Munc18-Syntaxin Complexes and Exocytosis in Human Platelets*

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The Sec1-Munc18 (SM)1 proteins are a conserved family of ~65-kDa molecules that play a central but still enigmatic role in exocytosis in yeast, neurons, and specialized secretory cells (reviewed in Ref. 1). Indeed, all types of intracellular trafficking appear to require both the SM proteins and SNAP receptors (SNAREs) (2). Three SM proteins have been identified in mammals: a neuronal form, Munc18a (Munc18-1), and two nonneuronal forms, Munc18b (18-2) and Munc18c (18-3, or platelet Sec1 protein) (3). Munc18a was originally identified in neurons by virtue of its binding to the SNARE protein syntaxin 1 (4). The original identification of different Munc18 genes suggested that unique Munc18-syntaxin interactions may play an important role in regulating the specificity of vesicle trafficking in cells, but more recent in vitro studies have suggested that interactions between these molecules are somewhat promiscuous (5, 6).

Munc18a expression is detected in early stem cells before syntaxin expression, and genetic ablation leads to embryonic lethality (7, 8). Embryonic neurons lacking Munc18a contain synaptic vesicles but are incapable of exocytosis, indicating that this SM protein is required for membrane fusion (7). The SM proteins interact with at least three classes of proteins, but the interaction that mediates their essential role in exocytosis is unknown (7). Early studies suggested that Munc18 inhibits exocytosis by binding to monomeric syntaxin molecules to block SNARE complex formation (9). In contrast to Munc18, yeast Sec1p binds to already assembled SNARE complexes, indicating that Sec1p may function after SNARE complex formation (10). Moreover, SM proteins appear to localize at sites of yeast vacuole fusion (11) or secretion (12). Experimental studies using genetic techniques have yielded contradictory results; overexpression of SM proteins has been shown to inhibit, as well as to stimulate, cellular exocytosis (13–18). Mutations in SM proteins also can either enhance or inhibit membrane fusion (reviewed in Ref. 19). These contradictory results may be due in part to the fact that alterations in Munc18 expression and/or structure may, over hours to days, influence the level of syntaxin or other secretory molecules in cells (7, 20). Indeed, SM proteins may act as chaperone-like molecules that deliver newly synthesized syntaxins to appropriate membrane sites (21). Finally, the stage of exocytosis at which the SM proteins act has yet to be clarified. In mouse chromaffin cells, Munc18 appears to function upstream of SNARE complex formation by promoting the docking of large dense core vesicles with the plasma membrane (18), but this is not seen in neurons at brain synapses (7). Taken together, genetic manipulation studies suggest that the SM proteins may have complex roles at several stages in vesicle trafficking (1).

Although structurally homologous, the Sec1 and Munc18 proteins display unanticipated differences in binding sites and function. In yeast, the Sec1 proteins appear to bind SNARE complexes via a short peptide motif in a manner that does not interfere with SNARE complex formation (10, 22, 23). In contrast, the mammalian Munc18 proteins bind with high affinity to a closed confirmation of syntaxin via several intermolecular contact sites in two domains in a manner that should prevent SNARE complex formation (24, 25). This suggests the possibility that the SM proteins, by virtue of different interacting partners and different binding specificities, may not have the...
same primary role in vesicle trafficking and exocytosis in all cell types (1).

Despite growing insights, the functional role of the SM-syntaxin interactions in regulated cellular secretion remains unclear from previous studies (1). This study used human platelets as a model system to examine the expression of SM proteins, the specificity of their interactions with the syntaxins, and the effect of syntaxin-SM complex formation on exocytosis. Because platelets are anucleate secretory cells that have almost no Golgi apparatus, they are well suited for examining the terminal stages of regulated exocytosis without the potential confounding effects of biosynthetic processes or vesicle trafficking (26). Platelets contain three types of specialized secretory organelles: α granules, dense granules, and secretory lysosomes (26). Recent studies have established that platelet exocytosis occurs through formation of SNARE complexes among proteins from three gene families: SNAP-23, vesicle-associated membrane proteins, and syntaxin 2 or 4 (3, 26–33). In these studies, we sought to identify the full complement of SM proteins in platelets and then to determine the specificity of their interactions with the syntaxins required for granule secretion. Anti-Munc18c monoclonal antibodies (mAbs) were generated that inhibit the SM-syntaxin complex in order to determine the acute effects of this complex on platelet granule secretion. Taken together, our results indicate that SM proteins selectively interact with specific syntaxins in platelets and through these interactions play a critical role in platelet exocytosis.

