Munc13-4 Is a GTP-Rab27-binding Protein Regulating Dense Core Granule Secretion in Platelets*

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Platelets store self-agonists such as ADP and serotonin in dense core granules. Although exocytosis of these granules is crucial for hemostasis and thrombosis, the underlying mechanism is not fully understood. Here, we show that incubation of permeabilized platelets with unprenylated active mutant Rab27A-Q78L, wild type Rab27A, and Rab27B inhibited the secretion, whereas inactive mutant Rab27A-T23N and other GTPases had no effects. Furthermore, we affinity-purified a GTP-Rab27A-binding protein in platelets and identified it as Munc13-4, a homologue of Munc13-1 known as a priming factor for neurotransmitter release. Recombinant Munc13-4 directly bound to GTP-Rab27A and -Rab27B in vitro, but not other GTPases, and enhanced secretion in an in vitro assay. The inhibition of secretion by unprenylated Rab27A was rescued by the addition of Munc13-4, suggesting that Munc13-4 mediates the function of GTP-Rab27. Thus, Rab27 regulates the dense core granule secretion in platelets by employing its binding protein, Munc13-4.

Upon stimulation, platelets secrete self-agonists, such as ADP and serotonin, which are stored in dense core granules (1). These secreted agonists contribute to the explosive activation of platelets. Although the molecular mechanisms underlying this regulated exocytosis remain largely unclear. Rab GTPases are localized to specific organelles and regulate several steps of vesicle transport (2, 3) and to date more than 50 members have been identified in mammals. Rab GTPases have been implicated in the regulation of the fusion of endocytic vesicles with the plasma membrane (4, 5). Rab27A and Rab27B are members of the Rab27 subfamily and are expressed in a variety of cell types. These two GTPases have been implicated in the endocytic and exocytic processes of melanosomes and melanolysosomes (6, 7).

Recently, the Ashen phenotype in mice has been demonstrated to be caused by a mutation in Rab27A (4). Ashen mice exhibit impaired coat color because of defective melanosome distribution, where Rab27A links a motor protein myosin Va to melanosomes, through its effector, melanoctin/Slc2-a (5, 6). In addition, in cytotoxic T lymphocytes and natural killer cells in ashen mice, lytic granule secretion is impaired presumably at the docking/fusion step (7, 8). Interestingly, it has been demonstrated that the bleeding time of ashen mice is prolonged because of platelet dysfunction caused by a lack of intact platelet dense core granules (4, 9). However, it is important to note that platelets also contain Rab27B, a homologue of Rab27A with 71% identity in the amino acid level, which may compensate for the defect of Rab27A in platelets in ashen mice (4). These studies emphasize the requirement of further investigation into the mechanisms by which Rab27 may control the biogenesis and exocytosis of dense core granules in platelets.

The final step of vesicle transport is docking and fusion with the target membrane, which is mediated by trans-SNARE complex between the vesicle SNARE and the target membrane SNARE (15). Accumulating evidence has revealed that Rab GTPases function upstream of SNARE pairing (2, 3). In addition to Rab GTPases, SNARE complex formation is directly regulated by several factors such as Munc18 and Munc13-1. In neuronal cells, Munc18 is thought to bind to the closed conformation of syntaxin1, a target membrane SNARE, to inhibit the trans-SNARE complex formation (16, 17). In contrast, Munc13-1 acts as a priming factor in neuronal secretion (18). Munc13-1 is suggested to promote formation of an open conformation of syntaxin1 and induce the assembly of trans-SNARE complexes. This is supported by the fact that transmission defects of unc-13 mutants in Caenorhabditis elegans could be rescued by expression of open form of syntaxin1 (19). Munc13-1 and its homologues, Munc13-2 and Munc13-3, are expressed exclusively in brain, except for ubMunc13-2, a splice variant of Munc13-2 (20, 21). In brain, each Munc13 isofrom expression pattern is spatially restricted, although there is some overlap between isoforms. Munc13-1-deficient mice exhibit partial defects in glutamatergic neuron neurotransmitter release (22), whereas Munc13-1/Munc13-2 double knockout mice have complete loss of synaptic transmission in not only glutamatergic neurons but also GABAergic neurons (23). These studies in mice have revealed the essential and general roles of Munc13s in neurotransmitter release. Recently, the fourth Munc13 ho-

