Small GTPase Rab4 Regulates Ca\textsuperscript{2+}-induced α-Granule Secretion in Platelets

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Upon activation, platelets release many active substances stored in α- and dense-core granules. However, the molecular mechanisms governing regulated exocytosis are not yet fully understood. Here, we have established an assay system using permeabilized platelets to analyze the Ca\textsuperscript{2+}-induced exocytosis of both types of granules, focusing on RabGTPases. Incubation with Rab GDP dissociation inhibitor, an inhibitory regulator of RabGTPases, reduced membrane-bound RabGTPases extensively, and caused strong inhibition of the α-subunit negative mutant of His-tagged Rab4S22N, but not that of [\textsuperscript{3}H]5-hydroxytryptamine (5-HT) in dense-core granules. Specifically, Rab4 co-fractionated with vWF and P-selectin (an α-granule marker) upon separation of platelet organelles by density gradient centrifugation. Incubation of the permeabilized platelets with cell extracts expressing the dominant negative mutant of His-tagged Rab4S22N, but not with those of similar mutant His-RabGTPases, inhibited the vWF secretion, whereas neither of the cell extracts affected the [\textsuperscript{3}H]5-HT secretion. Importantly, the inhibition of vWF secretion was rescued by depleting the cell extracts of His-Rab4S22N with nickel beads. Thus, in platelets, the regulatory mechanisms governing ρ- and dense-core granule secretions are distinct, and Rab4 is an essential regulator of the Ca\textsuperscript{2+}-induced exocytosis of α-granules.

Certain types of cells contain specialized granules, which are released in response to extracellular stimuli. The process is known as regulated exocytosis, which is often triggered by the increased concentration of intracellular calcium ions, followed by docking and fusion of granule membrane with plasma membrane (1). Platelets contain three types of such granules, dense-core granules, α-granules, and lysosomes, which are secreted upon activation of platelets (2, 3). Dense-core granules contain small molecules such as 5-hydroxytryptamine (5-HT)\textsuperscript{1} and α-granules contain newly synthesized proteins such as von Willebrand factors (vWF). Despite the biological significance of these secretions in platelet function (2), the molecular mechanism remains unclear.

Recent advances have shed light on the mechanism of intracellular membrane docking/fusion. An ATPase, N\textsubscript{-}ethylmaleimide-sensitive factor (NSF), and its binding protein, soluble NSF attachment protein (α-SNAP), are required for priming SNAP receptors (SNARE) (4) on vesicular (v-) and target (t-) membranes. Then, the cognate v- and t-SNARE (trans) complex on both sides of the membranes are formed, resulting in their docking/fusion (5, 6). Many v- and t-SNARE homologues have been identified from mammals to yeast, and the SNARE complex-mediated mechanism has been demonstrated to play essential roles in many pathways of intracellular vesicle transport (7). In the case of platelets, NSF (8) and α-SNAP (8, 9) have recently been shown to be required for the granule secretion. Furthermore, syntaxin2 and synaptosome-associated protein of 23 kDa (SNAP23), which are t-SNARE proteins, have been shown to be essential for the dense-core granule secretion (9), and syntaxin4 and SNAP23 function in the α-granule secretion (10).

However, the SNAREs are not the only molecules governing the regulation of intracellular membrane docking/fusion. The RabGTPases are also essential regulatory molecules, which are required at a step upstream of the formation of the SNARE complexes (11, 12). RabGTPase family is composed of more than 30 members and belongs to the Ras-related small GTPase superfamily (13, 14). Like other GTPases, Rab proteins have both GTP-bound active and GDP-bound inactive forms regulated by the GDP/GTP cycles. The active GTP-bound forms exert their functions on the specific organelle membranes through their effector molecules, with which only the active GTP-bound form can be associated (13–15). RabGTPases are also regulated by a unique negative regulator named Rab GDP dissociation inhibitor (GDI) (16, 17). RabGDI is a cytosolic protein, which forms a complex with GDP-bound Rab proteins to inhibit the GDP/GTP exchange (16, 17), and extracts Rab proteins from the membrane into cytosol (17). Moreover, RabGDI has recently been demonstrated to play also positive roles to accompany Rab proteins from cytosol to the proper membranes (17, 18).

The functional requirement of RabGTPases in vesicle docking/fusion has been demonstrated using genetics, morphology, and biochemistry (19). For example, mutations in Sec4, one of the yeast Rab proteins, cause accumulation of trans-Golgi network-derived vesicles, thereby demonstrating the requirement for this GTPase in docking vesicles to the plasma membrane...
(20). In mammals, Rab5 regulates early endosome vesicle docking through its effector protein EEA1 (21). The Rab5 effector proteins participate in oligomeric complexes regulating the priming and pairing of SNARE complexes, which ultimately drive bilayer fusion (22). Thus, RabGTPases are upstream regulators of SNARE complex formation.

