A role for Sec1/Munc18 proteins in platelet exocytosis

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A critical aspect of haemostasis is the release of clot-forming components from the three intra-platelet stores: dense-core granules, α granules and lysosomes. Exocytosis from these granules is mediated by soluble proteins [N-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs)] and integral membrane proteins [vesicle and target SNAP receptors (v- and t-SNAREs)]. Three Sec1/Munc18 proteins (SM proteins) are present in platelets (Munc18a, Munc18b and Munc18c) and they bind to and potentially regulate specific syntaxin t-SNAREs. In resting platelets, these SM proteins associate with granules and open canalicular system membranes predominantly but not with the plasma membrane. Munc18a binds to syntaxin 2 alone and does not associate with other members of the core SNARE complex. Munc18b associates with a larger complex that contains synaptosome-associated protein of 23 kDa (SNAP-23) and cellubrevin/vesicle-associated membrane protein 3. Munc18c associates with both syntaxins 2 and 4, with synaptosome-associated protein of 23 kDa (SNAP-23) and with a v-SNARE. On stimulation, most of the platelet SM proteins are still found in membrane fractions. Phosphorylation of each Munc18 increases in thrombin-treated cells and phosphorylated Munc18c remains associated with syntaxins 2 and 4, but its affinity for the SNAREs appears to be reduced. To determine the functional role of the platelet SM proteins, we examined the effects of Munc18-based peptides (Munc18a peptide 3 and Munc18c peptide 3). Addition of the peptides to permeabilized platelets inhibits secretion from all three platelet granules. These peptides also inhibit agonist-induced aggregation in saponin-permeabilized platelets. These studies demonstrate a clear role for SM proteins in platelet exocytosis and aggregation and suggest a dominant role for Munc18c in all three granule-release events.

Key words: secretion, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE), syntaxin.

INTRODUCTION

The primary role of platelets is to form a haemostatic plug in response to vascular lesions. This is accomplished by first detecting the lesion, adhering to the site of injury and then recruiting additional platelets [1]. Platelets have three granular stores (α, dense core and lysosomal granules) whose cargo must be released for efficient clot formation [2]. On activation by such agonists as thrombin and collagen, the three granules are moved to the centre of the platelet where their membranes fuse with the open canalicular system (OCS) [2,3]. Once the granule stores are released, clot formation begins as other platelets are recruited to the site of damage and begin to form a scaffold on which the clot will coalesce. The essential granule-to-OCS membrane-fusion events are the culmination of a complex cascade of protein–protein interactions [4].

Members of several protein families are essential for these membrane-fusion events. Soluble N-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptors (SNAREs) and Sec1/Munc18 proteins (SM proteins) [5] are two such families. According to the SNARE hypothesis [6], transport vesicles are engaged at their target membrane by the specific interaction of proteins on the vesicle (v-SNAREs) with cognate proteins on the target membrane (t-SNAREs). In platelets, v-SNAREs, thought to be important for membrane fusion, include synaptobrevin/VAMP

(vesicle-associated membrane protein)-2 [7], cellubrevin/VAMP-3 and endobrevin/VAMP-8 [8,9], whereas syntaxins 2 and 4 and synaptosome-associated protein of 23 kDa (SNAP-23; reviewed in [4]) represent the functional t-SNAREs. The SNAREs form a trans-membrane complex composed of four α-helical coiled coils. The v-SNAREs contribute one α helix, whereas the t-SNAREs contribute three α helices, one from syntaxin and two from SNAP-23/25 [10]. Two peripheral membrane proteins, α-SNAP and N-ethylmaleimide-sensitive fusion protein (NSF), are required to activate the SNAREs before their engagement and membrane fusion [6]. In the trans configuration, the SNARE complex spans the two fusing bilayers and is sufficient for membrane fusion [11]. The key step in controlling membrane fusion is the regulation of the assembly of these trans SNARE complexes.

SM proteins play a vital role in membrane fusion through their interactions with syntaxins. In vertebrates, there are less number of SM proteins (Munc18a, Munc18b, Munc18c, Sly1, Vps45, Vps33a and Vps33b) compared with the multiple SNARE isoforms [12]. The Munc18 subfamily associates with extracellular release events [13] through their specific binding to plasma-membrane syntaxins [14]. Absence of Munc18a in mice results in the complete elimination of neurotransmission [13] and large, dense-core vesicle exocytosis from chromaffin cells [15]. Addition of a squid homologue of Sec1 protein (s-Sec1) or a specific peptide from s-Sec1 [homologous with Munc18a

peptide 3 (18a/pep3)] into neurons inhibits evoked release of a neurotransmitter [16]. Munc18a and Munc18b exhibit selectivity in their association with syntaxins since they interact only with syntaxins 1a, 2 and 3, but not with syntaxin 4 [17–21]. Munc18a also responds to cell signalling via its phosphorylation on Ser^{313} by protein kinase C (PKC). This decreases its affinity for syntaxin but does not completely eliminate binding [22,23]. Less is known about the ubiquitously expressed Munc18c, which associates only with syntaxins 2 and 4 *in vitro* and localizes exclusively with membrane fractions [19,21,24]. In 3T3-L1 cells, Munc18c plays a role in insulin-stimulated mobilization of GLUT4 through its association with syntaxin 4 [19]. In platelets, Munc18c has also been shown to associate with syntaxin 4 and is phosphorylated after phorbol ester or thrombin treatment by PKC [25]. Munc18b, also ubiquitously expressed, controls membrane trafficking at the apical plasma membrane of epithelial cells through its dominant interaction with syntaxin 3 [20]. It associates with both cytosolic and membrane fractions [19] and is potentially involved in mast-cell granule exocytosis [21].

Much of our mechanistic understanding of SM protein–syntaxin interactions comes from studies of Munc18a, which was originally characterized as a key syntaxin 1 regulator with nanomolar affinity [26,27]. Syntaxin 1 cycles between 'open' and 'closed' conformations. In the more stable closed conformation, the N-terminal domain (Habc domain) folds back on to the SNARE motif, limiting the ability of syntaxin to interact with other SNARE partners [28]. Munc18a binds directly to this closed conformation, resulting in the formation of a stable complex [29]. Activation of the closed syntaxin 1–Munc18a complex through interactions with upstream regulators (i.e. Munc13 and DOC2 [30,31]) is thought to mediate the transfer of open syntaxin to the cognate SNAREs synaptobrevin/VAMP-2 and SNAP-25 (reviewed in [14]). Recent evidence suggests that this model for Munc18–syntaxin interactions may not generally apply to other members of the SM protein family.

Currently, there appears to be at least three different modes by which SM proteins interact with SNAREs (reviewed in [32]). The previously described direct Munc18a–syntaxin 1 interaction exemplifies the first class. Class 2 SM proteins bind to the open conformation of the corresponding syntaxin, only requiring a short sequence at the N-terminus of the t-SNAREs for a specific interaction. These class 2 SM protein–syntaxin interactions do not exclude SNARE–SNARE binding. In the third class, there is no direct binding of SM proteins to the corresponding syntaxin and, thus, complexes are formed through other soluble proteins. The exact role of SM proteins in exocytosis now appears to be dependent on the type of interaction with its respective syntaxin partner. However, SM proteins are clearly involved in the conformational cycle of the syntaxins and this role is directly required for membrane fusion.

In the present study, we investigate the roles of Munc18 isoforms expressed in platelets and characterize their intracellular localization in both resting and stimulated cells. We show that three of the seven SM proteins are expressed in platelets: Munc18a, Munc18b and Munc18c. In resting platelets, Munc18a is predominantly associated with syntaxin 2, whereas Munc18b and Munc18c associate with both t- and v-SNAREs. Upon stimulation with thrombin, phosphorylation levels of Munc18 increase. Phosphorylation of Munc18c may decrease its affinity for syntaxins 2 and 4. Using an inhibitory peptide that affects syntaxin interactions with Munc18, we show a dominant role for Munc18c in aggregation and in secretion from each of the three granule stores in platelets. Taken together, these results show a clear role for the SM family of proteins in the complex cascade of events that leads to platelet exocytosis.

