, the tyrosine phosphorylation of Munc18c increased by >2.5-fold, which corresponded to a 90% decrease in binding to Syntaxin 4 (data not shown). These results show that there are mechanistic parallels in the stimulus-induced dissociation of tyrosine-phosphorylated Munc18c from Syntaxin 4 between insulin-responsive adipocytes and insulin-secreting beta cells.

A) The N Terminus of Munc18c Is Required for Tyrosine Phosphorylation and Interaction with Syntaxin 4—To delineate the region of Munc18c required for modification by tyrosine phosphorylation, N-terminal deletion constructs were generated. Because truncated forms of Munc18c protein are generally poorly expressed or unstable, we generated deletions at internal Met sites that had reasonably good Kozak sequences. These deletions resulted in the truncation of the first 172, 255, or 332 residues. PCR products as well as Munc18c-WT were subcloned into pCDNA3/myc-His for expression in mammalian cells. B. DNA constructs were electroporated into MIN6 beta cells, and 48 h later, detergent lysates were prepared for immunoprecipitation (IP) with anti-Myc antibody. Myc-tagged proteins are denoted by asterisks. Proteins were subjected to 10% SDS-PAGE and initially immunoblotted (IB) for Myc to assess the size and level of expression of each truncation product (upper panel). Subsequently, the membrane was stripped and immunoblotted with anti-Phosphotyrosine (p-Tyr; middle panel) and anti-Syntaxin 4 (Syn4; lower panel) antibodies. Boxes in the phosphotyrosine immunoblot delineate the locations of the truncated protein bands detected by anti-Myc antibody.

All tyrosine residues essential for phosphorylation and that the N terminus of Munc18c is essential for its interaction with Syntaxin 4.

To identify candidate tyrosine residues within the N terminus of Munc18c, we used ClustalW (available at ca.expasy.org). Although no classic NPXY motifs were found, four tyrosine residues were selected as likely candidates for phosphorylation using a computer prediction program (available at www.cbs.dtu.dk) that searches for alternative motifs (59). Alternative motifs have at least one acidic residue in the four residues immediately upstream of the candidate tyrosine (Tyr = 0, P4 to P1). Among the sites within the two acidic residues, EE and ED are the most frequently occurring dipeptides at positions P4 and P3. Candidate sites were detected between amino acids 65 and 220. Alignment of mouse Munc18a and Munc18b, C. elegans UNC-18, D. melanogaster ROP, and S. cerevisiae Sec1 against residues 60–262 of mouse Munc18c is shown in Fig. 5. The four tyrosine residues meeting these criteria are located at positions 66, 103, 218, and 219 in the mouse Munc18c sequence (Fig. 5, shaded). Of these four sites, only Tyr103 is conserved among all SM proteins in the alignment.

Site-directed mutations of tyrosine to phenylalanine were made at residues 66, 103, 218, and 219 in the context of Munc18c-WT-Myc/His (Fig. 6A). Each construct was electroporated into MIN6 cells, and lysates were prepared for immunoprecipitation with anti-Myc antibody (Fig. 6B). Each mutant was expressed at a level below that of Munc18c-WT in the lysates as detected by Myc immunoblotting. Quantification of the Tyr/Myc ratio in each immunoprecipitation showed that there were no significant changes in modification (data not shown). Moreover, the phosphotyrosine level of each mutant correlated with its abundance in the lysate, suggesting that no single site confers the entire phosphorylation signal. Although each mutant retained the ability to co-immunoprecipitate Syntaxin 4, optical density scanning quantitation revealed that the Munc18c-Y219F mutant immunoprecipitated 2.5-fold more Syntaxin 4 than did Munc18c-WT (Fig. 6C). The amount of Syntaxin 4 immunoprecipitated by Munc18c-Y103F also increased, but failed to reach statistical significance. Of the four mutants, expression of Munc18c-Y103F was the most severely impaired relative to Munc18c-WT, and Syntaxin 4 levels, as well as total protein levels, tended to be reduced in lysates expressing Munc18c-Y103F. This suggests that Munc18c-Y103F is somehow toxic to cell viability. Altogether, these data suggest that Tyr219 is important for Syntaxin 4 binding.
To confirm that the increased binding of Syntaxin 4 to Munc18c-Y219F was independent of effects due to cell electroporation, MIN6 cells were transiently transfected by lipofection, and lysates were used for immunoprecipitation (Fig. 6D). Myc-tagged Munc18c-Y219F co-immunoprecipitated ~3-fold more Syntaxin 4 compared with Munc18c-WT, consistent with results obtained using electroporation to transfect cells. In addition, confocal immunofluorescence microscopy analysis demonstrated that Myc/His-tagged Munc18c-WT and Munc18c-Y219F were similarly localized to the cytosolic and membrane compartments, indicating that the increased affinity of Munc18c-Y219F for Syntaxin 4 was not due to altered localization of the Munc18c-Y219F protein (data not shown). These data suggest that the specific phosphorylation of Tyr219 may be pivotal in the dissociation of the Munc18c-Syntaxin 4 complex in response to stimuli such that mutation of the site results in reduced dissociation and enhanced binding of the complex.