**EXPERIMENTAL PROCEDURES**

**Antibody Production**—Female BALB/c mice (Charles River) were immunized intraperitoneally with 50 μg of recombinant Munc18c (r-Munc18c) in complete Freund’s adjuvant. They were subsequently boosted twice at 2–3-week intervals. The antibody titer was determined by solid phase radioimmunoassay 2 weeks after the final boost. The mice with the highest titers were hyperimmunized with r-Munc18c (5 μg intravenously and 10 μg intraperitoneally) 4 days prior to fusion. Somatic cell fusion was performed as described (34), and the fusion frequency was 50%. Hybridoma supernatants were tested for the production of anti-Munc18c mAbs by solid phase radioimmunoassay. Wells of a microtiter plate were coated with goat anti-mouse antibody (2 g/ml) for 1 h at 37 °C, and nonspecific protein binding was blocked with 1% bovine serum albumin. Hybridoma supernatants (50 μl) were added to the wells for 1 h. After washing, bound antibody was detected by [125I]-labeled sheep anti-mouse antibody (Amersham Biosciences) and quantitated by an Amersham Biosciences Storm 840 under conditions in which there was a linear relationship between intensity and pixel number as described (28).

**Immunoblotting and Immunoprecipitation**—Immunoprecipitation was performed as described previously (29). Solubilized bovine platelet membranes (typically 50 μl; see below) were preclariﬁed by incubation with rat anti-mouse κ chain or protein A-Sepharose beads for 1 h at 4 °C and centrifugation. The supernatants were incubated with murine mAbs or rabbit polyclonal antibody immobilized on anti-mouse κ chain or protein A-Sepharose beads, respectively (Zymed Laboratories, Inc.) for 2 h at 4 °C. Plates were washed twice with cushioned trypsination assay buffer (1% w/v Nonidet P-40, 1% Triton X-100, 10 mM sodium vanadate, 5 mM sodium fluoride, 100 units/ml aprotinin, pH 7.2). Immunoprecipitated proteins were solubilized in sample buffer and subjected to SDSPAGE. After electrophoretoblotting onto polyvinylidene diﬂuoride membranes, the immunoblotting was performed with the indicated antibodies. Bound antibodies were detected by an enhanced chemiluminescence (Fierce) protocol and chemiluminescence (Pierce) method (Pierce) and quantitated by an Amersham Biosciences Storm 840 under conditions in which there was a linear relationship between intensity and pixel number as described (28).

**Isolation of Platelet Cytosol and Membranes**—Citrated pooled human platelet-rich plasma (with 1 mM EDTA; Massachusetts General Hospital Blood Bank) was centrifuged at 200 × g for 20 min at 21 °C to remove aggregates and red blood cells. Platelets were centrifuged at 1500 × g for 20 min. The platelet pellet was suspended in 5 mM HEPES, pH 7.4, 140 mM NaCl, 4.8 mM KCl, 1 mM MgCl2, 5.5 mM glucose, 0.35% bovine serum albumin with 1 mM EDTA. Platelets were sonicated in the presence of 1 μM phenylmethylsulfonyl fluoride, 5 mM potassium vanadate, and 5 mM sodium fluoride with a Branson 450 Sonifier (large tip, output 60, 50% duty) briefly at room temperature (30 × 2) and then on ice (30 × 2). Large aggregates and unlysed cells were removed by centrifugation in a Sorvall H600A rotor at 4500 rpm for 20 min at 4 °C.