EXPERIMENTAL

Degenerate PCR of the platelet library for SM proteins

Degenerate PCR primers 5′-CGGGATCCAT(T/C/A)AA(A/G)-GA(T/C)AT(T/C/A)ATGGA(A/G)G and 5′-GGAATTCGT(G/A/T/C)AC(T/C)TC(A/G)TA(G/A/T/C)GC(A/G)CA(G/A/T/C)C were synthesized to highly conserved regions of Munc18a (amino acids 482–557) and other Munc18 homologues. These primers were used to screen a human platelet λZAP II cDNA library (gift from G. Roth, University of Washington School of Medicine, Seattle, WA, U.S.A.). Of the ten clones isolated, six encoded Munc18c, three encoded Munc18a and the remainder were unrelated. These PCR clones matched the human expressed sequence tag accession nos. R61138 (Munc18c-like) and H14397 (Munc18a-like) obtained from Genome Systems Inc. (St. Louis, MO, U.S.A.). These same PCR clones were utilized to generate non-degenerate primers against human Munc18a and Munc18c. Using Munc18c-specific primers 5′-CGGGATCCCATCGACA-TATTGCGGTTG and 5′-ACGCGTCGACCTATTCATCTTTAA-TTAAGG, another product was amplified from the platelet cDNA library and was subsequently used as a probe for filter hybridization screening of the same library. From this, two more independent clones encoding human Munc18c (amino acids 204–592) were identified.

Cloning of cDNAs and antibody production

A plasmid encoding Munc18a was a gift from Dr Richard Scheller (Stanford University, Stanford, CA, U.S.A.) and the coding region was cloned into pPROEX-HT$_b$ vector (Gibco BRL, Gaithersburg, MD, U.S.A.) for expression in *Escherichia coli*. Full-length soluble His$_6$–Munc18a was used to immunize rabbits for polyclonal antibody (pAb) production. Glutathione S-transferase (GST)–Munc18a was made in *E. coli* using the pGEX-KG (Amersham Biosciences) expression vector and was chemically coupled with glutathione–agarose [8]. An ammonium sulphate-precipitated immunoglobulin fraction was affinity-purified using GST–Munc18a/GSH–agarose to generate anti-Munc18a pAb. This antibody detects both Munc18a and Munc18b recombinant proteins and so was designated Munc18a/b pAb (Figure 1).

A full-length expressed sequence tag clone for Munc18b (AL542799) was obtained from Invitrogen (Carlsbad, CA, U.S.A.) and the coding region was amplified by PCR using primers (5′-CGGGATCCATGGCGCCCTCGGGGCTGAA and 5′-CGCAAGCTTTCAGGGCAGGGCAATGTCCTCCA). Initially, the PCR product was cloned into pBluescript II SK+ (Stratagene, La Jolla, CA, U.S.A.) for dideoxy sequencing and cloned into the pPROEX-HT$_b$ vector for recombinant protein production in *E. coli*. Full-length human Munc18c cDNA was generously provided by Dr Hitoshi Kitayama (Kyoto University, Kyoto, Japan) and was subcloned into the pQE-9 vector (Qiagen, Chatsworth, CA, U.S.A.) to produce a His$_6$–Munc18c-fusion protein.

Insoluble recombinant Munc18c in inclusion bodies was solubilized in 8 M urea buffered with 100 mM Tris/HCl (pH 7.5) before purification on an Ni^{2+}-nitrilotriacetate resin. This protein was used to immunize rabbits for antibody production. The anti-Munc18c antibody was then purified by affinity chromatography on a GST-Munc18c/GSH–agarose as above. Anti-Munc18a and anti-syntaxin 4 monoclonal antibodies (mAbs) were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-SNAP-23, anti-syntaxin 2, anti-syntaxin 4 and anti-rabGDI pAbs were produced as described previously [33,34]. VAMPX antibody, specific for synaptobrevin/VAMP-1, synaptobrevin/VAMP-2 and cellubrevin/VAMP-3 proteins, was obtained from
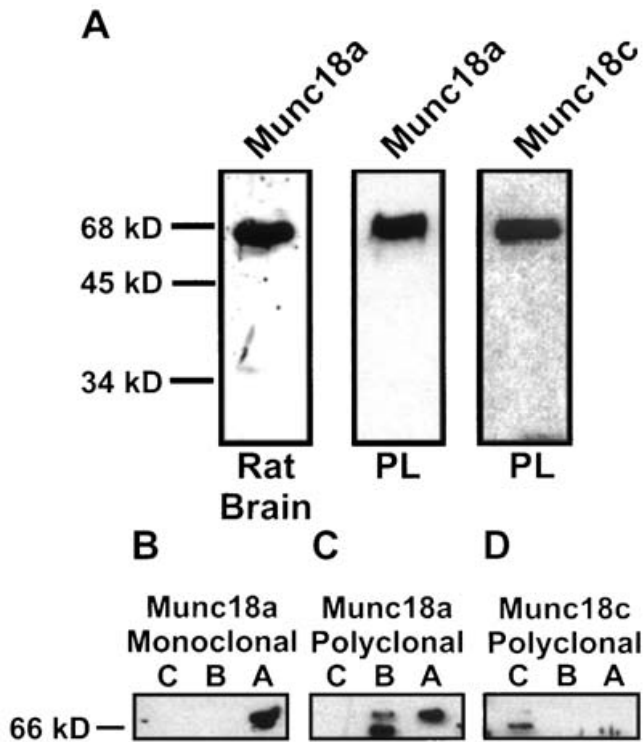
Figure 1   Presence of SM proteins in platelets

(**A**) Human platelets were solubilized in $1 \times$ SDS sample buffer in the presence of protease inhibitors and 100 μg of protein was loaded per lane for Western-blot analysis using anti-Munc18a mAb and anti-Munc18c pAb. Whole rat brain (10 μg) was used as a positive control for anti-Munc18a mAb. To determine the specificity of available antibodies, recombinant proteins (30 ng) of His$_6$–Munc18a (A), His$_6$–Munc18b (B) and His$_6$–Munc18c (C) were probed with anti-Munc18a mAb (**B**), anti-Munc18a/b pAb (**C**) and anti-Munc18c pAb (**D**).

Synaptic Systems (Goettingen, Germany). pTAP/p115 mAb was a gift from Dr M. G. Waters (Princeton University, Princeton, NJ, U.S.A.). Sheep anti-[human platelet factor 4 (PF4)] was purchased from Accurate Chemical (Westbury, NY, U.S.A.). All His$_6$-tagged fusion proteins were purified using Ni^{2+}-nitrilotriacetate resin (Qiagen).

Peptides

18a/pep3 (RKERISEQTYQLSRWTPIIKDIME) was synthesized by New England Peptide (Fitchburg, MA, U.S.A.). 18c/pep3 (RK-DRSAEETFQLSRWTPFIKDIME) and the scrambled peptide (RFKLWDKFESRTIDTSQPIMREEA) having the same amino acid composition but a different sequence compared with 18c/pep3 were synthesized at the Macromolecular Structural Analysis Facility (University of Kentucky).

Analysis of 20 S complexes in human platelets by matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry (MALDI–TOF-MS)

SNARE complexes were purified by the method of Söllner et al. [6]. The 20 S complexes, formed with 100 mg of detergent-solubilized platelet membrane proteins, α-SNAP and NSF*myc*, in the presence of adenosine $5'$-[β,γ-imido]triphosphate (AMP-PNP)/EDTA, were recovered by immunoprecipitation using 9E10 antibody coupled with Protein G–Superose. The eluted SNAREs

were concentrated, fractionated by SDS/PAGE and visualized by Coomassie Blue staining. The identified bands were excised, digested with trypsin and the resulting peptides were analysed by MALDI–TOF-MS at Biomolecular Mass Spectrometry Core Laboratory at the University of Louisville (www.louisville.edu/~wmpier01/biomassspec.htm).

Immunoelectron microscopy

Immunoelectron microscopy studies were performed as described previously [35]. The secondary antibodies used in these experiments were colloidal gold-conjugated goat anti-mouse (10 nm) and anti-rabbit (20 nm) secondary antibodies diluted in 1 % normal goat serum. Sections were counterstained with uranyl acetate and lead citrate and were viewed on a Hitachi H-7000 transmission electron microscope (Tokyo, Japan).