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1 The abbreviations used are: SNARE, soluble N-ethylmaleimidesensitive factor attachment protein receptor; PKC, protein kinase C; SLO, streptolysin-O; GST, glutathione S-transferase; GTPyS, guanosine 5′-O-thio)triphosphate; TOF-MS, time-of-flight mass spectrometry.
Here, we demonstrate that Rab27 regulates the Ca\(^{2+}\)-induced dense core granule secretion in platelets by showing that the addition of unprenylated Rab27 in an *in vitro* assay with permeabilized platelets inhibited the secretion. Furthermore, we identified a novel GTP-Rab27-binding protein in platelets as Munc13-4 and demonstrate that Munc13-4 mediates the function of GTP-Rab27 to promote the secretion.

**EXPERIMENTAL PROCEDURES**

**Antibodies, Constructs, Materials, and Others**—Anti-Na,K-ATPase rabbit polyclonal antibody (25) was a kind gift from Dr. K. Omori (Kansai Medical University, Kadoma, Japan). Anti-His\(_{6}\) and anti-Fc\(_{\gamma}\) monoclonal antibodies were purchased from Qiagen and Santa Cruz Biotechnology, respectively. Horseradish peroxidase-labeled antimouse and anti-rabbit IgG polyclonal antibodies were from Amersham Biosciences and were used as secondary antibodies for Western blot analysis visualized by enhanced chemiluminescence method (Amersham Biosciences). Unless otherwise specified, all of the other chemicals were purchased from Sigma, except for SLO, which was from Dr. S. Bhakdi (Mainz University, Mainz, Germany) (26).

The protein concentrations were determined by the Bradford method (Bio-Rad) or from the intensities of the bands in Coomassie Blue-stained SDS-PAGE gels using bovine serum albumin as a standard. cDNA encoding Rab27A was kindly provided by Dr. Y. Nozawa (Gifu International Institute of Biotechnology, Gifu, Japan) (27), and mutants Rab27A-Q78L and Rab27A-T23N were generated by PCR mutagenesis. cDNAs encoding Rab1B (28), Rab3B (28), and Rab3B(29) were from Dr. Y. Takai (Osaka University, Osaka, Japan), and Rab4B (30) and Rab5A (30) were from Dr. M. Zerial (Max Plank Institute, Dresden, Germany).

Human Rab27B cDNA was isolated from the Marathon-Ready human bone marrow cDNA (Clontech) by PCR. All of these cDNAs were subcloned into the prokaryotic expression vector pDEST17 (Invitrogen) for His\(_{6}\)-tagged recombinant protein production. GST fusion proteins of Rab3B, Rab4B, Rab5A, Rab27A, and Rab27B were produced by subcloning the cDNAs into pGEX-2T (Amersham Biosciences). These His\(_{6}\)-tagged and GST fusion proteins were produced in *Escherichia coli* strain BL21 and purified on nickel-nitritotriacetic acid-agarose (Qiagen) and glutathione-Sepharose (Amersham Biosciences), respectively. For full-length Munc13-4 recombinant protein production, the cDNA of Munc13-4 was subcloned into pDEST11 (Invitrogen), and a baculovirus strain BL21 and purified on nickel-nitrilotriacetic acid-agarose (Qiagen) or from the intensities of the bands in Coomassie Blue-stained SDS-PAGE gels using bovine serum albumin as a standard.

All of the purified recombinant proteins were extensively dialyzed against buffer A (50 mM Hepes/KOH, pH 7.2, 78 mM KCl, 4 mM MgCl\(_{2}\), 0.2 mM CaCl\(_{2}\), 2 mM EGTA, 1 mM dithiothreitol) and stored at −80 °C until use. All of the sequences of the PCR products were confirmed by sequencing using a 3100 Genetic Analyzer (Applied Biosystems).