To determine whether Tyr219 is indeed a site of phosphorylation, we first attempted to use two-dimensional phosphopeptide mapping. However, the tryptic cleavage sites flanking the peptide containing Tyr219 were too closely spaced and generated a fragment too small for analysis. As an alternative strategy, we isolated Tyr219 in a small fragment of Munc18c encoding residues 173–255 and performed site-directed mutagenesis using PCR-mediated site-directed mutagenesis in the context of the pcDNA3.1-Munc18c-WT/myc-His construct. DNA constructs were electroporated into MIN6 cells, and detergent cell lysates were prepared for immunoprecipitation with anti-Myc antibody. Immunoprecipitates were subjected to 10% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-Myc and anti-Syntaxin (Syn4) antibodies. The Myc blot was stripped and reblotted for phosphotyrosine (p-Tyr) to determine whether individual mutation of a particular site eliminates phosphorylation. C. Myc and Syntaxin 4 immunoblots from experiments in 8 were quantitated using optical density scanning to determine the ratio of Syntaxin 4 binding to each mutant. Data were normalized to Munc18c-WT = 1 and are shown as means ± S.E. for four independent experiments. *p < 0.02 versus Munc18c-WT. D. MIN6 cells at 50–70% confluence were transiently transfected using TransFectin lipid reagent with either Munc18c-WT or Munc18c-Y219F, and 48 h later, cells were harvested and used for immunoprecipitation with anti-Myc antibody as described for 8.

FIGURE 5. Sequence alignment of the SM protein family and identification of four candidate tyrosine residues for phosphorylation. ClustalW was used to align sequences of SM proteins relative to amino acids 60–262 of mouse Munc18c (available at ca.expasy.org/tools/align). Candidate residues for tyrosine phosphorylation (shaded) were identified based upon prediction (79) of non-NPX[T/Y] motifs using the prediction program from the Center for Biological Sequence Analysis at the Technical University of Denmark (available at www.cbs.dtu.dk). Amino acid positions are listed to the right of each sequence.

FIGURE 6. Mutation of Tyr219 results in enhanced binding of Munc18c with Syntaxin 4. A, four DNA constructs were generated with a single Tyr-to-Phe substitution at each candidate tyrosine residue (Y66F, Y103F, Y218F, and Y219F) using PCR-mediated site-directed mutagenesis in the context of the pcDNA3.1-Munc18c-WT/myc-His construct. B, DNA constructs were electroporated into MIN6 cells, and detergent cell lysates were prepared for immunoprecipitation with anti-Myc antibody. Immunoprecipitates were subjected to 10% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-Myc antibody. C, Myc and Syntaxin 4 immunoblots from experiments in 8 were quantitated using optical density scanning to determine the ratio of Syntaxin 4 binding to each mutant. Data were normalized to Munc18c-WT = 1 and are shown as means ± S.E. for four independent experiments. *p < 0.02 versus Munc18c-WT. D, MIN6 cells at 50–70% confluence were transiently transfected using TransFectin lipid reagent with either Munc18c-WT or Munc18c-Y219F, and 48 h later, cells were harvested and used for immunoprecipitation with anti-Myc antibody as described for 8.