So far, several RabGTPases including Rab3B, -4, -6, -8, and -27 have been demonstrated to be present in platelets (23, 24). However, it is still unclear whether or not Rab proteins regulate the regulated exocytosis of α- and dense-core granules. If it is the case, which Rab protein regulates the process? In order to address these questions, we have developed an in vitro assay system using permeabilized platelets for analyzing α- and dense-core granule secretions induced by Ca²⁺. Here, we show that Rab4 is an essential regulator of the α-granule secretion.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, Materials, and Others—His-tagged RabGDI (25) and His-tagged Rab5 (26) were purified from overexpressing Escherichia coli, kindly provided by Dr. M. Zerial (EMBL, Heidelberg, Germany), with nickel-agarose beads (Qiagen) according to the manufacturer’s instruction followed by extensive dialysis against Buffer A (50 mM Hepes/KOH, pH 7.2, 78 mM KCl, 4 mM MgCl₂, 0.2 mM CaCl₂, 2 mM EGTA, 1 mM dithiothreitol). In some experiments, the RabGDI was further purified using a MonoQ (Amersham Pharmacia Biotech) ion exchange column, followed by dialysis against Buffer B. Rab3B-containing plasmid (27) was a kind gift from Dr. Y. Takai (Osaka University, Osaka, Japan). Rab4B plasmid (28) was from Dr. M. Zerial. From these plasmids, mutant Rab3B36N and Rab4S22N were produced by polymerase chain reaction and confirmed that these DNA sequences were correct. Then, these mutants and wild type (wt)-Rab4 were cloned into mammalian expression vector, pcDNA3.1/His C (Invitrogen) at the BamHI-EcoRI site and transfected transiently using LipofectAMINE (Life Technologies, Inc.) into subconfluent HeLa cells in a 10-cm dish. The transfected cells were harvested after 24 h and extracted proteins by incubation of the cell pellets in Buffer A containing 0.1% Triton X-100 for 2 h at 4 °C. After centrifugation of the mixture at 100,000 × g for 30 min at 4 °C, the supernatant was used as cell extracts expressing the His-tagged Rab proteins. Anti-Na⁺-K ATPase rabbit polyclonal antibody (29) was a kind gift from Dr. K. Ohmori (Kansai Medical University, Kadoma, Japan), and anti-Rab5 monoclonal antibody and anti-RabGDI antisera (30) was from Dr. M. Zerial. Anti-vWF rabbit polyclonal antibody was purchased from Sigma; anti-His monoclonal antibody from Qiagen; anti-cathepsin-D, anti-Rab8, and anti-Rab27 monoclonal antibodies from Transduction Laboratories; anti-Rab4, anti-Rab7 goat polyclonal antibodies, and anti-Rab11 goat monoclonal antibody (ERK2-H) from Santa Cruz Biotechnology. Horseradish peroxidase-labeled anti-mouse, rabbit, and goat IgG monoclonal antibodies were from Amersham Pharmacia Biotech, which were used as secondary antibodies for Western blot analysis visualized by enhanced chemiluminescence method (Amersham Pharmacia Biotech). Unless otherwise specified, all the chemicals were purchased from Sigma, except for streptolysin O (SLO), which was from Dr. Bhakdi (Mainz University, Mainz, Germany).

Assay for the Ca²⁺-induced Secretions of α- and Dense-core Granules—The assay method will be described elsewhere (3). Briefly, freshly obtained washed platelets (5 × 10⁷ platelets/assay, counted with Coulter counter) were incubated with [³H]-5-HT (Amersham Pharmacia Biotech) to allow uptake into dense-core granules. The platelet plasma membrane was permeabilized in Buffer A containing 0.6 µg/ml SLO essentially as described (31). Approximately, 80% of the cytosol was lost by the procedure, as determined by quantifying the leakage of cytosolic lactate dehydrogenase measured with a kit (Waken Co., Osaka, Japan). Usually, the permeabilized platelets per assay contained approximately 20,000 cpm of [³H]-5-HT. Then, the platelets were stimulated with 0.6 µg/ml thrombin (data not shown). Although Ca²⁺ at 20 nm, the basal condition in this assay, only slightly induced β-granule secretions, Ca²⁺ at 2–200 µM efficiently induced these secretions (Fig. 2, A and B). Since it has been shown that the [Ca²⁺] in resting platelet cytosol is around 10 nM and it becomes increased to 1–10 µM upon platelet activation (35), the sensitivity of secretion in the permeabilized platelets to [Ca²⁺] was also similar to that in intact platelets. Thus, the secretions of α-
and dense-core granules in our in vitro assay system were similar to those in intact platelets, in terms of the time course and the Ca^{2+} sensitivity.

