Constitutive GDP/GTP Exchange and Secretion-dependent GTP Hydrolysis Activity for Rab27 in Platelets*

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We have previously demonstrated that Rab27 regulates dense granule secretion in platelets. Here, we analyzed the activation status of Rab27 using the thin layer chromatography method analyzing nucleotides bound to immunoprecipitated Rab27 and the pull-down method quantifying Rab27 bound to the GTP-Rab27-binding domain (synaptotagmin-like protein (Slp)-homology domain) of its specific effector, Slac2-b. We found that Rab27 was predominantly present in the GTP-bound form in unstimulated platelets due to constitutive GDP/GTP exchange. In permeabilized platelets, increase of Ca2+ concentration induced dense granule secretion with concomitant decrease of GTP-Rab27, whereas in non-hydrolyzable GTP analogue GppNHp (β-γ-imidoguanosine 5’-triphosphate)-loaded permeabilized platelets, the GTP (GppNHp)-Rab27 level did not decrease upon the Ca2+ induced secretion. These data suggested that GTP hydrolysis of Rab27 was not necessary for inducing the secretion. Taken together, Rab27 is maintained in the active status in unstimulated platelets, which could function to keep dense granules in a preparative status for secretion.

In eukaryotic cells, transport between distinct organelles is performed through vesicle trafficking. The final step of vesicle trafficking is regulated exocytosis in non-neuronal cells, such as lytic granule membrane (1). One of the key regulators for the SNARE complex formation is Rab GTPase (2, 3). So far, more than 60 members of Rab GTPases are identified in mammals, and they play critical roles in the specific transport pathways (2–4). Like other GTPases, the activity of Rab is regulated by its GDP/GTP cycle. Rab proteins have GTP-bound active and GDP-bound inactive forms. The activation process is performed by GDP/GTP exchange mediated by the GDP/GTP exchange factor. GTP-bound Rab execute their function by interaction with effector proteins. Then, GTP-Rab is inactivated into GDP-Rab by GTP hydrolysis that is mediated by the intrinsic GTPase activity and its enhancer, GTPase-activating protein. Furthermore, the Rab family has a unique regulatory protein named Rab GDP dissociation inhibitor (RabGDI), which extracts GDP-Rab from membrane into cytosol by forming a 1:1 complex and inhibits GDP/GTP exchange (3). RabGDI accompanies Rab in cytosol to the correct organelles, where they are reactivated by the function of GDI dissociation factor (5–8).

Although elucidation of the regulatory mechanism of the GDP/GTP cycle is crucial for understanding the functional mechanism of Rab GTPases, it has not been extensively investigated so far. Small GTPases belonging to Ras and Rho families are predominantly present in the GDP-bound forms under resting conditions and are transiently activated into GTP-bound forms upon stimulation (9), indicating that these GTPases function as “switches” that transduce extracellular signals. For Rab GTPases, most of Rab5, a regulator of endocytic pathway (10, 11), is in the GDP-bound form in unstimulated NR6 cells, and more than half of Rab5 rapidly and transiently becomes the GTP-bound active form upon epidermal growth factor stimulation (12). On the other hand, ~80% of Rab3D is in the GTP-bound form in unstimulated pancreatic acini (13). Since these two results are quite opposite, it is unclear whether RabGTPases are present in its GTP-bound or GDP-bound form. Furthermore, it is not known whether the activation status of other Rab GTPases is altered upon stimulation, like Rab5.

Rab27 is composed of two isoforms, Rab27A and Rab27B, that share ~70% identical amino acid residues (14–16). Accumulating evidence revealed that Rab27 is a general regulator for regulated exocytosis in non-neuronal cells, such as lytic granule.
secretion in cytotoxic T cells (17, 18), insulin secretion in pancreatic β-cells (19), and histamine-containing granule secretion in mast cells (20, 21). Rab27 also regulates melanosome traffic along actin cytoskeleton in melanocytes (22–25).

Dense granules in platelets contain self-agonists such as ADP and serotonin. Secreted ADP and serotonin play important roles for positive feedback activation of platelets at the site of thrombus formation (26, 27). We have demonstrated that Rab27 regulates the secretion in platelets by showing that the addition of non-prenylated Rab27A or Rab27B purified from *Escherichia coli* specifically inhibited the secretion in an assay using permeabilized platelets, possibly due to sequestering its putative effector molecules (28). We have identified a Rab27 effector in platelet cytosol as Munc13-4 (28). Munc13-4 is a non-neuronal homologue of Munc13-1, an essential priming factor in neuronal secretion.

In addition to Munc13-4, eight Rab27 effector molecules have been identified. Although a GTP-Rab27-binding minimal domain of Munc13-4 has not been determined, these eight molecules contain a common GTP-Rab27-binding structure named synaptotagmin-like protein (Slp)-homology domains (SHD) ~100 amino acid long at their N-terminal ends (16). These eight molecules are classified into two groups: Slp1~5 containing two C2 calcium-binding domains and Slp-lacking C2 domains (Slac2)-a~c (16). Although some SHDs potentially bind other Rab proteins in addition to Rab27, SHD of Slac2-b is specific for GTP-Rab27 among 20 tested Rab GTPases (16, 29).