Preparation of platelet cytosol and membranes

Platelets were isolated from concentrates (Central Kentucky Blood Center, Lexington, KY, U.S.A.) as described previously [36]. Platelet cytosolic and membrane extracts were prepared by sonication as described in [37]. Resting platelets were not treated with thrombin, whereas stimulated platelets were treated with 1 unit/ml thrombin. A $100 \times$ inhibitor cocktail (100 μM leupeptin, 400 μg/ml antipain, 1000 units/μl aprotinin, 100 μM pepstatin/400 μg/ml chymostatin/100 mM benzamidine/100 mM NaF/100 mM sodium vanadate/300 nM okadaic acid/100 mM EDTA/100 mM EGTA) was added to a final concentration of $1 \times$ immediately after sonication. After lysis, platelet extracts were cleared by centrifugation at 100 000 g for 1 h at 4 °C. The supernatant (cytosolic) and the pellet (membrane) fractions were separated by SDS/PAGE and analysed by Western blotting with the indicated antibodies. The enhanced chemiluminescent detection system (ECL®; Pierce, Rockford, IL, U.S.A.) with secondary antibodies covalently coupled with horseradish peroxidase was used to visualize the immunodecorated proteins.

Assay of platelet exocytosis

Platelets were metabolically labelled with [^{3}H]hydroxytryptamine (5-HT), permeabilized with streptolysin O (SLO) and assayed for secretion of granule contents as described previously [33,35]. Briefly, 50 μl of platelets (approx. 10^7–10^8) in assay buffer was mixed with 50 μl of assay buffer containing 8 mM ATP, 1.6 units/ml SLO and 18a/pep3, 18c/pep3 or scrambled peptide for 10 min at 25 °C. The reaction mixtures were further incubated on ice for 30 min. After the samples were warmed to 25 °C for 5 min, 10 μM CaCl$_2$ (final) was added and the mixtures were incubated at 25 °C for 5 min. The reactions were stopped by centrifugation at 13 000 g for 1 min and [^{3}H]5-HT secretion was measured by scintillation counting. Hexosaminidase secretion was measured as described previously [35] and PF4 secretion was measured by ELISA [38].

Aggregation of saponin-permeabilized human platelets

Aggregation experiments were performed by the method originally described by Authi and co-workers [39]. Briefly, banked platelets were treated with 10 ng/ml prostaglandin I$_2$, harvested by centrifugation at 5000 g for 4 min and washed in an equal volume of Ca^{2+}-free Hepes/Tyrode's buffer containing 0.2 unit/ml apyrase. The washed platelets were centrifuged at 5000 g for

5 min and resuspended in cytosolic-type KCl medium (described in [39]). Aggregation tests were performed in an optical aggregometer (Chrono-Log Corp., Havertown, PA, U.S.A.) using 0.5 ml of platelet suspension treated with 14 μg/ml saponin [40] for 2 min. Peptides were added immediately after treatment with saponin and aggregation was induced with 0.2 unit/ml thrombin. Cytosolic-type KCl medium served as the 100 % reference for aggregation.

Immunoprecipitation of phosphorylated platelet extracts

Platelet concentrates (5×10^{10} cells/ml) in citrate were centrifuged at 5000 *g* and the pelleted cells were resuspended in the same volume of phosphorylation buffer (154 mM NaCl/2.7 mM KCl/1 mM MgCl$_2$/5.6 mMglucose/7 mM NaHCO$_3$/5 mM Pipes/NaOH) containing 0.2 unit/ml apyrase and 2 mM EGTA. Platelets were labelled with 0.2 mCi of [^{32}P]P$_i$ for 2 h at 25 °C. After incubation, resting platelets were resuspended in phosphorylation buffer (pH 6.5) containing 10 ng/ml prostaglandin I$_2$. Stimulated platelets were resuspended in phosphorylation buffer (pH 7.4) with 2 mM CaCl$_2$. After incubation for 5 min at 37 °C, platelets were stimulated with 1 unit/ml thrombin. Both resting and stimulated cells were solubilized with 0.8 % (w/v) Triton X-100 in solubilization buffer containing 100 mM Tris (pH 7.4), 2 mM EGTA and EDTA, 2 × Complete protease inhibitor (Roche Diagnostics Corp., Indianapolis, IN, U.S.A.) and 10 mM sodium orthovandate for 1 h at 4 °C. The insoluble material was removed by centrifugation at 16000 *g* for 5 min. All subsequent steps were performed at 4 °C with rotation. Extracts were used immediately or stored at −80 °C. To remove non-specific material, radio-labelled extracts (100 μl) from both resting and stimulated cells were incubated with Protein A–Sepharose beads (25 μl of packed beads) in solubilization buffer for 30 min at 4 °C. The beads were cleared at 1000 *g* for 1 min and the supernatants were incubated in the presence of antibodies for 0.1 mg/ml anti-Munc18a/b pAb, 0.2 mg/ml anti-Munc18c, 0.2 mg/ml anti-syntaxin 2 or 0.2 mg/ml anti-syntaxin 4 pAb for 2 h at 4 °C. Protein A–Sepharose beads (50 μl) were added and incubated for 1.5 h at 4 °C. The beads were recovered by centrifugation for 10 s and the supernatants were collected. The beads were washed four times in solubilization buffer, followed by a final wash in 100 μl of PBS with 2 mM sodium orthovandate. Then, 1 × SDS sample buffer was added and the beads were incubated at 95 °C for 5 min. Detection of immunoprecipitated Munc18a, Munc18c, syntaxin 2 and syntaxin 4 proteins was accomplished by SDS/PAGE and the proteins were transferred to nitrocellulose membranes followed by Western blotting with the indicated antibodies. The presence of ^{32}P in immunoprecipitates was measured using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) with ImagQuant software.

As an alternative to this procedure, immunoprecipitations were performed using Seize Primary Immunoprecipitation Kit (Pierce) following the manufacturer's instructions. This procedure allowed more efficient immunoprecipitation of proteins and was required to examine Munc18a-containing complexes in resting platelets. To remove amines from the antibody solution, anti-Munc18a mAb and anti-Munc18c pAb were dialysed in PBS overnight at 4 °C. Anti-Munc18a mAb (100 μg) and 230 μg of Munc18c were coupled with the AminoLink® Coupling Gel. Platelet extracts (1 mg), prepared as described previously, were diluted in PBS and added to the columns for 2 h at 25 °C. The unbound sample was removed by microcentrifugation at 300 *g* for 1 min. Samples were washed and eluted with the low pH ImmunoPure® elution buffer. Eluted proteins were precipitated by treatment with 12 %

(w/v) trichloroacetic acid for 20 min at 4 °C. Precipitated proteins were washed three times with ethyl ether and the pelleted protein was resuspended in 1 × SDS sample buffer for 5 min at 95 °C. Samples were analysed by Western blotting as described above.

RESULTS

Human platelets contain three SM proteins

Molecular biology techniques and Western blotting were initially used to determine which SM proteins were present in platelets. PCR screening of a platelet cDNA library using degenerate PCR primers detected cDNAs encoding both Munc18a and Munc18c (see the Experimental section). Recombinant Munc18a, Munc18b and Munc18c were produced from cDNA expression constructs and used as antigens for antibody production and also as standards to assess the specificity of the antibodies used in the present study. Both recombinant Munc18b and Munc18c required denaturation for their purification. As shown in Figure 1(B), the commercially available anti-Munc18a mAb reacted specifically with Munc18a. Anti-Munc18a pAb, generated against recombinant protein, detected both Munc18a and Munc18b (Munc18a/b pAb). Anti-Munc18c pAb was specific for Munc18c. Using anti-Munc18c pAb, it was possible to demonstrate that Munc18c was present in platelets. This finding agrees with a previously published report [25]. Munc18a was also detected in platelets, using the commercially available mAb (Figure 1A). Taken together, these two experiments demonstrate that Munc18a and Munc18c are present in platelets.

In an attempt to identify proteins that make up 20 S SNARE complexes in platelets, the method described by Söllner et al. [6] was used. NSF*myc* and α-SNAP were added to platelet membrane extracts to form 20 S SNARE complexes. These SNARE complexes were purified by anti-*myc* affinity chromatography and the SNAREs were then specifically eluted by adding ATP/Mg^{2+}. The eluted proteins were analysed for the presence of SNARE proteins and SNARE regulatory proteins. Two known platelet SNARE proteins, SNAP-23 and cellubrevin/VAMP-3 [41], were used as controls for this analysis and were detected either by Western blotting (Figure 2A) or by Coomassie Blue staining and MS (Figures 2B and 2C) respectively. One syntaxin-binding protein, Munc18b, was also detected using MS (Figures 2B and 2C). The peptides identified from the excised proteins (cellubrevin/VAMP-3 and Munc18b) showed broad coverage and were derived from regions that clearly allow identification of the specific isoform (Figure 2C). Strikingly, the syntaxin-binding protein Munc18b was found associated with platelet SNARE complexes (Figure 2B), indicating that it behaves as a class 2 SM protein [32]. Taken together, the Western blotting, PCR and MS experiments conclusively show that platelets contain at least three of the seven human members of the SM protein family. Owing to the specificity of the available reagents, the bulk of the present study focuses on Munc18a and Munc18c.