The sonicate was centrifuged in a Beckman SW28 rotor at 28,000 rpm for 1 h at 4 °C to separate cytosol (supernatant) from platelet membranes. Membranes were dissolved in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM sodium vanadate, 10 μg/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride, pH 7.4) at 4 °C for 30 min. After centrifugation at 15,000 rpm in a Sorvall SS-34 rotor for 30 min, the supernatants were used for co-immunoprecipitation.

**PCR**—Recombinant human Munc18c fragments comprising domain 1 (amino acids 1–134) or domains 2 and 3 (amino acids 135–592) were generated by PCR. Both strands of the DNA were sequenced and ligated into pBK-CMV vector. Human Munc18c was amplified from human platelet cDNA using standard techniques as described (33). The primers mimicked sequences unique to Munc18c: 5’-GAGATTCCACCTG-CCTCCCTCC and 5’-TGTGAGTGGCGCCTGTCGCC. A coupled transcription and translation reaction was performed with a T3 primer in reticulocyte lysate using [35S]methionine (1000 Ci/mmol; Amersham Life Sciences) as described by the manufacturer (Promega). The labeled Munc18c domains were puriﬁed on a G25 column (Roche Applied Science) and used in a binding assay. Wells of a microtiter plate were coated with goat anti-mouse antibody (2 μg/ml) for 60 min at 37 °C, and nonspeciﬁc protein binding sites were blocked with 1% albumin in Tris-buffered saline. Culture supernatants containing Mab 889 or an anti-digoxin mAb of the same isotype (control) were added to the wells for 60 min at room temperature. After washing, the labeled Munc18c domain 1 or domains 2 and 3 fragments were added to the wells (200,000 cpm/50 μl) for 60 min at room temperature. Cells were washed and the amount of bound Munc18c domains. Specific binding for each fragment was determined by subtracting the background binding to the anti-digoxin mAb (negative control). The effects of mAbs on syntaxin 4-Munc18c binding were examined in a similar fashion. Wells of a microtiter plate were coated with syntaxin 4 mAb (5 μg/ml). After nonspeciﬁc binding sites were blocked (0.2% gelatin, 0.05% Tween 20) for 30 min, platelet membranes were added (50 μl, diluted 1:3 in PBS, 3 h). After washing, [35S]Munc18c was added to the wells in the presence of various concentrations of puriﬁed mAbs. After a 40-min incubation, wells were washed and counted and the percent binding was determined by reference to wells containing no inhibitor (0% inhibition) and wells containing saturating amounts of inhibitor (100% inhibition). The purified polyclonal anti-Munc18c antibodies, 100% inhibition). The direct binding of mAbs to r-Munc18c was examined in a capture tag assay. Wells of a microtiter plate were coated with puriﬁed mAbs (10 μg/ml) and then nonspeciﬁc binding sites were blocked with 1% bovine serum albumin. Puriﬁed r-Munc18c (10 μg/ml) was added to wells for 1 h, and the wells were washed. Bound r-Munc18c was detected by the addition of 125I-labeled polyclonal anti-Munc18c antibodies (100,000 cpm/well) followed by washing and scintillation counting.

**Cell Permeabilization and Secretion Assays**—Human platelet-rich plasma was prepared from freshly drawn blood, and platelet dense granules were loaded with [3H]serotonin as described previously (30). Platelets were centrifuged at 1500 × g for 8 min, the supernatant was carefully removed, and the cells were resuspended in a Ca2+-buffering solution containing 20 mM PIPES, pH 7.4, 150 mM potassium glutamate, 5 mM glucose, 2.5 mM EDTA, 2.5 mM EGTA, 0.05% bovine serum albumin (buffer A). The platelet count was adjusted to 8 × 106/ml with buffer A.