The Assay for Secretion of Dense Core Granules—The standard assay method for the Ca\(^{2+}\)-induced dense core granule secretion was essentially described previously (31) except that human platelet cytosol was used (32) instead of rat brain cytosol. Unless otherwise specified, the standard assay was following: freshly obtained washed platelets (1 × 10\(^{9}\) platelets/assay, counted with the Coulter Counter) were incubated with [\(^{3}H\)]serotonin (32) instead of rat brain cytosol. Unless otherwise specified, all of the other chemicals were purchased from Sigma, except for SLO, which was from Dr. S. Bhakdi (Mainz University, Mainz, Germany) (26). The protein concentrations were determined by the Bradford method (Bio-Rad) or from the intensities of the bands in Coomassie Blue-stained SDS-PAGE gels using bovine serum albumin as a standard. cDNA encoding Rab27A was kindly provided by Dr. Y. Nozawa (Gifu International Institute of Biotechnology, Gifu, Japan) (27), and mutants Rab27A-Q78L and Rab27A-T23N were generated by PCR mutagenesis. cDNAs encoding Rab1B (28), Rab3B (28), and Rab3B(29) were from Dr. Y. Takai (Osaka University, Osaka, Japan), and Rab4B (30) and Rab5A (30) were from Dr. M. Zerial (Max Plank Institute, Dresden, Germany). Human Rab27B cDNA was isolated from the Marathon-Ready human bone marrow cDNA (Clontech) by PCR. All of these cDNAs were subcloned into the prokaryotic expression vector pDEST17 (Invitrogen) for His\(_{6}\)-tagged recombinant protein production. GST fusion proteins of Rab3B, Rab4B, Rab5A, Rab27A, and Rab27B were produced by subcloning the cDNAs into pGEX-2T (Amersham Biosciences). These His\(_{6}\)-tagged and GST fusion proteins were produced in *Escherichia coli* strain BL21 and purified on nickel-nitritotriacetic acid-agarose (Qiagen) and glutathione-Sepharose (Amersham Biosciences), respectively. For full-length Munc13-4 recombinant protein production, the cDNA of Munc13-4 was subcloned into pDEST11 (Invitrogen), and a baculovirus strain BL21 and purified on nickel-nitrilotriacetic acid-agarose (Qiagen) or from the intensities of the bands in Coomassie Blue-stained SDS-PAGE gels using bovine serum albumin as a standard.

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prepared by incubation of glutathione-Sepharose beads with GST or pretreated GST-Rab27A at 4°C for 1 h, followed by washing the beads with Buffer A three times. The coated beads were incubated with the platelet cytosol (30 mg of proteins) at 4°C for 1 h, followed by washing the beads with Buffer A three times, and the bead-associated proteins were extracted by the SDS sample buffer. The samples were analyzed in a Coomassie Blue-stained SDS-PAGE gel as described under “Experimental Procedures.” A protein band at 120 kDa (asterisk) was specifically detected in lane 4. B, the 120-kDa protein was analyzed by TOF-MS and identified as human Munc13-4 as described under “Experimental Procedures.” Underlining indicates the peptides whose masses were matched with peptide masses detected by the TOF-MS analysis. C, the domain structures of Munc13-1–3 and Munc13-4 are shown.

Cloning of Munc13-4—The TOF-MS analysis of the GTP-Rab27A-binding protein at 120 kDa was performed by Kyoto Science Co. (Kyoto, Japan). The report first showed that a candidate protein could be FLJ00067, which contained an incomplete sequence with possible introns and C-terminal deletion. We reconstituted the full-length sequence with several expressed sequence tags overlapping with FLJ00067 and identified it as a human homologue of rat Munc13-4 (24). Using the sequences, we designed primers and amplified the full-length cDNA by PCR using the Marathon-Ready human bone marrow cDNA (Clontech). The PCR product was cloned into pENTR (Invitrogen) and verified by sequencing using a 3100 Genetic Analyzer.

Anti-Munc13-4 and -Rab27 Antibodies—cDNA encoding the N-terminal region of Munc13-4 (residues 1–262) was obtained by PCR and subcloned into pDEST17 (Invitrogen). The His6-Munc13-4 (1–262) protein was produced and purified as described above. Anti-Munc13-4- and anti-Rab27A rabbit polyclonal antibodies were generated using His6-Munc13-4 (1–262) and His6-Rab27A as antigens, respectively. The anti-Rab27A antibody recognized both Rab27A and Rab27B. For transient expression of Munc13-4, HeLa cells were transfected with pcDNA

FIG. 2. Identification of a GTP-Rab27A-binding protein in platelet cytosol as Munc13-4. A, GST-loaded (lane 3) and GTP-S-bound (lane 4) and GDP-bound (lane 5) GST-Rab27A-loaded glutathione beads were incubated with platelet cytosol at 4°C for 1 h. GST-loaded (lane 1) and GST-Rab27A-loaded (lane 2) beads were also treated in the same way without the cytosol. After washing the beads, the bead-associated proteins were analyzed in a Coomassie Blue-stained SDS-PAGE gel as described under “Experimental Procedures.” A protein band at 120 kDa (asterisk) was specifically detected in lane 4. B, the 120-kDa protein was analyzed by TOF-MS and identified as human Munc13-4 as described under “Experimental Procedures.” Underlining indicates the peptides whose masses were matched with peptide masses detected by the TOF-MS analysis. C, the domain structures of Munc13-1–3 and Munc13-4 are shown.