Munc18 proteins are associated with platelet membranes

The significance of SM protein localization is highlighted by reports showing enrichment of these proteins at sites of membrane fusion [42,43]. Recent results indicate that SM proteins can bind to syntaxins that are in three different states: 'closed', 'open' or 'in SNARE complex' (as reviewed in [32]). Based on this, one might expect that different SM proteins cycle between a bound and an unbound state (cytosolic versus membrane-associated)
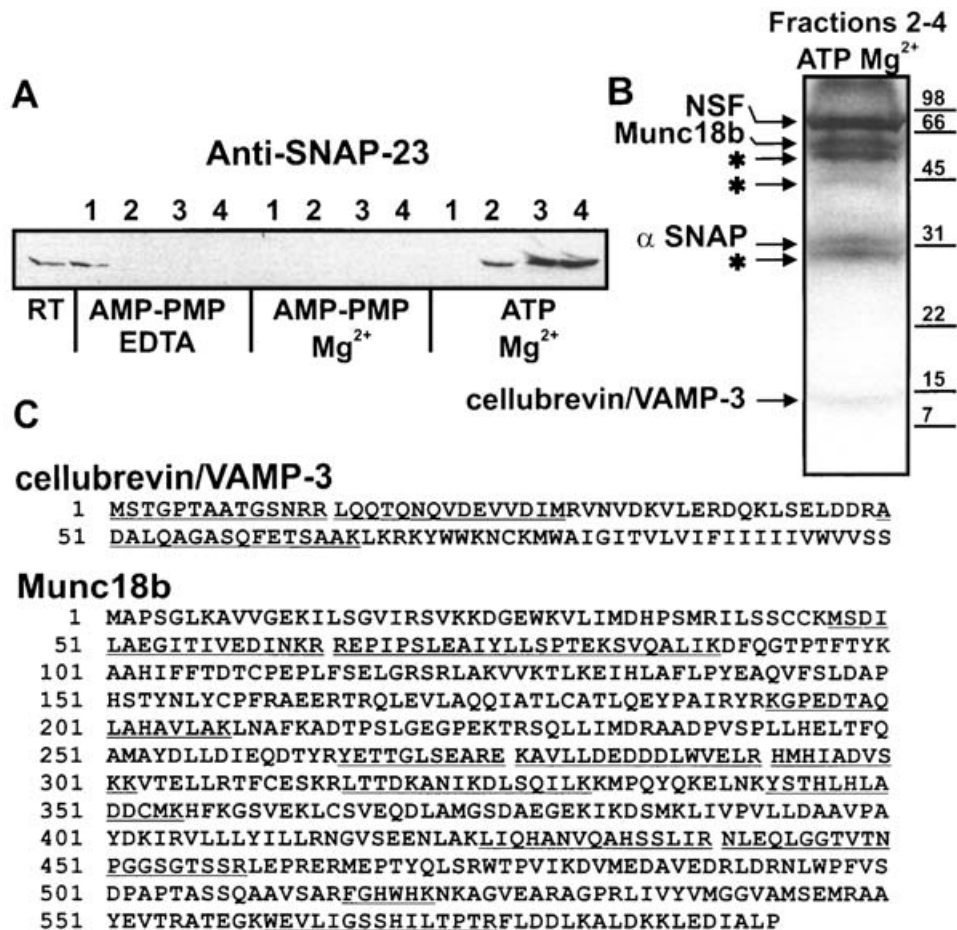
A