Streptolysin O (Sigma) was dissolved in buffer A at a concentration of 25,000 units/ml, reduced with 2 mM dithiothreitol at 4 °C for 30 min, aliquoted, and stored at −70 °C. Platelet suspensions (20 μl) in 1.5 ml plastic tubes were mixed with 25 μl of buffer A containing various agents and 200–400 units/ml streptolysin O. The samples were incubated at 25 °C for 10 min on ice for 30 min. Then ATP (5 μM, 50 mM) and calcium (100 mM) were added, and the samples were incubated at 25 °C for 10 min. Granule secretion was induced by increasing the free calcium ion concentration (0.1–10 μM as indicated) by adding 2.5–10 μl of CaCl2 in buffer A. The free calcium ion concentration was determined by the calcium ion titration curves de-
The expression of Munc18 proteins in human platelets. A, Coomassie-stained, reduced SDS-PAGE gels containing detergent extracts of proteins from human brain (50 μg) and an equivalent amount (by protein staining) of proteins extracted from human platelet membranes. The first lane contains protein standards (STD), and the molecular masses are indicated in kDa. Immunoblots of the same samples with antibodies (Ab) against recombinant Munc18c (B), a peptide unique to Munc18a (C), or a peptide sequence present in both Munc18a and Munc18b (Munc18a/b) (D) are shown. After SDS-PAGE, proteins were electroblotted to polyvinylidene difluoride membranes and incubated with the indicated antibodies (1:1000 dilution). After washing, the bound antibody was detected by alkaline phosphatase-labeled anti-rabbit antibody (1:15,000 dilution), followed by chemiluminescence. The relative migration of prestained molecular mass standards in kDa is indicated. The arrow indicates the position of Munc18 proteins; lower molecular weight bands may arise from partial degradation of the protein (see “Results”).

SM-Proteins in Human Platelets—The expression of Munc18 proteins in human platelets was examined by immunoblotting. An anti-Munc18c monoclonal antibody readily detected Munc18c in human platelets, but minimal amounts were found in human brain (Fig. 1B), despite comparable amounts of total protein in each sample as detected by Coomassie staining (Fig. 1A). Munc18c was also detected in human platelets by an antibody raised to a unique peptide sequence in that protein (not shown). Munc18a was readily detected in protein extracts from human brain by a polyclonal antibody generated against a peptide sequence unique to that Munc18 protein (Fig. 1C). Lower amounts of Munc18a were detected in protein extracts from human platelets (Fig. 1C). No polyclonal antibody existed that was unique for Munc18b, but a polyclonal antibody generated against a peptide sequence found in both Munc18a and Munc18b (Munc18a/b) identified Munc18a/b in both human brain and human platelets (Fig. 1D). Because the immunoblotting experiments did not directly identify the Munc18b in platelets, we performed reverse transcription-PCR with human platelet mRNA. A PCR product of the appropriate size was obtained from both human platelet and human brain samples with specific Munc18b primers (not shown). Taken together, these immunoblotting and PCR experiments provided evidence that platelets contain Munc18a, Munc18b, and Munc18c. The members of the Munc18 family migrated with the same apparent molecular mass of ~70 kDa. Trace amounts of Munc18 immunoreactivity were also observed at lower apparent molecular sizes, which may represent partial degradation or alternatively spliced transcripts. Previous studies have shown that syntaxins 2 and 4 mediate platelet granule secretion (27, 31). Polyclonal antibodies were generated against recombinant syntaxins 2 and 4, which share 49% amino acid identity. The specificity of these antisera for their cognate syntaxin was examined by immunoblotting. In side by side experiments, the syntaxin 2 antisera bound preferentially to recombinant syntaxin 2 and showed minimal cross-reactivity with recombinant syntaxin 4 (Fig. 2A). Similarly, the syntaxin 4 antisera showed highly selective binding to recombinant syntaxin 4 and minimal if any binding to recombinant syntaxin 2 (Fig. 2A). Immunoblotting experiments with these antibodies confirmed the presence of both syntaxin 2 and 4 in platelets that migrated with different relative molecular masses (Fig. 2B). Syntaxin 4 was found exclusively in platelet membranes, whereas syntaxin 2 was detected in both platelet membranes and cytosol (Fig. 2C). In contrast, Munc18c and Munc18a/b proteins were detected in both the cytoplasm and platelet membrane (Fig. 2D).