A.  

| Mr (kD) | 1 | 2 | 3 | 4 | 5 |
|--------|---|---|---|---|---|
|        | 175 | 47.5 | 32.5 | 16.5 | *p120 |

GST-Rab27A

B.  

| C1 | C2 | MBD1 | MBD2 | C2 |
|----|----|------|------|----|
| C1 | C1 | MBD1 | MBD2 | C2 |
| C1 | C1 | MBD1 | MBD2 | C2 |
| C1 | C1 | MBD1 | MBD2 | C2 |

Munc13-1
Munc13-2
Munc13-3
Munc13-4

C.  

| C1 | C2 | MBD1 | MBD2 | C2 |
|----|----|------|------|----|
| C1 | C1 | MBD1 | MBD2 | C2 |
| C1 | C1 | MBD1 | MBD2 | C2 |
| C1 | C1 | MBD1 | MBD2 | C2 |
| C1 | C1 | MBD1 | MBD2 | C2 |

Munc13-1
Munc13-2
Munc13-3
Munc13-4

Anti-Munc13-4 and -Rab27 Antibodies—cDNA encoding the N-terminal region of Munc13-4 (residues 1–262) was obtained by PCR and subcloned into pDEST17 (Invitrogen). The His6-Munc13-4 (1–262) protein was produced and purified as described above. Anti-Munc13-4- and anti-Rab27A rabbit polyclonal antibodies were generated using His6-M unc13-4 (1–262) and His6-Rab27A as antigens, respectively. The anti-Rab27A antibody recognized both Rab27A and Rab27B. For transient expression of Munc13-4, HeLa cells were transfected with pcDNA
(Invitrogen) containing full-length Munc13-4 using LipofectAMINE (Invitrogen).

Assay Analyzing Direct Interaction of Munc13-4 with Rab27—Binding of Munc13-4 with small GTPases was carried out by affinity chromatography. Glutathione-Sepharose beads coated with GTPγS- or GDP-bound Rab GTPases (each 1 μg) were prepared by incubation for 1 h at 4 °C in Buffer A. Then the prepared beads were incubated with purified 0.5 μg of His₅-Munc13-4 for 1 h at 4 °C in Buffer A and washed three times with Buffer A at 4 °C. Bead-associated His₅-Munc13-4 was analyzed by immuno-blotting with anti-Munc13-4 antibody.

Density Gradient Separation of Platelet Organelles—We first loaded [³H]serotonin into dense core granules of platelets from 50 ml of freshly obtained blood and permeabilized the platelets with SLO. Then, after centrifugation to remove most of cytosol and resuspension in 1 ml of Buffer A containing the ATP regeneration system, the platelets were disrupted by sonication and centrifuged at 600 × g for 5 min to remove unbroken platelets. The supernatant was layered on the top of metrizamide stepwise gradient (each 1.0-ml layer at 1.30 g/ml) in density decreasing by each 0.03 g/ml from the bottom) in Buffer A containing the ATP regeneration system and centrifuged at 28,000 rpm with Beckman Rotor SW40 for 2 h at 4 °C as described (31). Aliquots of the fractions were analyzed by Western blot with indicated antibodies and counting radioactivity of [³H]serotonin. The separation of the membrane and cytosolic fraction was performed by centrifugation at 300,000 × g for 30 min at 4 °C after disrupting platelets by sonication or at 600 × g for 5 min after the SLO permeabilization of platelets.

RESULTS

Unprenylated Rab27 Inhibits the Ca²⁺-induced Dense Core Granule Secretion—In the present study, we used a previously established in vitro assay system using SLO-permeabilized platelets by monitoring secreted [³H]serotonin preloaded into dense core granules (31, 32, 35). Agonists promote granule secretions by increasing intracellular calcium ion concentrations in platelets (36). Upon permeabilization of platelets the intracellular and extracellular concentrations of calcium are equal; therefore we used calcium chloride as a stimulus. In the assay, the secretion of the granules was reconstituted by the addition of ATP and exogenous platelet cytosol, and the response observed was equivalent to intact platelets in the time course and the Ca²⁺ sensitivity (31, 32, 35).