Anti-SNAP-23

| | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |

RT | AMP-PMP EDTA | AMP-PMP Mg^{2+} | ATP Mg^{2+}

B

Fractions 2-4
ATP Mg^{2+}

NSF
Munc18b
*
*
α SNAP
*

cellubrevin/VAMP-3 →

98
66
45
31
22
15
7

C

cellubrevin/VAMP-3

```
  1 MSTGPTAATGSNRR LQQTQNQVDEVVDIMRVNVDKVLERDQKLSELDDRA
 51 DALQAGASQFETSAAKLKRKYWWKNCKMWAIGITVLVIFIIIIVWVVSS
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Munc18b

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  1 MAPSGLKAVVGEKILSGVIRSVKKDGEWKVLIMDHPSMRILSSCCKMSDI
 51 LAEGITIVEDINKR REPIPSLEAIYLLSPTEKSVQALIKDFQGTPTFTYK
101 AAHIFFTDTCPEPLFSELGRSRLAKVVKTLKEIHLAFLPYEAQVFSLDAP
151 HSTYNLYCPFRAEERTRQLEVLAQQIATLCATLQEYPAIRYRKGPEDTAQ
201 LAHAVLAKLNAFKADTPSLGEGPEKTRSQLLIMDRAADPVSPLLHELTFQ
251 AMAYDLLDIEQDTYRYETTGLSEARE KAVLLDEDDDLWVELR HMHIADVS
301 KKVTELLRTFCESKRLTTDKANIKDLSQILKKMPQYQKELNKYSTHLHLA
351 DDCMKHFKGSVEKLCSVEQDLAMGSDAEGEKIKDSMKLIVPVLLDAAVPA
401 YDKIRVLLLYILLRNGVSEENLAKLIQHANVQAHSSLIR NLEQLGGTVTN
451 PGGSGTSSRLEPRERMEPTYQLSRWTPVIKDVMEDAVEDRLDRNLWPFVS
501 DPAPTASSQAAVSARFGHWHKNKAGVEARAGPRLIVYVMGGVAMSEMRAA
551 YEVTRATEGKWEVLIGSSHILTPTRFLDDLKALDKKLEDIALP
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Figure 2   Identification of Munc18b and cellubrevin/VAMP-3 in platelets

SNARE complexes were purified by the method of Söllner et al. [6]. The 20 S complexes, formed with 100 mg of detergent-solubilized platelet membrane proteins α-SNAP and NSF*myc* in the presence of AMP-PNP/EDTA, were recovered by immunoprecipitation using 9E10 antibody coupled with Protein G–Superose. The beads were washed first with 4 column vol. of a buffer containing AMP-PNP/EDTA, then with AMP-PNP/MgCl$_2$, and the SNAREs were eluted with ATP/MgCl$_2$. Purification of platelet SNAREs was followed by Western blotting an aliquot of each fraction with anti-SNAP-23 antibody (**A**). RT indicates the initial run-through of the affinity column. The eluted SNAREs were concentrated, fractionated by SDS/PAGE and visualized by Coomassie Blue staining (**B**). The indicated bands (marked with arrows) were excised and digested with trypsin and the resulting peptides were analysed by MALDI–TOF-MS. Labels associated with the bands reflect their identity. Bands marked with an asterisk were actin or keratin or were not conclusively identified. Two SNARE proteins were conclusively identified using this technique, namely cellubrevin/VAMP-3 and Munc18b. Their protein sequence is shown (**C**), and the peptides identified by mass spectra results are underlined.

depending on whether they are class 1 or not. Analysis of crude membrane and cytosolic fractions from resting and thrombin-stimulated platelets showed that Munc18c associated with the membrane fraction (Figure 3A). Munc18a and Munc18b were found in both the cytosolic and membrane fractions, although most of them associated with the membranes (Figure 3B). The distribution of proteins between cytosolic and membrane fractions did not significantly change after stimulation. As a control, most of the known membrane proteins, syntaxins 2 and 4 (Figure 3), were found to be present in the membrane fractions. The distribution of known cytosolic proteins (pTAP and rabGDI) demonstrated the quality of the fractionation procedure (Figure 3).

To analyse further the subcellular distribution of the Munc18s, platelets were probed by immunoelectron microscopy. Resting platelets were prepared for microscopy by fixation, dehydration and embedding in LR White resin. Platelet-containing grids were prepared by thin sectioning and immunolabelled with anti-Munc18a or anti-Munc18c primary antibodies followed by incubation with the appropriate gold-conjugated secondary antibodies. Examination revealed a predominant localization of gold particles on α granules and lysosomes with labelling also

A

Cytosol | Membranes | Cytosol | Membranes

pTAP/p115
Munc18c
Syntaxin-4

Resting   Stimulated

B

Cytosol | Membranes | Cytosol | Membranes

rabGDI
Munc18a/b
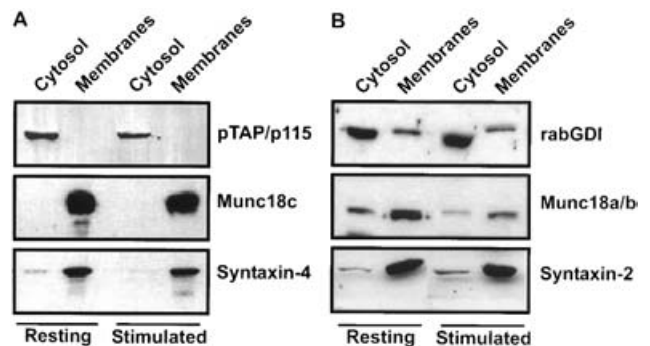Syntaxin-2

Resting   Stimulated

Figure 3   Localization of Munc18s in resting and thrombin-stimulated platelets

Resting and stimulated platelet extracts were separated into cytosolic and membrane fractions in the presence of protease inhibitors. Protein fractions were probed by Western blotting using anti-Munc18c antibody (**A**) along with antibodies for a known cytosolic marker (pTAP/p115) and a known membrane-associated protein (syntaxin 4). Protein fractions were also probed by Western blotting using anti-Munc18a/b pAb (**B**), a known cytosolic protein (rabGDI) and a membrane-associated protein (syntaxin 2).
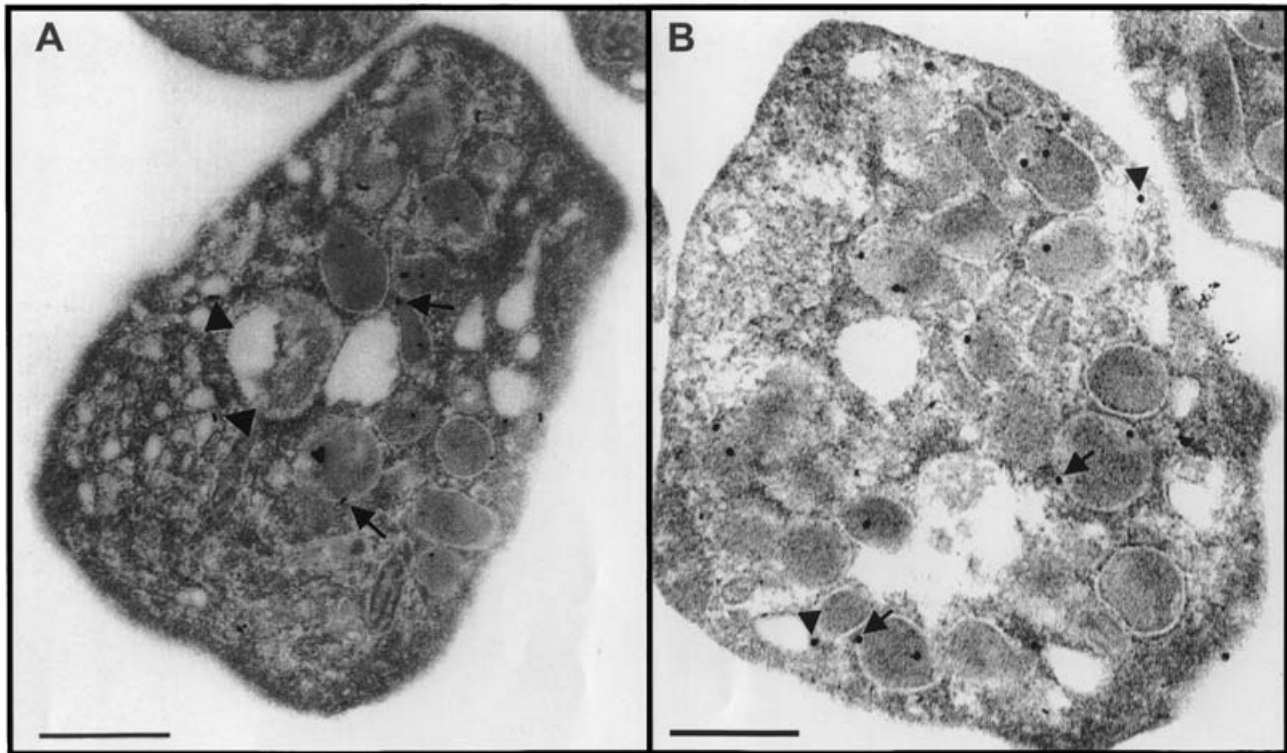
Figure 4   Subcellular localization of Munc18s by electron microscopy

For immunoelectron microscopy, platelets were fixed, dehydrated in a series of ethanol washes and embedded in LR White resin. Grids were blocked with goat serum and incubated with primary antibodies for anti-Munc18a mAb (**A**) and anti-Munc18c pAb (**B**). The grids were rinsed and incubated with colloidal gold-conjugated goat anti-mouse (10 nm) or goat anti-rabbit (20 nm) antibodies and counterstained with uranyl acetate and lead citrate. Arrows indicate labelling of the granule. Arrowheads refer to labelling of the OCS. Scale bar, 0.5 μm.

observed on the OCS and the plasma membrane (Figures 4A and 4B). It should be noted that distinguishing between the three types of granules is difficult in this preparation due to an inability to differentiate between α granules and lysosomes and due to the loss of dense-core granule morphology during fixation. No staining was seen in the absence of primary antibodies or when preimmune antisera were used (results not shown). The distribution of grains was quantified by counting the grains that were localized to the granules, the OCS and the plasma membrane. An effort was made to assign grains to the proper category; therefore, grains that could not be definitively determined were labelled as 'other', resulting in an under-representation of the specific localization of Munc18a and Munc18c. The quantitative data for the distribution of Munc18a, Munc18c and the previously published syntaxin 2 [35,44] (Table 1) indicate that the largest portion of these proteins associates with granule membranes and the remainder are divided evenly between OCS and plasma membranes. Syntaxin 4 [35,44] was found most abundantly associated with OCS and granule membranes, and less with the plasma membrane. These results demonstrate that Munc18 proteins appear to co-localize with the two syntaxin t-SNAREs that are required for granule-to-OCS membrane fusion [33,35,36,45].

Association of Munc18 proteins with SNARE proteins

From the localization results, it is clear that Munc18s co-localize with syntaxins and potentially with other SNARE proteins. Next, experiments were performed to determine whether any