SM-Syntaxin Complex Formation in Human Platelets—To determine the specificity of SM-syntaxin interactions in platelets, co-immunoprecipitation experiments were performed with antibodies against Munc18a/b and Munc18c. The anti-Munc18a/b antibody precipitated Munc18a/b but not Munc18c from platelets (Fig. 3A). Conversely, the anti-Munc18c antibody precipitated Munc18c but not Munc18a/b from platelets (Fig. 3A). These results confirmed the specificity of these antibodies for their respective Munc18. When Munc18c was precipitated from platelets, it was found to be complexed with syntaxin 4, and minimal if any complex formation with syntaxin 2 was detected (Fig. 3B and C). When Munc18a/b were precipitated from platelets, no complex formation was detected with either syntaxin 2 or syntaxin 4 (Fig. 3B, B and C). Taken together, these experiments indicated that Munc18c preferentially bound to syntaxin 4 versus syntaxin 2. No significant binding interactions were found with Munc18a/b and either syntaxin 2 or syntaxin 4.

SM-Syntaxin Complex Formation and Exocytosis—Platelets secrete their granule contents in response to cellular activation by physiologic agonists such as thrombin or nonphysiologic agents such as phorbol esters (28). In contrast, physiologic agents such as prostaglandin I₂ (PGI₂) that passivate platelets inhibit cell activation and prevent secretion. If SM-syntaxin complex formation plays a functional role in platelet exocytosis, it may be modulated by cell activation. Consequently, the relative amounts of Munc18c-syntaxin 4 complex formation were examined in cells treated with PGI₂, thrombin, and PMA by quantitative immunoblotting. When compared with PGI₂-treated cells, cell activation by thrombin or PMA decreased Munc18c-syntaxin 4 complexes in platelet membranes (Fig. 4B, p < 0.05). However, there was no change in the distribution of Munc18c from membrane to cytosol with cell activation (Fig. 4A), which suggested that this hydrophilic protein remained
membrane-associated in activated cells through other, nonsyntaxin interactions.

These experiments indicated that cell activation was linked to dissociation of the Munc18-syntaxin complex but did not clarify how complex formation per se affected platelet secretion. To determine whether dissociation of the SM-syntaxin complex lead to exocytosis required molecular probes that could interfere with or separate this complex. The crystal structure of the SM-syntaxin complex reveals extensive intermolecular contacts between these two proteins (24). Consequently, four peptides were synthesized to mimic the sites on Munc18c that are projected by crystal structure studies to form intermolecular contacts with syntaxins (24). These peptides inhibited the binding of [35S]Munc18c to syntaxin 4 in vitro (Fig. 5A). The peptides were introduced into permeabilized platelets, and their effect on Ca2+-triggered exocytosis was determined. Low levels of Ca2+ triggered small amounts of dense granule secretion (Fig. 5B). Secretion was modestly enhanced by the addition of peptides in a stoichiometric combination (total 1 mM; Fig. 5B). To verify that this stimulatory effect was sequence-specific and not simply related to peptide charge or amino acid content, the peptides were pretreated with proteinase K. Digestion of the peptides by proteinase K attenuated the stimulatory effect of the peptides on secretion, whereas proteinase K alone had no effects (Fig. 5B). In addition, the individual Munc18c peptides that mimicked only one contact site with syntaxin 4 did not stimulate secretion (even at the same 1 mM concentration), providing further evidence that the stimulatory effect of the peptide combination (total 1 mM) was specific. Still, the combination of peptides had no effect on α granule or lysosomal secretion (not shown). To examine this more critically, we sought other molecular approaches to interfere with or dissociate the Munc18c-syntaxin 4 complex. Soluble recombinant syntaxin 4 molecules or syntaxin 4 antibodies might inhibit platelet syntaxin 4-Munc18c binding interactions or interfere with formation of platelet syntaxin 4-SNARE complexes; this approach was ruled out because the potential dual effects of these inhibitors would confound experimental interpretation (27, 32). Consequently, monoclonal antibodies were generated against Munc18c to specifically inhibit or dissociate the platelet SM-syntaxin complex. Two mAbs, 889 and 132, bound to recombinant Munc18c (Fig. 6A), whereas a control mAb (antidigoxin), which does not bind to a secretion molecule, did not specifically bind to Munc18c. In immunoblotting experiments, mAb 889 recognized a single band of the appropriate molecular size (70 kDa) in platelet lysates (Fig. 6B). In contrast, mAb 132 did not react with denatured Munc18c in immunoblots. In vitro, mAb 889 was a potent inhibitor of complex formation between [35S]Munc18c and syntaxin 4, whereas mAb 132 had only mild inhibitory effects (Fig. 6C). Studies with recombinant fragments revealed that mAb 889 bound specifically to domains 2 and 3 of Munc18c and not to domain 1 (Fig. 6D). When added to platelet lysates, mAb 889 dissociated
Munc18-syntaxin 4 complexes, whereas a control (anti-digoxin) monoclonal antibody of the same serotype had no effect (not shown).