Small GTPases produced in E. coli are not modified by the addition of prenyl groups at their C termini (37), which is essential for the correct localization and activity (2, 3, 38). Incubation of permeabilized platelets with Rab27A and Rab27B purified from E. coli inhibited the Ca²⁺-induced dense core granule secretion in a concentration-dependent manner (Fig. 1A). Incubation of permeabilized platelets with other small GTPases such as Rab3B, Rab4B, Rab5A, or Rap1B, a Ras family small GTPase whose GTP-bound form has been shown to be increased upon platelet activation (39), had no effect (Fig. 1B), indicating that the effect of Rab27 is specific. We prepared and purified mutant Rab27A-T23N, which preferentially binds GDP, and Rab27A-Q78L, which lacks GTPase activity (40). Incubation of permeabilized platelets with Rab27A-Q78L, wild type Rab27A, and Rab27B could be due to sequestration of putative Rab27 effector molecules from endogenous membrane-associated GTP-Rab27 by forming nonfunctional complexes with effector proteins. These data demonstrate that Rab27 is involved in the regulation of dense core granule secretion in platelets.

Identification of Munc13-4 as a GTP-Rab27-binding Protein in Platelet Cytosol—To elucidate the mechanism of action of Rab27, we attempted to identify GTP-Rab27-binding proteins that might mediate the function of Rab27 in the granule secretion, from platelet cytosol by affinity chromatography. As shown in Fig. 2A, we detected one major protein migrating at ~120 kDa on GTPγS-GST-Rab27A beads (lane 4) but not on GDP-GST-Rab27A- (lane 5) or GST beads (lane 3). TOF-MS analysis of the protein and a data base search revealed that the 120-kDa protein was the human homologue of rat Munc13-4 (24) because most of the peptide masses obtained by the TOF-MS analysis were detected all over the human Munc13-4 molecule (Fig. 2B). Human Munc13-4 consists of 1,090 amino acids, and the primary structure is 88% identical to that of rat Munc13-4. As is the case with Munc13-1–3, Munc13-4 contains two calcium-binding C2 domains and Munc13 homology do-

FIG. 3. Anti-Munc13-4 antibody recognizes a single band at 120 kDa in platelet lysate. A, Munc13-4- and mock-transfected HeLa cell lysates were immunoblotted with the anti-Munc13-4 antibody as described under “Experimental Procedures.” B, platelet lysates were immuno-blotted with preimmune serum and the anti-Munc13-4 antibody as described under “Experimental Procedures.” The data shown are the representative of three independent experiments with similar results.

FIG. 4. Direct interaction of recombinant Munc13-4 with GTPγS-Rab27 in vitro. A, recombinant His₅-Munc13-4 was produced and purified from the over-expressing Sf9 cells and analyzed by SDS-PAGE gel stained by Coomassie Blue as described under “Experimental Procedures.” B, glutathione beads coated with GTPγS- or GDP-bound various GST-Rab GTPases (each 1 μg) were incubated with His₅-Munc13-4 (0.5 μg) in vitro, and the bead-associated His₅-Munc13-4 was detected by Western blotting with anti-His₅ antibody as described under “Experimental Procedures.” Purified Munc13-4 used for the affinity analysis is also shown (10% of load). The data shown are representative of three independent experiments with similar results.
mains, whereas Munc13-4 lacks the long N-terminal region containing a phorbol ester-binding C1 domain present in Munc13-1–3 (21, 24) (Fig. 2C).

We generated an antibody against the N-terminal region of human Munc13-4 (residues 1–262). The antibody recognized a 120-kDa protein in HeLa cells transfected with Munc13-4 but not in mock transfected cells (Fig. 3A). The antibody recognized a single band at 120 kDa in platelet lysate (Fig. 3B) and also the 120-kDa protein eluted from the GTPγS-GST-Rab27A-loaded affinity chromatography column (data not shown), confirming that the 120-kDa protein that we identified is indeed Munc13-4.