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Tyrosine Phosphorylation of Munc18c

Determination of phosphorylation at Tyr218 and Tyr219

Figure 7. A fragment of Munc18c containing only candidate sites Tyr218 and Tyr219 within residues 173–255 (Munc18c-(173–255)-WT) was linked to EGFP. Within the context of this construct (Munc18c-(173–255)-WT-EGFP), Tyr218 and Tyr219 were individually or doubly mutated to yield constructs Munc18c-(173–255)-Y218F-EGFP, Munc18c-(173–255)-Y219F-EGFP, and Munc18c-(173–255)-Y218F/Y219F-EGFP. BDNAs were transiently transfected into MIN6 cells, and lysates were prepared from unstimulated cells for immunoprecipitation (IP) with anti-phosphotyrosine antibody (p-Tyr). Immunoprecipitates were subjected to 10% SDS-PAGE, transferred to PVDF membrane, and immunoblotted (IB) with anti-EGFP antibody. Ponceau S staining showed equivalent protein loading of immunoprecipitates on the gel.

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Confers Glucose-induced Phosphorylation to Munc18c—Because Tyr219 was the only conserved tyrosine to have a significant impact upon Syntaxin 4 affinity in mutational analyses (Fig. 6), we sought to determine whether Tyr219 is responsible for mediating the increased tyrosine phosphorylation in response to glucose. First, we tested whether the glucose-induced increase in abundance of tyrosine-phosphorylated Munc18c could be recapitulated by Munc18c-(173–255)-WT-EGFP. MIN6 cells were transiently transfected with Munc18c-(173–255)-WT-EGFP DNA and, after 48 h of incubation, were either left unstimulated or stimulated with glucose for 5 min, and lysates were prepared (Fig. 8). Anti-phosphotyrosine antibody immunoprecipitated Munc18c-(173–255)-WT-EGFP from unstimulated lysates, and 2 ± 0.2-fold (p < 0.005) more Munc18c-(173–255)-WT-EGFP was immunoprecipitated from glucose-stimulated lysates (Fig. 8, lanes 5 and 6). Ponceau S staining showed equal loading of immunoprecipitation reactions of total lysate proteins as well. In addition, Munc18c-(173–255)-WT-EGFP was equivalently expressed in unstimulated and glucose-induced lysates (Fig. 8, lanes 1 and 2). Thus, the fragment of Munc18c containing Tyr219 conferred the majority of the glucose-induced phosphorylation observed in the endogenous full-length Munc18c protein.

In parallel experiments, we tested whether mutation of Tyr219 disrupts the ability of glucose to increase the level of phosphorylation of
Phosphorylation of Tyr219 Is Functionally Important for Syntaxin 4 Binding to VAMP2—We have shown previously that glucose stimulation for 5 min increases the co-immunoprecipitation of VAMP2 with SNAP-25 or Syntaxin 1A from islet cell lysates (46). Because Munc18c-Y219F showed an increased affinity for Syntaxin 4 (Fig. 6) and because Munc18c-(173–255) Y219F-EGFP failed to show glucose-enhanced tyrosine phosphorylation (Fig. 8), we questioned whether expression of Munc18c-Y219F inhibits the association of endogenous Syntaxin 4 with VAMP2. Lysates prepared from MIN6 cells transiently transfected with vector DNA (pcDNA3) and left unstimulated or glucose-stimulated for 5 min were used for immunoprecipitation with Syntaxin 4. Anti-Syntaxin 4 antibody coprecipitated ~30 ± 2.9% (p < 0.005) more VAMP2 from glucose-stimulated lysates compared with unstimulated lysates (Fig. 9A, lanes 1 and 2). However, expression of full-length Munc18c-Y219F or Munc18c-WT dramatically inhibited the interaction between endogenous Syntaxin 4 and VAMP2 under unstimulated and stimulated conditions (Fig. 9A, lanes 3–6), consistent with the notion that overabundant Munc18c sequesters Syntaxin 4 from VAMP2 to inhibit vesicle fusion.