To determine how SM-syntaxin interactions may affect the terminal phases of Ca$^{2+}$-triggered exocytosis, we examined the effects of these inhibitory antibodies on secretion in permeabilized platelets. Permeabilized platelets stimulated with threshold amounts of Ca$^{2+}$ (200 nM) showed a small increase in platelet dense granule secretion when compared with platelets without added Ca$^{2+}$ (Fig. 7A). In platelets stimulated with threshold amounts of Ca$^{2+}$, the control mAb had no effect. mAb 132, the weaker inhibitor of Munc18c-syntaxin 4 interactions, mildly stimulated dense granule secretion (Fig. 7A). mAb 889, the more potent inhibitor of Munc18c-syntaxin 4 interactions, strongly increased dense granule secretion (Fig. 7A). This stimulatory effect was specific, because an anti-SNAP-23 antibody strongly inhibited dense granule secretion (Fig. 7A). Further evidence for specificity came from the observation that the stimulatory effects on dense granule secretion of the anti-Munc18c mAb 889 were dose-related (Fig. 7B). In addition to amplifying dense granule secretion, this anti-Munc18c antibody also significantly enhanced exocytosis from α granules and lysosomes in permeabilized platelets stimulated with threshold concentrations of Ca$^{2+}$ (Fig. 7, C and D). When platelets were stimulated with 10 μM Ca$^{2+}$, which induces maximal secretion, mAb 889 had minimal stimulatory effects when compared with the control mAb. In contrast, a polyclonal anti-SNAP-23 antibody was a potent inhibitor of Ca$^{2+}$-induced exocytosis (Fig. 7E), as expected.