Table 1   Immunoelectron microscopy of Munc18s and syntaxins in platelets

Antibody	Total grains	Plasma membrane (%)*	OCS (%)*	Granule (%)*	Others (%)*†
Munc18a	1464	12	15	40	33
Munc18c	1683	9	13	42	36
Syntaxin 2‡	1283	14	16	28	42
Syntaxin 4‡	1163	6	33	25	35

 * Percentage of the grains associated with the indicated structure.
 † Structures that were not readily identifiable.
 ‡ Previously published results [12] shown only for comparison.

Munc18 or syntaxin protein formed complexes in platelets. Resting platelet extracts were subjected to immunoprecipitation with anti-Munc18a and anti-Munc18c antibodies and both bound and unbound fractions were analysed by Western blotting for Munc18 and other SNARE proteins. Munc18a was detected in both bound and unbound fractions, suggesting that 100 μg of the commercial antibody was not completely sufficient to recover all the Munc18a proteins from the extracts (Figure 5A). However, of the syntaxins analysed in this precipitate, only syntaxin 2 was found associated with Munc18a in significant quantities. The association of Munc18a with syntaxin 2, but not syntaxin 4, is consistent with previous *in vitro* experiments using recombinant proteins [18,26]. Using anti-Munc18c antibodies, syntaxins 2 and 4 along with other SNARE proteins (SNAP-23 and VAMP) were found to associate with Munc18c (Figure 5B). This association of both syntaxins 2 and 4 with Munc18c is consistent with previous *in vitro* experiments [46]. The results presented in this paper
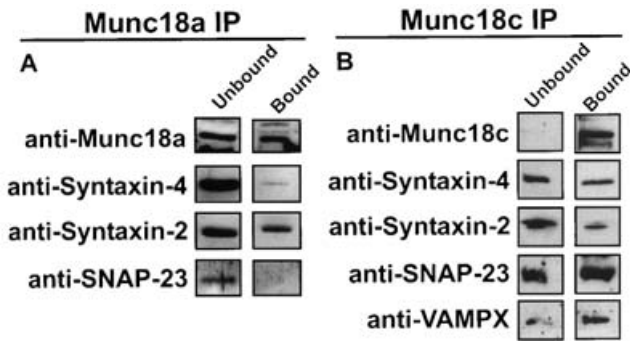
Figure 5   Munc18s interact with specific syntaxins

Platelets were resuspended in Triton X-100 solubilization buffer in the presence of protease inhibitors. Platelet extracts were incubated with 100 μg of anti-Munc18a mAb (**A**) or 235 μg of anti-Munc18c pAb (**B**) cross-linked to Protein G–Sepharose beads. The unbound and bound fractions were collected. Samples were probed by Western blotting using anti-Munc18a/b pAb, anti-Munc18c pAb, anti-syntaxin 2 pAb, anti-syntaxin 4 mAb, anti-SNAP-23 pAb and anti-VAMPX mAb.

show that Munc18–syntaxin interactions occur in platelets and the *in vitro* defined specificity [17–21,24] is maintained in these cells. What these results also demonstrate is that Munc18a and Munc18c use different modes of interaction to bind to their respective syntaxins. Munc18a binds exclusive of other SNAREs (class 1), whereas Munc18c binds in complex with other SNAREs (class 2). Finding other SNARE proteins associated with Munc18c, but not Munc18a, is not entirely surprising, since recent results suggest that multiple classes of SM protein–syntaxin interactions are possible (reviewed in [32]).

Munc18 proteins function in platelet exocytosis and clot formation

Previous experiments revealed that three of the seven SM proteins in humans are present in platelets and they localize to mem-

branes in both resting and stimulated platelets. Munc18s also bound specifically to two of the known platelet syntaxins. Next, we determined whether Munc18s play a role in platelet function. To address this question, we made use of two inhibitory peptides that were based on an evolutionarily conserved region of Munc18, termed 18a/pep3 and 18c/pep3 (Figure 6A). Based on the crystal structure of Munc18a in complex with syntaxin [28], 18a/pep3 mimics a looped region that is surface-exposed and opposite to the syntaxin-binding domain. These peptides disrupt the association of s-Sec1 with s-syntaxin (the squid homologue of syntaxin 1) [16] and that of syntaxin 4 with Munc18c [24]; however, it is not clear whether it disrupts extant Munc18–syntaxin complexes. The peptides also inhibit neurotransmission [16] and GLUT4 mobilization [24].

SLO-permeabilized platelets were incubated with increasing concentrations (0.2–0.8 mM) of 18a/pep3 and 18c/pep3 and assayed for release from all three granule stores of platelets after stimulation with 10 μM CaCl$_2$. Secretion from all three granule stores was inhibited by the addition of 18c/pep3 (Figures 6B–6D). To determine if the same region of Munc18a (amino acids 464–487; Figure 6A) would have similar effects, permeabilized platelets were treated with increasing amounts of 18a/pep3. Inhibition of dense-core and lysosomal granules by 18a/pep3 was significantly less when compared with inhibition by 18c/pep3. Release from α granules was inhibited to a similar extent. This may suggest that Munc18a plays a critical role only in release from α granules. However, due to the sequence similarity between these two peptides, it is difficult to discern whether the differential inhibition was strictly due to differences between the Munc18 isoforms required for the secretion events or just cross-reactivity between the two peptides. The inhibitory effect was sequence-specific, since a scrambled peptide had no significant effect on release (Figure 6A).

Platelet aggregation in response to stimulus requires the release of contents from the three granule stores and ultimately results in clot formation. From the previous experiments, it was clear that both 18a/pep3 and 18c/pep3 inhibited release from the three
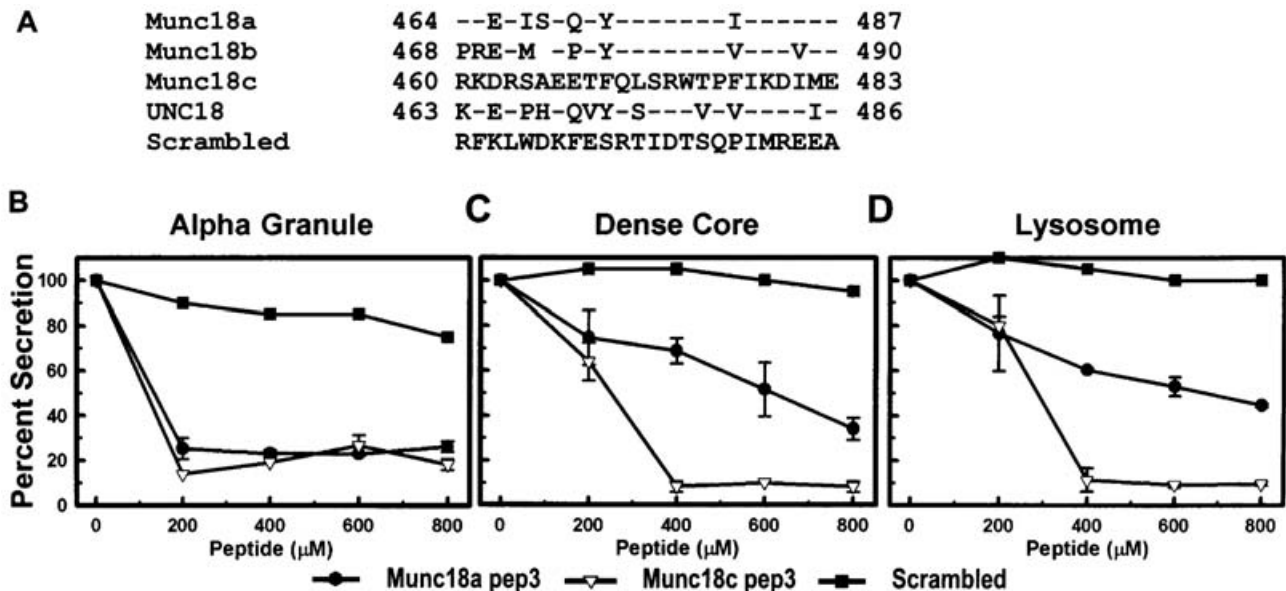

Figure 6   Munc18-derived peptides inhibit platelet granule exocytosis

Sequence comparison of various Munc18 isoforms within the region of 18a/pep3 [16] and 18c/pep3 (**A**). The permeabilized platelet exocytosis assay was performed in the presence of increasing amounts of 18c/pep3 ($\triangledown$), 18a/pep3 ($\bullet$) or a scrambled peptide with the same composition as 18c/pep3 ($\blacksquare$) (**B**–**D**). After stimulation with 10 μM CaCl$_2$, platelet supernatants were assayed for the presence of PF4 (**B**), [^{3}H]5-HT (**C**) or hexosaminidase (**D**).
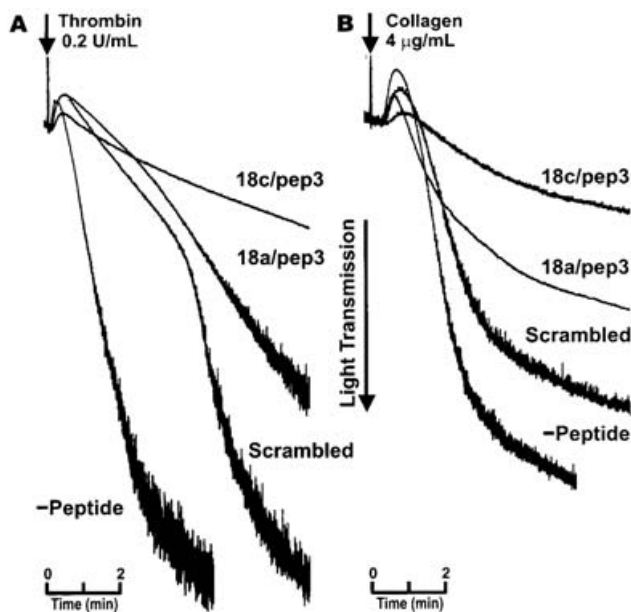
Figure 7 Munc18-derived peptides inhibit platelet aggregation

Washed platelets were incubated at 37 °C for 5 min and then treated with saponin, 18c/pep3 and 18a/pep3 for 2 min. After incubation, platelets were treated with thrombin (**A**) or collagen (**B**) and light transmittance aggregation was measured. Scrambled peptide and samples without peptide treatment (−Peptide) were run as specificity controls.