The utility of this as an assay system to test the importance of Tyr219 for function in SNARE complex assembly lies in the ability of Syntaxin 4 overexpression to release the inhibition caused by Munc18c-WT expression. We have demonstrated previously that inhibition of GLUT4 translocation in 3T3L1 adipocytes caused by overexpression of Munc18c can be reversed if Syntaxin 4 levels are coordinately increased (24). In this, Syntaxin 4 is used as a "molecular sponge" to bind excess Munc18c. In the present study, Syntaxin 4 overexpression in Munc18c-WT-expressing cells restored the level of VAMP2 coprecipitated by anti-Syntaxin 4 antibody in unstimulated lysates to ~90% of the control level (pcDNA3-transfected cells) and to ~80% of the control level in glucose-stimulated lysates (Fig. 9B, lanes 1 and 2 and lanes 5 and 6, respectively). However, Syntaxin 4 overexpression in Munc18c-Y219F-expressing cells failed to restore the glucose-induced increase in Syntaxin 4–VAMP2 association (Fig. 9B, lane 4 versus lane 2). Overexpression of the Munc18c-WT and Munc18c-Y219F proteins was confirmed by immunoblotting for the Myc epitope, although no decrease in Munc18c-WT binding was observed in glucose-stimulated lysates. This may be a reflection of the transient nature of the dissociation between Munc18c and Syntaxin 4 or of transient phosphorylation caused by alterations in the balance of kinase and phosphatase activities. Overall, these results showed that the increased affinity of the Munc18c-Y219F mutant for Syntaxin 4, combined with the inability of the mutant to undergo glucose-induced phosphorylation, resulted in the failure of Munc18c-Y219F to release Syntaxin 4 upon glucose stimulation and impaired the glucose-stimulated increase in Syntaxin 4–VAMP2 association.

Phosphorylation of Tyr219 Is Functionally Important for Glucose-stimulated Secretion—To determine whether the effects of Munc18c-Y219F expression on Syntaxin 4–VAMP2 association correlate with functional inhibition of glucose-stimulated insulin secretion, we used the human C-peptide reporter assay. Human C-peptide (synthesized from a human proinsulin cDNA) is packaged in an identical fashion to mouse C-peptide and serves as a reporter from transfected cells. MIN6 cells were cotransfected with pcDNA3 control DNA plus human proinsulin cDNA and left unstimulated or glucose-stimulated for 1 h. Human C-peptide secreted into the buffer was quantitated by radioimmunoassay and normalized for total cellular protein content (ng of C-peptide secreted per mg of cellular protein). Data in each of five independent experiments were normalized to basal = 100 and are shown as means ± S.E. Munc18c-WT with Syntaxin 4 overexpression was significantly increased relative to other transfected cells. * , p < 0.05.
Tyrosine Phosphorylation of Munc18c

insulin DNA exhibited 160 ± 5.2% (p < 0.005) of basal human C-peptide secretion in response to glucose, an appropriate increase in human C-peptide secretion in response to glucose in this assay system (55, 56). In contrast, overexpression of Munc18c-WT or Munc18c-Y219F reduced glucose-stimulated secretion to 115 and 121% of the basal level, respectively (Fig. 10, bars 1 and 3), consistent with their inhibition of endogenous Syntaxin 4 association with VAMP2. The inhibitory effect of Munc18c overexpression is also consistent with inhibition of glucose-stimulated insulin secretion in islets from Munc18c-overexpressing transgenic mice (31). Overexpression of Syntaxin 4 with Munc18c-WT partially and significantly increased human C-peptide secretion to 132% of the basal level (Fig. 10, bar 2). This partial restoration of human C-peptide secretion correlates with the partial restoration of Syntaxin 4-VAMP2 binding induced by overexpression of Syntaxin 4 with Munc18c-WT in Fig. 9B. However, Syntaxin 4 overexpression with Munc18c-Y219F failed to increase glucose-stimulated insulin secretion at all. These data suggest that phosphorylation of Tyr219 is essential for glucose-regulated dissociation, which is important for the function of the Munc18c-Syntaxin 4 complex in regulating glucose-stimulated exocytosis and secretion.