**DISCUSSION**

Experiments utilizing cellular overexpression of syntaxin and SM mutants have yielded conflicting insights into the mechanistic role of the SM proteins. This may be due to secondary effects that overexpression has on cellular trafficking and biosynthesis of these molecules (1). As experimental cells, platelets are well suited to examine the role of SM-syntaxin complex formation on exocytosis, relatively separate from the potential roles SM proteins may play in other vesicle trafficking processes. We found that human platelets contain all three members of the Munc18 family. These Munc18 proteins were distributed in both the cytoplasmic and membrane fractions of human platelets. Syntaxin 4 expression was limited to the membrane, whereas syntaxin 2 was found in both the cytosol and membrane fractions. Syntaxin 2 is alternately spliced at the C terminus to yield variants with hydrophilic sequences or variants with hydrophobic transmembrane domain sequences (39). The presence of syntaxin 2 in the classical membrane and nonmembrane fractions suggests that platelets may contain more than one syntaxin 2 splice variant. Co-immunoprecipitation studies indicated that Munc18c preferentially complexes with syntaxin 4 in platelets but had minimal if any interactions with syntaxin 2. Under the same experimental conditions, no binding was seen between Munc18a/b and syntaxin 2 or 4 in platelets. Agents that activate platelets and trigger secretion enhanced the dissociation of the Munc18c-syntaxin 4 complex, suggesting that the complex may act to inhibit secretion.
Fig. 7. mAbs that inhibit Munc18c-syntaxin 4 complexes amplify platelet exocytosis. A, mAbs 132 and 889 increase platelet dense granule secretion at low [Ca\(^{2+}\)]. Platelets permeabilized with streptolysin were incubated with Ca\(^{2+}\) (0 or 0.2 \(\mu\)M) in the absence or presence of various antibodies (150 \(\mu\)g/ml; anti-Munc18c mAb 132 or 889, a control mAb (anti-digoxin; 150 \(\mu\)g/ml), a purified polyclonal anti-SNAP-23 antibody, or a nonimmune IgG. The secretion of \(^{14}\)C-serotonin (mean \(\pm\) S.E.) from dense granules was measured by \(\beta\)-scintillation counting. In these experiments, the maximum secretion induced by thrombin (1 unit) in nonpermeabilized cells was 4709 cpm. B, anti-Munc18c mAb 889 increases dense granule secretion in a dose-dependent fashion at low [Ca\(^{2+}\)]. Platelets permeabilized with streptolysin were incubated with Ca\(^{2+}\) (0 or 0.2 \(\mu\)M) in the presence of no mAb, anti-Munc18c mAb 889 (25–150 \(\mu\)g/ml), or a control mAb (anti-digoxin; 150 \(\mu\)g/ml), and the secretion of \(^{14}\)C-serotonin (mean \(\pm\) S.E.) from dense granules was measured. In these experiments, the maximum secretion induced by thrombin (1 unit) in nonpermeabilized cells was 10,700 cpm. Shown are the effects of anti-Munc18c mAb on \(\alpha\) granule (P-selectin) (C) and lysosomal (hexosaminidase) secretion (D) in permeabilized platelets in the presence of low [Ca\(^{2+}\)]. Permeabilized platelets were incubated with Ca\(^{2+}\) (0 or 0.2 \(\mu\)M) in the presence of no mAb, anti-Munc18c mAb 889 (150 \(\mu\)g/ml), or a control mAb (150 \(\mu\)g/ml). A granule secretion was determined by flow cytometry with a phycoerythrin-coupled anti-P-selectin antibody (Ab). Lysosomal secretion was determined by measuring the release of hexosaminidase. The maximum secretion in these experiments by thrombin (1 unit) in nonpermeabilized platelets for P-selectin was 784 arbitrary units (AU), and for hexosaminidase it was 4263 arbitrary units. Data represent mean \(\pm\) S.E.

Experimental approaches were taken to determine the effect of SM-syntaxin binding interactions on exocytosis. Peptides that mimic the contact sites of Munc18c with syntaxin 4 augmented dense granule secretion induced by Ca\(^{2+}\) in permeabilized platelets. Similarly, mAbs that inhibit Munc18c-syntaxin 4 binding significantly amplified Ca\(^{2+}\)-induced exocytosis. To our knowledge, these are the first data to show that acute inhibition of the SM-syntaxin complex directly amplifies Ca\(^{2+}\)-induced exocytosis in cells. Taken together, these studies establish that Munc18c forms complexes with syntaxin 4 in platelet membranes that are modulated by cell activation and that play important functional roles in platelet exocytosis.