granule stores. To test the effects of these peptides on platelet aggregation, platelets were washed and then permeabilized with saponin [40]. Saponin-treated platelets were incubated with both 18a/pep3 and 18c/pep3, scrambled peptides and compared with a no peptide control. The permeabilized platelets were stimulated with both thrombin and collagen with aggregation measured by light transmission (Figure 7). The most drastic effects occurred after the addition of 18c/pep3. 18a/pep3 and 18c/pep3 significantly inhibited aggregation compared with the no-peptide samples in both the thrombin- and collagen-stimulated platelets. Inhibitory effects seen with 18c/pep3 were dose-dependent and required the addition of saponin (results not shown). Platelets treated with 18a/pep3 showed less of an effect on aggregation when compared with those treated with 18c/pep3. This is consistent with the differences in the inhibition of secretion discussed above. Treatment with scrambled peptide did not alter aggregation to the levels seen with either 18a/pep3 or 18c/pep3. Samples treated with buffer alone aggregated to expected levels, given the stimulus.

Taken together, these results show that the Munc18 proteins clearly play a role in platelet secretion and confirm the importance of the secretory process in platelet aggregation. However, because these two peptides are derived from highly conserved regions, it is difficult to use the results presented to assign specific Munc18 isoforms to specific secretion events. Unfortunately, antibodies to the specific Munc18 proteins have not proved to be effective inhibitors of secretion; therefore, specific assignment of isoform function will require further experimentation.

Munc18 proteins are phosphorylated after stimulation with thrombin

Given the importance of Munc18–syntaxin interactions in platelet function, we next examined mechanisms that could alter these two proteins during platelet stimulation. Munc18a is phosphoryl-

ated on Ser[313] by PKC and this phosphorylation affects the rate of neuroendocrine cell exocytosis [22]. Munc18c was also shown to be phosphorylated in a PKC-dependent manner after thrombin stimulation of platelets [25]. To show first that Munc18s were phosphorylated after stimulation, pAbs for Munc18a/b and Munc18c were used to immunoprecipitate complexes from [32]P-labelled extracts from resting or thrombin-stimulated platelets. As shown in Figure 8(A), Munc18a/b and Munc18c are phosphorylated after stimulation with thrombin. Munc18c showed significant phosphorylation in resting cells, which increased 2.5-fold after stimulation with thrombin. Munc18a/b had detectable levels of phosphorylation, which also increased after stimulation with thrombin. Levels of phosphorylation of Munc18a/b detected were significantly lower than that of Munc18c.

To address whether syntaxins are phosphorylated and if Munc18 is phosphorylated in complex with syntaxins, co-immunoprecipitation experiments were performed using antibodies to syntaxins 2 and 4 (Figure 8B). Under resting conditions, phosphorylated Munc18 (phospho-Munc18c) did appear to associate with syntaxin 4. Lower levels of phospho-Munc18 were seen associated with syntaxin 4 in thrombin-stimulated cells. Detectable levels of phospho-Munc18 were not co-precipitated with syntaxin 2 (Figure 8A). Western blotting these precipitates with antibodies for both syntaxins 2 and 4 showed significant levels of each syntaxin recovered from these samples (results not shown); however, neither syntaxin 2 nor syntaxin 4 appears to be significantly phosphorylated in resting platelets or after thrombin stimulation (Figure 8B). These results suggest that phosphorylation of Munc18s has an effect on Munc18–syntaxin interactions, but they do not support a role of Munc18s in phosphorylation of syntaxins.

Association of Munc18c with syntaxins after stimulation

To determine more directly whether the levels of Munc18–syntaxin complexes are altered after stimulation, syntaxins were immunoprecipitated from resting or stimulated platelet extracts and the precipitates were probed with Munc18c antibodies. Munc18c was chosen as the focus for these experiments because of its abundance and ease of detection by Western blotting (see Figure 9A, upper panel). As in previous experiments (Figure 5), Munc18c was associated with syntaxins 2 and 4 under resting conditions (Figure 9A). On stimulation with thrombin, Munc18c still co-precipitated with syntaxins 2 and 4 although in reduced amounts when compared with resting conditions. Levels of precipitated syntaxins 2 and 4 were determined by quantitative Western blotting and they remained constant in both samples (Figure 9A and results not shown). Quantification of the Munc18c co-precipitated (normalized to syntaxin recovered) showed that stimulation with thrombin led to approx. 40% reduction in Munc18c associated with syntaxin 2 and approx. 25% reduction in Munc18c associated with syntaxin 4 (Figure 9B). The fact that lower levels of Munc18c were detectable suggests that the Munc18c–syntaxin complex is still present, but is less stable when derived from the stimulated cell extracts. Based on the results shown in Figures 8 and 9, one possible explanation for the decrease in Munc18c–syntaxin association is that phosphorylation of Munc18c may destabilize the complex.

DISCUSSION

In the present study, we show that, of the seven SM proteins in vertebrates, at least three are present in human platelets and these
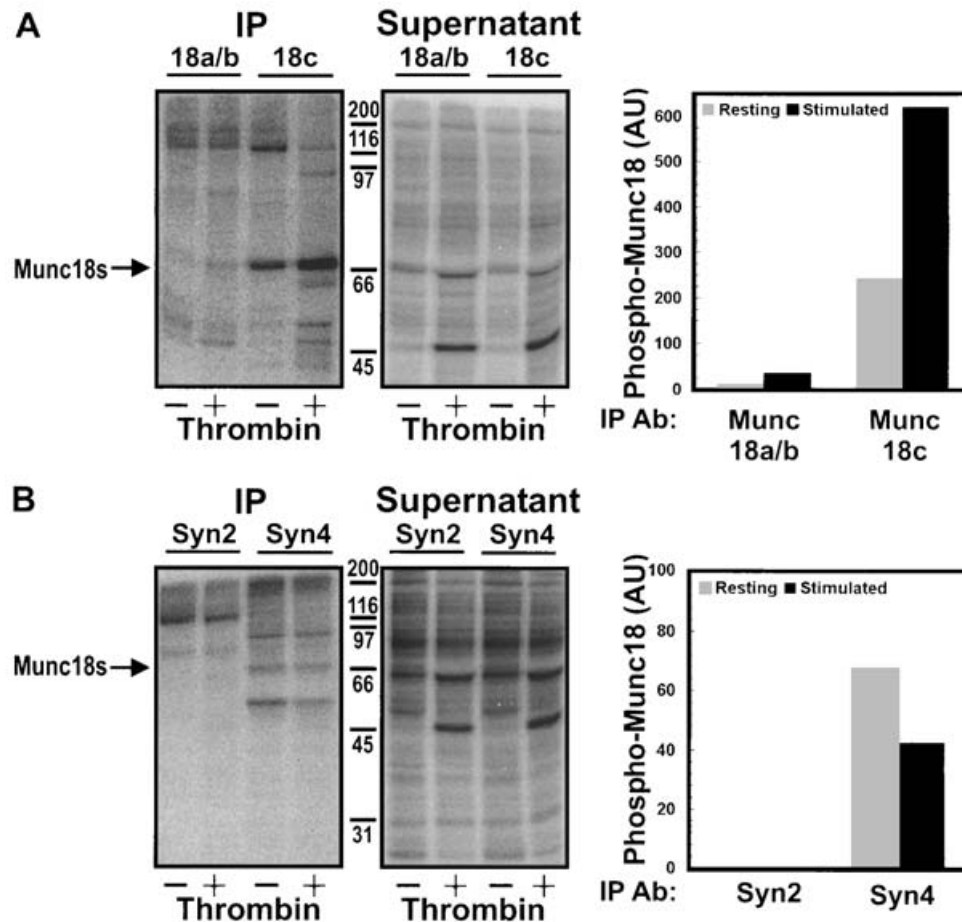
Figure 8   Changes in phosphorylation of Munc18 protein after stimulation

Platelets washed in Hepes/Tyrode's buffer were labelled by incubating with [^{32}P]P$_i$ (0.2 mCi/ml) for 2 h. Resting platelets and thrombin-treated (1 unit/ml) platelets were both solubilized in 0.8 % Triton X-100 solubilization buffer containing 10 mM sodium vandate. Proteins were immunoprecipitated from platelet extracts using anti-Munc18a/b pAb (18a/b), anti-Munc18c (18c) (**A**), anti-syntaxin 2 (Syn2) or anti-syntaxin 4 pAb (Syn4) (**B**). After SDS/PAGE fractionation, a PhosphorImager was used to visualize the proteins. Immunoprecipitated (IP) samples were compared with supernatants after antibody incubation (supernatant). Quantification of the radiolabel was accomplished by comparing the volume densities of the phospho-Munc18 in the Munc18 and syntaxin immunoprecipitates. The results are presented in arbitrary units (AU).

are from the Munc18 subfamily (Figures 1 and 2). Munc18c is associated exclusively with platelet membranes, whereas Munc 18a and Munc18b are present in both the cytosolic and membrane fractions. This distribution does not change when platelets are stimulated with thrombin (Figure 3). At the subcellular level, Munc18a and Munc18c localize to OCS and granule membranes in resting cells (Figure 4 and Table 1). This corresponds to the previously reported [35,45] localization of syntaxins 2 and 4, two t-SNAREs required for platelet exocytosis [33,35,38,45], and is consistent with the role of Munc18 as a syntaxin-binding protein. Three different Munc18–syntaxin complexes were isolated from platelet extracts: Munc18c–syntaxin 4, Munc18c–syntaxin 2 and Munc18a–syntaxin 2 (Figure 5). The Munc18a–syntaxin 2 complex is a class 1 SM protein–syntaxin complex that excludes other SNAREs. The Munc18c-containing complexes could be either class 1 or 2 complexes. Since SNAREs excluding syntaxins 2 and 4 are present in the Munc18c immunoprecipitates, at least one of the complexes (Munc18c–syntaxin 2 or Munc18c–syntaxin 4) must be a class 2 complex. This distinction is significant since it implies divergent control of the syntaxins' conformational cycle (closed to open), which may ultimately affect the kinetics of each of the three platelet exocytosis events.