To examine whether the interaction of Munc13-4 with Rab27 is direct, we produced and purified full-length His6-Munc13-4 using the baculovirus expression system in Sf9 insect cells (Fig. 4A). As shown in Fig. 4B, recombinant Munc13-4 could bind directly to GTPγS-Rab27A but only slightly to GDP-Rab27A in vitro. Furthermore, Munc13-4 demonstrated a weaker interaction with GTPγS-Rab27B, compared with GTPγS-Rab27A. Importantly, Munc13-4 did not bind to GTPγS-Rab3B, -Rab4B, or -Rab5A (Fig. 4B). Thus, the interaction of Munc13-4 with Rab27 is direct, GTP-dependent, and specific.

Localization of Munc13-4—Munc13-1 has been demon-

![Fig. 5. Localization of Munc13-4 in platelet organelles. A and B, after centrifugation of isolated platelets permeabilized with SLO for 30 min (A) or sonicated directly (B), comparable amounts of the supernatant (sup) and pellets (ppt) were analyzed by immunoblotting with anti-Munc13-4, anti-PKCα, and anti-Rab27 antibodies as described under “Experimental Procedures.” The data shown are the representative of three independent experiments with similar results. C, after separation of organelles of [3H]serotonin-loaded permeabilized platelets by density gradient, [3H]serotonin was measured by a liquid scintillation counter and Munc13-4, Rab27, and Na,K-ATPase were detected by immunoblotting in each fraction as described under “Experimental Procedures.” The data shown are the representative of three independent experiments with similar results.

![Diagram](http://www.jbc.org/Downloaded from)
stated to localize specifically to the presynaptic plasma membrane, although it does not contain a membrane-spanning region (20). Munc13-4 also lacks a transmembrane region. We examined the subcellular localization of Munc13-4 in platelets. As shown in Fig. 5A, Munc13-4 was equally distributed between the cytosolic and membrane fractions in SLO-permeabilized platelets, whereas Rab27 was exclusively membrane-associated. As expected, PKCα was found predominantly in the soluble supernatant fraction (Fig. 5A). When isolated platelets were directly sonicated and centrifuged at 300,000 × g, localization of Munc13-4, Rab27, and PKCα was similar to the results in the SLO-permeabilized platelets (Fig. 5B). Namely, ∼50% of Munc13-4, most of Rab27, and a small part of PKCα were recovered in the pellet after the high speed centrifugation (Fig. 5B), suggesting a strong affinity of Munc13-4 to the membrane.

Next, we biochemically examined the localization of Rab27 and membrane-associated Munc13-4 in platelet organelles. We first loaded [3H]serotonin into dense core granules of platelets and permeabilized the platelets with SLO. Then, after centrifugation to remove cytosol, the cytosol-depleted platelets were disrupted by sonication, and the low speed supernatant containing platelet organelles was separated by a density gradient method (31). As shown in Fig. 5C, [3H]serotonin was recovered in two peaks. The lighter peak is presumably due to [3H]serotonin leaking from the dense core granules. The heavier fractions of [3H]serotonin indicate the presence of dense core granules. The majority of Rab27 was recovered together with fractions of dense core granules containing [3H]serotonin, whereas some Rab27 was detected in the low density fractions where a plasma membrane marker Na,K-ATPase was recovered (Fig. 5C). Under these conditions, Munc13-4 was recovered in the lighter fractions together with Na,K-ATPase but not in the vesicle fractions (Fig. 5C), suggesting that Munc13-4 is on the plasma membrane but not on the dense core granules in platelets.

Involvement of Munc13-4 in the Regulation of Dense Core Granule Secretion—We finally examined whether Munc13-4 regulates dense core granule secretion in platelets using the semi-intact secretion assay. In basal conditions, SLO-permeabilized platelets retained a residual amount of membrane-associated Munc13-4 (Fig. 5A), and exogenously added platelet cytosol contained 80 nM Munc13-4. Under these conditions, the addition of purified Munc13-4 (Fig. 4A) enhanced the Ca\(^{2+}\)-induced dense core granule secretion in a concentration-dependent manner (Fig. 6A). The activity of Munc13-4 was abolished when Munc13-4 was denatured (Fig. 6A), suggesting that the activity was due to a nonspecific effect of the buffer. The dense core granule secretion was time-dependent, and the addition of Munc13-4 not only accelerated the kinetics but also increased the amounts of the secretion (Fig. 6B). Importantly, the inhibition of secretion by unprenylated Rab27A was rescued by the addition of recombinant Munc13-4 in a concentration-dependent manner (Fig. 6C).