Whether the different Munc18s have specific functional interactions with syntaxins has been unclear. The SM proteins are structurally homologous, but there is only 47–63% sequence identity between human Munc18a, Munc18b, and Munc18c (3). It was originally hypothesized that unique interactions between specific Munc18 isoforms and syntaxins might provide a combinatorial mechanism for the regulation of vesicle transport in different mammalian cells (6). However, subsequent in vitro studies indicated that the interactions of the SM proteins with syntaxins are moderately promiscuous. In vitro, Munc18a and Munc18b show a similar spectrum of binding with syntaxins 1A, 2, and 3 (5, 40, 41). Structure-function studies of Munc18b indicated that specific residues in the molecule may mediate interactions with one syntaxin but not another (42). In vitro studies with Munc18c showed that it can bind both syntaxin 2 and syntaxin 4 (40). Surprisingly, however, co-immunoprecipitation studies with anti-Munc18c antibodies indicated that platelet Munc18c preferentially complexed with syntaxin 4 despite the presence of significant amounts of syntaxin 2 in platelets. This confirmed cellular overexpression studies identifying complex formation between Munc18c and syntaxin 4 (15). The mAbs generated against Munc18c provided unique tools for investigating the role of the SM-syntaxin complex on Ca\(^{2+}\)-induced secretion. When compared with a control (anti-digoxin) antibody, mAb 132 weakly inhibited and mAb 889 strongly inhibited syntaxin 4-Munc18c binding in vitro. In permeabilized platelets, the effect of the antibodies on Ca\(^{2+}\)-induced secretion was clearly related to their inhibitory effects on syntaxin 4-Munc18c binding. The control mAb had no effect, the weaker inhibitor mAb 132 had modest stimulatory effects, and the potent inhibitor mAb 889 had strong, dose-dependent stimulatory effects on dense granule secretion. These stimulatory effects were specific, since an anti-SNAP-23 antibody inhibited dense granule secretion un-
Under the same conditions. The potent inhibitor of syntaxin 4-Munc18c interactions, mAb 889, consistently augmented secretion from dense (~2–4-fold) and α granules (~3-fold) and lysosomes (~2.5-fold), in the presence of threshold amounts of intracellular Ca^{2+}. Similarly, a combination of peptides that mimicked the projected sites on Munc18c that contact syntaxin 4 also inhibited syntaxin 4-Munc18c binding and enhanced dense granule secretion (~1.7-fold), although to a lesser extent than mAb 889 in parallel experiments. The stimulatory effects of these peptides appeared specific, because they were lost after digestion of proteinase K and were not seen with the individual peptides alone. Still, the combination of these contact site peptides had minimal or no effects on α granule or lysosomal secretion, and the individual peptides alone were inert. The failure of these contact site peptides to enhance secretion from α and lysosomal granules probably reflects the fact that they are less potent inhibitors of Munc18c-syntaxin interactions than mAb 889 (Figs. 5 and 6). These peptides may only have augmented dense granule secretion because dense granule exocytosis is more readily induced at a lower threshold of cell activation than α or lysosomal secretion. Indeed, dense granule secretion is triggered by lower levels of intracellular Ca^{2+}, requires smaller doses of thrombin, proceeds at a faster rate, and requires less energy than α and lysosomal granule secretion (43–48).

Expression studies in Chinese hamster ovary cells have indicated that Munc18b can complex with both syntaxin 2 and 3 (42); through these interactions, Munc18b may mediate exocytic transport in epithelial cells. However, we detected no complex formation between Munc18a/b and platelet syntaxin 2 or syntaxin 4 in co-immunoprecipitation studies. This suggests a lack of interactions between Munc18a/b and these syntaxins in platelets. An alternate explanation is that the Munc18a/b immunoprecipitating antibody, because of steric hindrance or competitive binding, was unable to interact with Munc18a/b-syntaxin complexes. We were unable to explore the potential functional role of Munc18a/b in platelet exocytosis, because there are no specific inhibitors of these molecules.

The present studies with PGL2-treated and thrombin-activated cells indicate that the amount of Munc18c complexed with syntaxin 4 decreases with cell activation, although the amount of Munc18c in the membrane does not. How cell activation regulates SM-syntaxin interactions in vitro is still unclear, although in vitro studies have suggested that phosphorylation of these proteins may play a key role (3, 28, 49). Munc18c is a hydrophilic protein, and its persistence in the membrane of activated platelets, while not bound to syntaxin 4, is likely to be mediated by interaction with another membrane protein or membrane-associated molecule (perhaps the molecule(s) that dissociate(s) the SM-syntaxin complex). The SM proteins bind directly with Doc2 (50) and Mint (51) and interact indirectly with Munc13 in Caenorhabditis elegans (52) and Rab homologues in yeast (53). It has been proposed that the SM proteins are fusogens (7), that they regulate the dynamics of the fusion pore (54) during exocytosis, and that they modulate the docking of dense core vesicles with the membrane (18). Thus, in addition to their effects on triggered exocytosis mediated through syntaxin interactions, the SM proteins may well have additional functional roles in cellular vesicle trafficking processes that are specified by cellular location, interacting partners, and specific intracellular signals.

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