Discussion

All types of SNARE-mediated vesicle/granule exocytosis require an SM protein (57). Using insulin-stimulated GLUT4 vesicle translocation as our model system, we have shown previously that Munc18c-Syntaxin 4 complexes are important for the fusion step of vesicle exocytosis (25), although the mechanism for this has remained unknown. Here, we have presented evidence of a novel and conserved mechanism for Munc18c-Syntaxin 4 dissociation, a key step that is required for vesicle fusion to occur. This model mechanism entails stimulus-induced tyrosine phosphorylation of Munc18c, which dissociates it from Syntaxin 4, enabling Syntaxin 4 to bind with VAMP2.

The conserved nature of this model is supported by evidence showing that Munc18c becomes increasingly tyrosine-phosphorylated in response to stimuli that initiate the exocytosis of vesicles/granules in two distinct cell types, pancreatic beta cells and adipocytes. Increased tyrosine phosphorylation of Munc18c correlated with its diminished interaction with Syntaxin 4. Tyrosine phosphorylation was mapped within the N-terminal 255 residues of Munc18c, and this region was found to be essential for its interaction with Syntaxin 4. Analysis of this region revealed the presence of four candidate tyrosine residues for phosphorylation; however, only mutation of Tyr219 was found to alter Munc18c binding affinity for Syntaxin 4. Tyr219 was found to be required to confer the glucose-induced phosphorylation to a fragment of Munc18c containing only this and a second candidate tyrosine residue (Tyr218). Our studies using the Y219F mutant of Munc18c indicated that phosphorylation of Tyr219 was functionally important for Syntaxin 4 binding to VAMP2 and also for stimulus-induced secretion, altogether suggesting that phosphorylation of Tyr219 is crucial for the subsequent function of Syntaxin 4 in granule fusion and insulin release.

Munc18c was found to be phosphorylated in the basal state at multiple tyrosine residues because the individual mutation of Tyr66, Tyr103, Tyr218, or Tyr219 in full-length Munc18c failed to eliminate phosphotyrosine immunoreactivity. Munc18c-(173–255)-WT, which contained only the candidate sites Tyr218 and Tyr219, was able to recapitulate the glucose-stimulated increase in tyrosine phosphorylation of endogenous Munc18c, suggesting that one or both of the two included candidate tyrosines was phosphorylated preferentially in response to glucose stimulation. Our data further showed that mutation of Tyr219, but not Tyr218, resulted in loss of this glucose-induced phosphorylation, suggesting Tyr219 as the primary site for stimulus-induced phosphorylation. Although Tyr66 and Tyr103 were not similarly tested in the context of a smaller fragment of Munc18c for the ability to confer stimulus-enhanced phosphorylation, neither the Y66F nor Y103F mutation exerted a significant alteration in affinity for Syntaxin 4, suggesting against either site as crucial for Syntaxin 4 function with VAMP2 in stimulus-induced exocytosis. However, it is unclear as to why any molecules of the Munc18c-(173–255)-Y218F-EGFP mutant, which had only Tyr219 intact, were immunoprecipitated by anti-phosphotyrosine antibody from unstimulated lysates. In fact, Munc18c-(173–255)-Y218F-EGFP was immunoprecipitated in slightly greater abundance than Munc18c-(173–255)-WT-EGFP. One possible explanation could be that the mutation in the context of this small fragment of Munc18c induced a conformational change that enhanced the accessibility of the site for modification by phosphorylation. In addition, it is possible that the non-candidate site, Tyr196, was somehow made active as a site of phosphorylation and contributed to the tyrosine phosphorylation. Other proteins such as S100 and NFA1 (nuclear factor of activated T cells) are phosphorylated at multiple sites, at which a threshold level of phosphorylation serves as a binary switch (58–60). Alternatively, the transcription activator Ets-1 is phosphorylated at multiple sites, which act additively to produce graded DNA binding affinity, shifting Ets-1 from a dynamic conformation poised to bind DNA to a well folded inhibited state (61). However, it is clear from the double mutant that elimination of Tyr218 and Tyr219 together eliminated all phosphotyrosine immunoreactivity, indicating that they were indeed phosphorylated. Future studies will be required to determine whether Munc18c phosphorylation proceeds via a binary switch or a “graded rheostat” mechanism.