**RabGDI Inhibited the α-Granule Secretion, but Not the Dense-core Granule Secretion**—Next, we tried to investigate whether RabGTPases are involved in the regulation of the Ca^{2+}-induced exocytosis of α- and dense-core granules. We first analyzed the effects of RabGDI (16, 17), a general inhibitor of RabGTPases. We purified His-RabGDI from the overexpressing E. coli with nickel-agarose beads to more than 90% purity (Fig. 3A), followed by dialysis as described under “Experimental Procedures.” When permeabilized platelets were incubated with the RabGDI, more than 80% of Rab4, -5, -6, and -8 were extracted from the membrane of the permeabilized platelets (Fig. 3B). Although RabGDI has been shown to extract all the tested RabGTPases from the membrane (25), Rab27 remained on the membrane (Fig. 3B). The data suggested that there was some difference in sensitivity of RabGTPases to extraction by RabGDI from the membrane in platelets, similarly as observed in a fibroblast cell line (36). On the other hand, both vWF stored in α-granules and P-selectin which is a cell adhesion molecule stored on α-granule membrane (37) were not extracted by the RabGDI treatment, suggesting that α-granule membrane remained intact by the RabGDI treatment.

Under these conditions, incubation with RabGDI inhibited the Ca^{2+}-induced secretion of vWF strongly (Fig. 3C), whereas the secretion of [3H]5-HT measured in the same samples was not affected (Fig. 3D). The effects of the RabGDI treatment were obtained specifically due to the RabGDI function. First, the purified RabGDI was extremely pure (Fig. 3A) and extensively dialyzed against the same buffer used in the reaction. Second, the inhibition was abolished when the RabGDI sample was boiled (Fig. 3, B and C), suggesting the effect was not due to the buffer. Third, the RabGDI further purified with a MonoQ anion exchange column also inhibited the Ca^{2+}-induced secretion of vWF (data not shown), suggesting that the effect was not due to any contaminating proteins.

These data suggested that the regulatory mechanisms of α- and dense-core granule secretions were distinct and that certain Rab protein(s) were required for α-granule secretion, whereas RabGTPases sensitive to the RabGDI treatment might not be involved in dense-core granule secretion. It has been demonstrated that there are certain vesicle populations, such as those transported to apical domain of an epithelial cell line (38) and vesicles containing influenza virus hemagglutinin in a fibroblast cell line (39), whose transports are resistant to RabGDI treatments. Since the RabGDI treatment did not affect the dense-core granule secretion (Fig. 3D), the dense-core granules could be classified into such a RabGDI-resistant group of vesicles. However, we cannot exclude a possibility that RabGDI-insensitive Rab proteins such as Rab27 (Fig. 3B) might regulate the dense-core granule secretion.

**Rab4 Co-fractionated with vWF in α-Granules by Density-gradient Centrifugation**—Next, in order to determine which Rab proteins were associated with α-granules, platelet organelles were separated by 5–40% stepwise metrizamide-density gradient (Fig. 4A). In the separation, both vWF (2) and P-selectin (37) co-fractionated mainly in the 20–25% layer and less in the 25–30%, indicating the position of α-granules. Rab4 was recovered in two peaks, the 0–5% layer and in the high density fractions, which were overlapped with the fractions of vWF and P-selectin, suggesting that some Rab4 could be associated with α-granules.

Although Rab6 and Rab8 have been suggested to be associated with α-granules (23), they were recovered widely in the gradient and some parts of them were recovered in the fractions of vWF and P-selectin (Fig. 4A). Rab27 has also been suggested to be associated with α-granules (40). However,
and simple centrifugation at 100,000 g for 60 min (B) as described under "Experimental Procedures." Fractions were analyzed by Western blot as shown in the figures. The results shown are representative of five independent experiments with similar results.

Rab27 was recovered in the slightly lighter fractions than those of vWF and P-selectin (Fig. 4A). Rab3B was recovered in the lighter fractions of the 0% and the 0–5% layer. Rab5 was recovered largely in different fractions from those of Rab4 (Fig. 4A), although both of them have been shown to be localized to early endosomes in fibroblast cell lines (41, 42). The reason of the difference was unclear. In the gradient separation, plasma membrane marker Na-K ATPase (29) and lysosomal cathepsin-D (43) were recovered in the lighter fractions, and mitochondrial HSP60 (44) was in the heavier fractions (Fig. 4A), compared with those of α-granules. According to the results using a similar separation shown previously (23), dense-core granule could be recovered in the 35–40% layer or the bottom of the tube in the condition.