**DISCUSSION**

Here we have demonstrated that Rab27 regulates the Ca\(^{2+}\)-induced dense core granule secretion in platelets by showing that the addition of unprenylated dominant active Rab27A, wild type Rab27A, and Rab27B but not other GTPases inhibited the secretion in permeabilized platelets. In addition, we identified Munc13-4 as a novel GTP-Rab27-binding protein. We further demonstrated that Munc13-4 mediates the function of GTP-Rab27 in the regulation of the secretion by showing that the addition of Munc13-4 enhanced the secretion and rescued the inhibition of secretion by unprenylated Rab27.

We have shown that the addition of unprenylated Rab27 purified from *E. coli* inhibited the Ca\(^{2+}\)-induced dense core granule secretion. In the secretion assay, added unprenylated Rab27 is theoretically unable to localize to the membrane and therefore would sequester Rab27 effector molecules by forming

![Fig. 6. Munc13-4 enhances the dense core granule secretion and rescues the inhibition by unprenylated Rab27A.](image)
nonfunctional complexes. Unprenylated Rab27A-T23N with preferential GDP binding would act as a dominant negative protein when expressed in cells (41). However, here we observed almost no effect of Rab27A-T23N on the dense core granule secretion in our semi-intact system (Fig. 1C). On the other hand, unprenylated GTPase-deficient mutant Rab27A-Q78L inhibited the secretion to a similar extent as wild type Rab27A and Rab27B. This could depend upon the ability of association with effector molecules of Rab27. The mechanism might be similar to that seen in the inhibition of the insulin signaling in adipocytes by overexpression of prenylation-deficient Rab4 (42). Thus, GTP-Rab27 is essential for dense core granule secretion in platelets once the granules are normally generated, although there is controversy concerning the role of Rab27 in dense core granule biogenesis (4, 13, 14).

To identify specific GTP-Rab27-binding protein(s) that may function in dense core granule secretion in platelets, we performed Rab27 affinity chromatography using platelet cytosol as the source for interacting proteins. We detected a GTP-bound Rab27A-binding protein at 120 kDa and identified it as the human homologue of rat Munc13-4 (24) by TOF-MS analysis and a database search. Recombinant Munc13-4 bound to purified Rab27A and Rab27B in their GTPyS-bound forms in vitro, indicating that their interaction is direct and nucleotide-dependent.

So far, eight proteins have been identified as GTP-Rab27A-binding molecules (43, 44). Five of them contain the C-terminal tandem calcium-binding C2 domains (45) and are designated as synaptotagmin-like proteins (Slp 1–6 tandem calcium-binding C2 domains (45) and are designated as binding molecules (43, 44). Five of them contain the C-terminal nucleotide-dependent. vitro, indicating that their interaction is direct and nucleotide-dependent.

We have demonstrated that the addition of recombinant Munc13-4 in the secretion assay accelerated the kinetics and increased secretion, indicating that Munc13-4 positively regulates dense core granule secretion in platelets. Importantly, the inhibitory effect of unprenylated Rab27A was rescued by the addition of recombinant Munc13-4. Exogenously added unprenylated Rab27A is presumed to form nonfunctional complexes with putative Rab27 effectors, thereby inhibiting secretion. Because exogenously added Munc13-4 overcomes inhibition by unprenylated Rab27, it is most likely that Munc13-4 mediates the function of GTP-Rab27 in the dense core granule secretion in platelets. Interestingly, the inhibitory effect of unprenylated Rab27A on the secretion was stronger than that of unprenylated Rab27B (Fig. 1A), which may reflect distinct roles of each isoform within the regulation of dense core granule secretion in platelets. Our data suggest that this difference is due to the stronger affinity of Rab27A to Munc13-4 than that of Rab27B (Fig. 4B).