Here, to analyze the GDP/GTP-bound status of Rab27 in platelets, we utilized two assays, a GTP-Rab27 pull-down assay using SHD of Slac2-b and an assay using thin layer chromatography analyzing GDP/GTP associated with immunoprecipitated Rab27. By these two methods, we demonstrated that Rab27 in unstimulated platelets was predominantly in the GTP-bound form, a state that was maintained by constitutive GDP/GTP exchange activity. The GTP-bound form of Rab27 drastically decreased upon granule secretion, due to enhanced GTP hydrolysis activity that was secretion-dependent. Furthermore, we showed that GTP hydrolysis of Rab27 might not be required for the induction of secretion in platelets, namely hydrolysis would be a consequence of secretion.

**EXPERIMENTAL PROCEDURES**

*Materials*—Rabbit polyclonal anti-Rab27A (28) and -Rab27B (30) antibodies were generated using His6-Rab27A and glutathione S-transferase (GST)-Rab27B as antigens, respectively. For Rab27 immunoprecipitation, we used both antibodies in combination. Mouse monoclonal anti-His6 antibody was purchased from Sigma. Horseradish peroxidase-labeled anti-rabbit and anti-mouse IgG polyclonal antibodies were from Amersham Biosciences and used as secondary antibodies for Western blot analysis visualized by the enhanced chemiluminescence method (Amersham Biosciences). Streptolysin-O was from Dr. S. Bhakdi (Mainz University, Mainz, Germany) (31). [α-32P]GTP (3,000 Ci/mmol), and [γ-32P]GTP (3,000 Ci/mmol) were purchased from PerkinElmer Life Sciences, and [32P]phosphorus (200 μCi/mmol) was from Amersham Biosciences. Unless otherwise specified, all the other chemicals including nucleotides, N-ethylmaleimide (NEM), and thrombin were purchased from Sigma.

cDNA encoding rat Rab27A was kindly provided by Dr. Y. Nozawa (Gifu International Institute of Biotechnology, Gifu, Japan) (14). Human Rab27B cDNA was cloned from the Marathon-Ready human bone marrow cDNA (Clontech) by PCR. SHD of Slac2-b (KIAA0624) (amino acids 1–79) was generated by PCR using KIAA0624 clone provided by Kazusa DNA Research Institute as a template. All the sequences of PCR products were confirmed by a 3100 genetic analyzer (Applied Biosystems). cDNAs were subcloned into the prokaryotic expression vector pDEST17 (Invitrogen) for His6-tagged protein and pGEX-2T (Amersham Biosciences) for GST fusion proteins. These His6-tagged and GST fusion proteins were produced in *E. coli* strain BL21 and purified according to the manufacturers’ instructions. All the purified recombinant proteins were extensively dialyzed 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) and stored at −80 °C until use. 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.

**Platelet Dense Granule Secretion Assays**—Freshly obtained washed platelets (5 × 10^7 platelets/assay, counted with the Coulter counter) were incubated with [3H]serotonin (Amer sham Biosciences) to allow uptake into dense core granules (~20,000 cpm/assay) followed by washing with Buffer A. For the assay using intact platelets, they were stimulated with 0.5 units/ml thrombin (Sigma) at 30 °C for the indicated periods, and secreted [3H]serotonin was measured by a liquid scintillation counter (Beckman) after removing platelets by centrifugation. The secretion levels of [3H]serotonin were expressed as percentages of the total [3H]serotonin in the platelets before the final incubation.

The method of dense granule secretion assay using platelets permeabilized by streptolysin-O was described previously (28, 32–35). Briefly, plasma membrane of [3H]serotonin-loaded platelets was permeabilized with streptolysin-O in Buffer A, where the calculated free Ca^{2+} concentration was ~20 μM (36). The permeabilized platelets were incubated with ATP, human platelet cytosol at 2.0 mg of proteins/ml, and tested materials at 4 °C for 15–30 min followed by further incubation at 30 °C for 3 min. Finally, the platelets were stimulated with 20 μM Ca^{2+} (36) at 30 °C for the indicated periods, and the reaction was stopped by the addition of ice-cold Buffer A containing 10 mM EGTA.