Tyrosine phosphorylation results from the net effects of protein-tyrosine kinases balanced by the effects of protein-tyrosine phosphatases (PTPs) (62). The data presented here suggest that Munc18c can be tyrosine-phosphorylated under basal conditions. One candidate protein-tyrosine kinase may be the Fes/Fer non-receptor protein-tyrosine kinase, which has been shown to participate in SNARE-mediated vesicle fusion (63). Moreover, glucose stimulation increased the level of Munc18c tyrosine phosphorylation, and this effect was mimicked by inhibition of PTPs using pervanadate, suggesting that glucose may enhance phosphorylation via inactivation of PTPs. If analogous in adipocytes, inactivation of PTPs would be triggered by insulin stimulation. Although it was clearly shown that increased phosphorylation of Munc18c correlated with its dissociation from Syntaxin 4, significant loss of Syntaxin 4 binding was observed only when cells had been treated with the PTP inhibitor pervanadate. This suggests that the concurrent activities of protein-tyrosine kinases and PTPs at the multiple sites of phosphorylation may contribute to our diminished ability to detect these transient changes in Munc18c-Syntaxin 4 binding. In support of a role for glucose in the inactivation of PTPs, it has been shown that glucose stimulation can produce H2O2, which causes oxidative stress in beta cells (64), and that oxidative stress can inactivate PTPs (65). Thus, glucose stimulation may induce the inactivation of PTPs, which causes a net increase in Munc18c tyrosine phosphorylation. Future studies will be focused on identification of the protein-tyrosine kinases and PTPs involved in the tyrosine phosphorylation of Munc18c.

One very important finding from this study is that the inhibition of exocytosis caused by overexpression of Munc18c correlated with a marked decrease in endogenous Syntaxin 4-VAMP2 interaction. Although we (16, 54) and others (28) have shown previously that overexpression of Munc18c in islet cells, adipocytes, and skeletal muscle tissue results in significantly impaired regulated exocytosis, the under-
lying cause of this inhibition was unknown. The data presented here suggest that the underlying mechanism for the inhibitory effect of Munc18c overexpression involves Munc18c sequestration of endogenous Syntaxin 4, which reduces Syntaxin 4-VAMP2 association. This was further demonstrated by the partial rescue of both Syntaxin 4-VAMP2 binding and functional exocytosis upon addition of exogenous Syntaxin 4. Because the restoration of binding and exocytosis was indeed only partial, it remains possible that overexpression of Munc18c impairs/sequestrers molecules other than just Syntaxin 4.

In fact, dissociation of the Munc18-syntaxin complex is also thought to be catalyzed by additional SNARE-interacting proteins. For example, the dissociation of yeast homologs Sly1p (Munc18 homolog) and Sed5p (syntaxin homolog) has been shown to require the small GTPase protein Ypt1p (66). Similarly, in adipocytes, the Munc18c-Syntaxin 4 complex has been proposed to be dissociated upon activation of the Rab4 GTPase (67) or the phosphatidylinositol 3-kinase/Akt-dependent phosphorylation of the Rab GTPase-activating protein AS160 (68, 69). In support of this, the crystal structure of Munc18-1 revealed a region that is very similar to the Ypt1p-binding site of Sly1p (70). Moreover, we (71) and others (72) have targeted this region of Munc18-1 and Munc18c with small interfering peptides to demonstrate the importance of this loop region to vesicle fusion. However, other than Ypt1p in yeast, no direct interactions between mammalian Munc18 and Rab proteins have been demonstrated to date, suggesting that the mechanisms for dissociation of the mammalian Munc18-syntaxin complexes may differ from those of their yeast counterparts.