Because SNAREs and Rab GTPases are key molecules in the regulation of vesicle transport, elucidation of the molecular mechanism of their cooperation could provide a clue for further understanding of vesicle docking/fusion. There have been several examples of Rab effector proteins interacting with the components of the SNARE machinery. For example, the Rab5 effector Rabenosyn-5 interacts directly with Vps45, a member of the Munc18/Sec1 family, in the regulation of endocytic membrane traffic (46). Similarly, the Ypt7 effector complex that controls vacuolar fusion in yeast interacts directly with Vps33, which is also a Munc18/Sec1 family protein (47). Thus, a group of Rab effectors appear to regulate SNARE pairing through Munc18/Sec1 family proteins. Another group of Rab effectors has been shown to bind SNAREs directly, as shown for the Rab5 effector EA1A1 interacting with syntaxin6 (48) and syntaxin13 (49).

It has also been demonstrated that Munc13-1 could be a direct regulator of syntaxin1 (50, 51) and that a GTP-Rab3A-binding protein, RIM1, which is a cytomatrix protein at the active zones of synapses, interacts with Munc13-1 (52). Thus, Rab3A might regulate Munc13-1 indirectly through interaction with RIM1. On the other hand, we here report the first direct link between Rab GTPases and a member of the Munc13 family, which may provide a novel mechanism for the control of SNARE activity by regulatory Rab GTPases. It will now be essential to explore the mechanisms of how Munc13-4 regulates the SNARE pairs that function in dense core granule secretion. In platelets, it has been shown that syntaxin2 mediates dense core granule secretion (53), suggesting that Munc13-4 might regulate syntaxin2.

We detected Munc13-4 in both cytosolic and membrane fractions, whereas Rab27 is exclusively in the membrane fraction (Fig. 5, A and B). Furthermore, by a density gradient separation, Munc13-4 was recovered in the plasma membrane fraction, whereas a major part of Rab27 was recovered in the granule fraction (Fig. 5C). Thus, the localization of Rab27 and Munc13-4 is not overlapping to a great extent. Munc13-4 on the plasma membrane may mark the target site for the dense core granule docking by interacting directly with activated Rab27 on the vesicle membrane. Because Rab3A is present on the synaptic vesicles (54) and its effector RIM1 and RIM1’s partner Munc13-1 are on the presynaptic membrane in neurons (20), there might be a common regulatory mechanism used by the Rab GTPase-Munc13 system.

Taken together, the work presented here demonstrates that Rab27 regulates dense core granule secretion in platelets by employing the GTP-Rab27-binding protein, Munc13-4. Our current findings could provide a novel mechanism by which a Rab GTPase controls the regulated exocytosis through direct interaction with a Munc13 family protein. To contribute to further understanding of the regulation of vesicle transport, it is essential to elucidate the molecular mechanism of how the Rab27-Munc13-4 system promotes regulated exocytosis.

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Note Added in Proof—Feldmann et al. (Feldmann, J., Callebaut, I., Raposo, G., Certain, S., Bauc, D., Dumont, C., Lambert, N., Ouache, M., Chardin, M., Chedeville, G., Tamary, H., Minard-Colin, V., Vilmer, E., Blache, S., Le Deist, F., Fisher, A., and de Saint Basile, G. (2003) Cell 115, 461–473) have published that mutation in Munc13-4 causes familial hemophagocytic lymphohistiocytosis (FHL3) where cytolytic granule fusion to the plasma membrane in cytotoxic T lymphocytes is impaired similar to mutation in Rab27A (11, 12), suggesting that the Rab27-Munc13-4 regulatory system may also function in regulated secretion in the cells.

REFERENCES
1. Brass, L. F. (2000) in Hematology: Basic Principles and Practice (Hoffman, R. B., Benz, E. J., Furie, B., Cohen, H., and Silberstein, L. E., ed) 3rd Ed., pp. 1753–1770, Churchill Livington, New York
2. Takai, Y., Sasaki, T., and Matsumura, T. (2001) Physiol. Rev. 81, 153–208
3. Zerial, M., and McBride, H. (2001) Nat. Rev. Mol. Cell Biol. 2, 107–117
4. Wilson, S. M., Yip, R., Swing, D. A., O’Sullivan, T. N., Zhang, Y., Novak, E. K., Swank, R. T., Russell, L. B., Copeland, N. G., and Jenkins, N. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7935–7938
5. Wu, S.-F., Luo, R., Zhang, H., Wang, F., Sellers, J. R., Mateis, L. E., Copeland, N. G., Jenkins, N. A., and Hammer, J. A., III (2002) Nat. Cell Biol. 4, 271–278
Munc13-4 Is a GTP-Rab27-binding Protein Regulating Dense Core Granule Secretion in Platelets

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