The results shown in Figures 6 and 7 clearly demonstrate that Munc18 proteins are required for platelet exocytosis and therefore for platelet function. Given that Munc18c is associated with both functionally relevant t-SNAREs (syntaxins 2 and 4 [33,35,38,45]), it could be central to all three release events in platelets. This is consistent with the potency of the inhibitory effects of 18c/pep3 peptide. However, given the high sequence conservation between the two peptides (see Figure 6A), it is not clear whether the effectiveness of the 18c/pep3 is truly isoform-specific. Additionally, the exact mechanism of the effect of the peptides on Munc18–syntaxin complexes is not defined. It has been shown previously that addition of peptide 3 disrupts the association of syntaxin and Munc18 *in vitro* [16] and in a plasma-membrane competition assay [24], suggesting that the peptides compete for syntaxin binding. However, this is confusing based on the recent crystal structures of three different SM proteins [28,47,48]. From the crystallography results, Munc18 peptide 3 is derived from a region opposite to the proposed syntaxin-binding site. It is probable that this region of SM proteins does not directly contact the syntaxin, but represents an effector-binding site whose conformation is critical to syntaxin binding. Consistent with this theory is the close proximity of the sly1-20
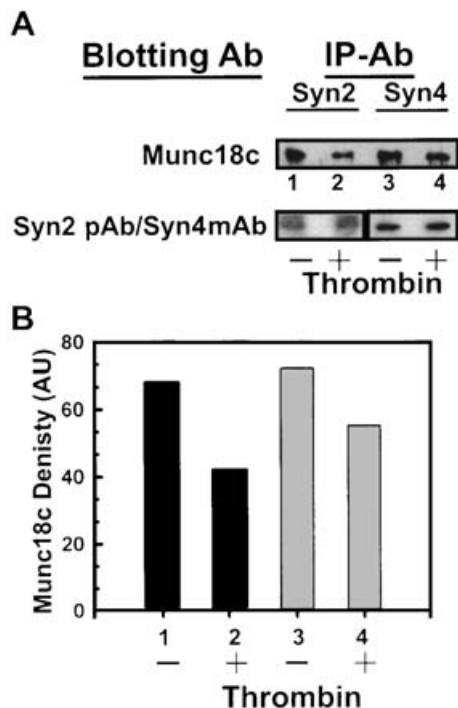
Figure 9   Reduced levels of Munc18c associated with syntaxins after thrombin stimulation

Platelet samples were prepared as described in Figure 8. (**A**) Immunoprecipitation (IP) reactions with 30 μg of anti-syntaxin 2 pAb (Syn2) and 30 μg of anti-syntaxin 4 pAb (Syn4) were conducted and IP fractions were probed by Western-blot analysis using anti-Munc18a/b and anti-Munc18c pAbs. Anti-syntaxin 2 and 4 mAbs were used in Western-blot experiments to show successful precipitation of the protein and comparison of protein amounts for each sample. (**B**) Volume densities of the Munc18c Western blots were calculated (NIH Image; National Institutes of Health, U.S.A.) and normalized to the corresponding level of syntaxin from each sample. Numbers under each bar correspond to the indicated lanes in (**A**). Results are presented in arbitrary units (AU).

mutation (Glu^{532} → Lys). This mutation, in the yeast SM protein, alleviates the requirement of Ypt1 (a rab GTP-binding protein) in endoplasmic reticulum to Golgi transport [48,49], suggesting that the sly1-20 mutant does not require activation of upstream regulators. Although this point will require further investigation, it is clear that Munc18 peptide 3 serves as a useful disrupter of SM protein function. In the present study, we demonstrate the functional significance of Munc18s in platelet exocytosis and aggregation.

On stimulation, the level of Munc18 bound to syntaxin decreases by approx. 25–40 % (Figure 9). This may represent a release of syntaxins to allow for interactions with other SNAREs and thus form the fusogenic SNARE complex [11]. Although this is the probable pathway for class 1 SM protein–syntaxin interactions [32], it may not be essential for the class 2 Munc18c–syntaxin interactions, which are not exclusive of other SNAREs. We propose that the reduction in the levels of Munc18c bound to syntaxin (Figure 9) could be the result of a conformational change in the complex, making it less stable to co-immunoprecipitation. Such a change may reflect a key step in the cascade of protein–protein interactions that ultimately lead to membrane fusion.

One potential modification that could account for the decrease in Munc18–syntaxin interactions is Munc18 phosphorylation. Reed et al. [25] originally showed that Munc18c was phosphorylated by PKC in activated platelets. The results presented in this paper confirm this observation and further demonstrate

that Munc18a/b is also phosphorylated in activated cells, but to a much lower level than Munc18c. Reed et al. [25] proposed that phosphorylation leads to release of syntaxin 4 from Munc18c to promote syntaxin interactions with other SNAREs. The results presented in this paper are partly consistent with this proposal, although the disassembly of the complex is not complete (see Figures 8 and 9). Given that the two Munc18c–syntaxin complexes could be class 2 interactions, the role of Munc18c phosphorylation may not be as clear as proposed originally. Consistent with this idea are the studies of Barclay et al. [22] showing that phosphorylation of Munc18a affects secretion dynamics and release kinetics but not the number of events. This suggests that Munc18a phosphorylation controls the membrane fusion process, but may not play a role in SNARE complex assembly. Barclay et al. [22] demonstrated further that Munc18a phosphorylation decreased its affinity for syntaxin 1a by approx. 100-fold, which agrees with an *in vitro* binding assay reported previously [23]. Despite the uncertainty over the mechanistic role of Munc18 phosphorylation, it is clear that syntaxin 2 or syntaxin 4 phosphorylation is not critical for controlling Munc18–syntaxin interactions, since neither was detected in stimulated platelets (Figure 8).

Based on previously published results [9,33,35,38,44,45], it is now established that platelet exocytosis is mediated by SNARE proteins. The present challenge is to identify the potential SNARE regulators present in platelets and to assign functions to specific secretion events. In the present study, we have partially characterized another step in the cascade of protein–protein interactions required for platelet exocytosis by showing that Munc 18 proteins are required for release. We have outlined the possible interactions between the platelet Munc18s and the functionally relevant syntaxins (syntaxins 2 and 4). We have provided evidence to suggest that regulation of Munc18–syntaxin interactions may not strictly follow the paradigm of class 1 SM protein–syntaxin interactions exemplified by Munc18a and syntaxin 1. It is clear, however, that more work will be required to determine how these interactions determine the specificity of the platelet release reaction.

During the preparation of this manuscript, an electronic publication by Houng et al. [50] appeared describing the roles of SM protein interactions in platelet exocytosis.

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