Although our demonstration of the regulation of the Munc18-syntaxin complex by tyrosine phosphorylation of Munc18c is a novel concept, modification of Munc18 and syntaxin proteins by serine and threonine phosphorylation has been investigated previously. For example, serine phosphorylation of Munc18-1 by protein kinase C disrupts interaction with Syntaxin 1A (35, 38), and threonine phosphorylation of Munc18-1 by Cdk5 results in the disassembly of the Munc18a-Syntaxin 1A complex (37). Furthermore, serine phosphorylation of Syntaxin 1A by the death-associated protein kinase DAPK significantly decreases interaction with Munc18-1 (73). In addition, Syntaxins 4 and 1A can be phosphorylated by multiple kinases; however, this has not been shown to occur in adipocytes or islet beta cells (73–76). Moreover, in our beta cell metabolic labeling studies, immunoprecipitation of Munc18c did not co-immunoprecipitate phosphate-incorporated Syntaxin 4 (data not shown). These data suggest two possibilities: 1) Syntaxin 4 is not phosphorylated in beta cells, or 2) Munc18c cannot bind to Syntaxin 4 that is phosphorylated. Thus, the sites of serine/threonine phosphorylation in the Munc18c and Syntaxin 4 proteins remain unknown and require future investigation. In all, although the serine phosphorylation of other Munc18 isoforms has been demonstrated, it has yet to be demonstrated that this can fully account for the mechanism underlying alterations in the Munc18-syntaxin complex in response to stimuli. It may be that the phosphorylation of Munc18 proteins at serine, threonine, and tyrosine residues coordinate regulates this interaction.

Munc18c and Syntaxin 4 are ubiquitously expressed proteins that are emerging as key regulators of additional exocytotic events that impact human health other than the diabetes field. For example, Syntaxin 4-based complexes regulate hemostasis through platelet exocytosis, implicating their importance in cardiovascular disease (43, 77). In addition, Syntaxin 4 and Munc18c have been shown to be regulated during macrophage activation to function in membrane traffic and tumor necrosis factor-α cytokine secretion (78), suggesting a role for the Munc18c-Syntaxin 4 complex in macrophage-mediated host defense and inflammatory disease. Finally, the exocytosis of Weibel-Palade bodies containing von Willebrand factor, P-selectin, and interleukin-8 within minutes after stimulation has been shown recently to be regulated by Syntaxin 4 in endothelial cells (42), showing the potential importance of Munc18c-Syntaxin 4 complexes in vascular homeostasis.

Significant progress has been made in defining signaling cascades that lead to the secretion of insulin from islet beta cells and the uptake of glucose into skeletal muscle and adipose tissues via GLUT4 vesicle translocation; however, each cascade is rate-limited by the distal steps of vesicle exocytosis. The functional regulation of Syntaxin 4 by Munc18c is thought to be crucial in the fusion event, although the details have remained unclear. Given the evidence showing that the same Munc18c-Syntaxin 4 complex regulates insulin secretion and insulin action, therapies that target or even affect secondarily these SNARE and SNARE-associated proteins will have consequences in regulation of glucose homeostasis. This could be beneficial if the regulation occurs in parallel, but hazardous if the complex regulation is opposing (i.e., up-regulation of Syntaxin 4 results in decreased glucose uptake but increased insulin secretion). Thus, the mechanistic data presented here begin to fill an important gap in our understanding of the etiology of insulin resistance and diabetes.

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