Since cytosolic protein RabGDI (17) was recovered in both the 0% and the 0–5% fractions, the 0–5% fraction might possibly contain both cytosol and low density organelles. Therefore, to clarify the cytosolic amounts of organelle markers and Rab proteins, the low speed supernatant of the sonicated platelets was centrifuged at 100,000 g for 60 min to separate the cytosol from the membrane fraction. Then, comparable amounts of both fractions were analyzed by Western blot (Fig. 4B). As expected, vWF, P-selectin, cathepsin-D, and HSP60 were recovered in the membrane fraction. Although Na-K ATPase was recovered in the 0–5% fraction in the density-gradient separation (Fig. 4A), it was detected exclusively in the membrane fraction. As shown previously, Rab3B (23) and RabGDI (17) were recovered mainly in the cytosolic fraction (Fig. 4B). Rab4, -5, -6, -8, and -27 were recovered mainly in the membrane fraction (Fig. 4B). The detection of Rab4 in the 0–5% fraction in the density-gradient separation (Fig. 4A) could reflect the presence of Rab4 on lighter density organelles.

**Dominant Negative Rab4 Inhibited α-Granule Secretion—** Since the inhibition of Ca2+-induced α-granule secretion by RabGDI (Fig. 3C) suggested involvement of Rab protein(s) in the regulation, we were prompted to determine which Rab(s) were responsible for it. Because of the possible association of Rab4 with α-granules (Fig. 4A), we focused upon Rab4, using Rab3B as a control. First, we produced cell extracts expressing His-tagged Rab3BT36N and Rab4S22N (45) using Triton X-100 as described under "Experimental Procedures" (Fig. 6A). These mutants correspond to H-RasS17N (46) and Rab5S34N (26), which have been shown to bind GDP preferentially, but not GTP, and demonstrated to act as dominant negative proteins. The concentrations of the His-tagged mutant Rab proteins were determined by quantitative Western blot with the anti-His antibody using His-tagged Rab5 purified from overexpressing E. coli as a standard (Fig. 6A). Then we analyzed the effects...
His-Rab4S22N (extracts did not inhibit the vWF secretion (Fig. 6A) at 50 nM or with the mock-transfected cell expressing His-tagged wt-Rab4 at 50 nM. wt-Rab4 (Fig. 6B) specifically the inhibition of Rab4 function. In summary, a permeabilized platelet system established here revealed that small GTPase Rab4 could positively regulate the Ca^{2+}-induced secretion of vWF in platelets, although we cannot exclude the possibility of the involvement of Rab6, Rab8, Rab27, or yet unidentified RabGTPases.

Rab4 has been demonstrated to be localized to early endosomes and regulate the recycling vesicle transport in a fibroblast cell line (41). Recently, it has also been shown to positively regulate the transport of vesicles containing glucose transporter 4 in adipocytes in response to insulin since a C-terminal peptide of Rab4 and an anti-Rab4 antibody inhibited it (47). In contrast to these data, Ohnishi et al. (48) have shown that the same Rab4 C-terminal peptide and the anti-Rab4 antibody enhanced the amylase secretion in response to cholecytokinin in pancreatic acinar cells. The authors concluded that Rab4 has functions as an inhibitor in regulated secretion of amylase (48). Thus, although Rab4 might be involved in regulation of certain regulated exocytosis, the function of Rab4 has not yet been concluded to be a positive or negative regulator. Since our results have shown that the dominant negative mutant Rab4 inhibited the α-granule secretion in platelets, Rab4 could positively regulate the α-granule secretion in platelets.

The Rab3 isoforms have been noted as regulators of Ca^{2+}-induced exocytosis (13, 14). For example, in Rab3A-deficient mice, long term potentiation of mossy fibers is abolished, demonstrating the involvement of Rab3A in the regulation of the neurotransmitter release (49). Furthermore, inhibition of Rab3B expression has been shown to attenuate Ca^{2+}-induced exocytosis in rat anterior pituitary cells (50). However, in our results, Rab3B did not seem to be involved in the Ca^{2+}- or dense-core granule secretion in platelets. In summary, a permeabilized platelet system established here revealed that small GTPase Rab4 could positively regulate the Ca^{2+}-induced α-granule secretion and that Rab proteins which are sensitive to the RabGDI-treatment might not play important roles in the dense-core granule secretion. Further investigation is required for elucidation how Rab4 regulates the α-granule secretion.

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