**Assay Analyzing Specific Binding of SHD of Slac2-b with GTP-Rab27**—Binding of SHD of Slac2-b with Rab27 was performed by affinity chromatography. First, a non-hydrolyzable GTP analogue, GTPγS, and GDP-bound His6-Rab27A and His6-Rab27B were prepared by incubation of these GTPases (0.2 μM) with 1 mM GTPγS and GDP in the presence of 4 mM MgCl2 and 10 mM EDTA at 30 °C for 30 min followed by the addition of 15 mM MgCl2 to quit the reaction as described (7). Then, GDP- and GTPγS-preloaded Rab27 (1 μg) was incubated with glutathione-Sepharose beads (Amersham Biosciences) coated with GST-SHD of Slac2-b at 4 °C for 1 h in Buffer A and washed three times with the same buffer. Bead-
associated His$_6$-Rab27 was analyzed by immunoblotting with anti-His$_6$ antibody.

**GTP-Rab27 Pull-down Assay with GST-SHD of Slac2-b**—The amount of GTP-bound Rab27 in platelets was measured by affinity pull-down using the SHD. The standard assay was as follows. Platelets isolated from freshly obtained whole blood were lysed in Buffer A containing 0.5% Triton X-100 and protease inhibitor mixture (P8340, Sigma) at 4 °C for 5 min followed by centrifugation at 300,000 x g for 5 min. This procedure completely extracted Rab27. Then, the supernatants were incubated with glutathione beads coated with 10 μg of GST-SHD at 4 °C for 30 min. The beads were washed three times with Buffer A containing 0.1% Triton X-100 and the protease inhibitor mixture, and bead-associated Rab27 was analyzed by immunoblotting with anti-Rab27 antibody. Densitometric analysis of appropriately exposed film was performed, and the signals were quantified using Image J 1.33u software (National Institutes of Health).

To calculate the ratio of GDP/GTP, the standards were produced as described previously (13). The same amount of aliquots of the Triton X-100 extract was incubated at 30 °C for 90 min in the presence of 10 mM EDTA and 4 mM MgCl$_2$ with 1 mM GppNHp (a non-hydrolyzable GTP analogue) or GDP followed by the addition of 15 mM MgCl$_2$ to quic the reaction and determination of GTP-bound Rab27 by the pull-down method. The amount of pulled down Rab27 in the platelet lysate after incubation with GppNHp was defined as 100%, and that with GDP was defined as 0%.

**Evaluation of Nucleotide Bound to Rab27**—Washed platelets ($2 \times 10^8$ platelets/assay) were incubated with 300 μCi of [³²P]phosphorinus (200 μCi/mmol; Amersham Biosciences) in Buffer B (138 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl$_2$, 12 mM NaHCO$_3$, 0.49 mM MgCl$_2$, 5.5 mM glucose, 50 mM HEPES/KOH, pH 7.4) at 37 °C for 3 h. After the platelets were washed with Buffer B twice followed by incubation at 30 °C for 3 min, they were stimulated with 0.5 units/ml thrombin at 30 °C for 3 min. Then, platelets were lysed with Buffer A containing 0.5% Triton X-100 and the protease inhibitor mixture at 4 °C for 5 min. Supernatants after centrifugation were immunoprecipitated using anti-Rab27 antibodies at 4 °C for 30 min. Bound nucleotides were eluted in 25 μl of Buffer A containing 0.2% SDS, 10 mM EDTA, 1 mM GTP, and 1 mM GDP at 85 °C for 3 min. The samples (6 μl of each) were then spotted onto a polyethyleneimine-cellulose TLC plate (Merck) and developed for 90 min in 1 M LiCl$_2$ and 1 M formic acid. The plates were dried at −80 °C for 36–48 h for autoradiography. Densitometric analysis of appropriately exposed film was performed, and the signals were quantified using Image J 1.33u software (National Institutes of Health).

**Statistical Analysis**—All values presented are means ± S.E. Student’s t test was used in Fig. 7A. Values of $p < 0.05$ were considered statistically significant.

**RESULTS**

**Characterization of the SHD of Slac2-b as the GTP-Rab27-interacting Domain**—We first characterized the SHD of a Rab27-specific effector, Slac2-b. We examined whether GST-SHD specifically bound GTP-bound Rab27A and Rab27B, both of which are expressed in platelets (37). We incubated GTPγS (a non-hydrolyzable GTP analogue)-loaded and GDP-loaded His$_6$-Rab27 with GST-SHD-coated glutathione beads, and bead-associated Rab27 was analyzed by immunoblotting with anti-His$_6$ antibody. As shown in Fig. 1A, GST-SHD efficiently bound GTPγS-loaded Rab27A and Rab27B, with minimal binding to GDP-Rab27A and -Rab27B (Fig. 1A), indicating that the SHD specifically interacted with both GTP-Rab27A and GTP-Rab27B.

Next, we analyzed the involvement of Rab27 in the secretion using the SHD, which would sequester GTP-bound Rab27. In the assay, more than 50% of [³H]serotonin preloaded into dense granules in permeabilized platelets was secreted within 1 min in response to Ca$^{2+}$ stimulus at 30 °C, whereas the background secretion was 5–10% (Fig. 1B). The addition of GST-SHD, but not GST, in the assay efficiently inhibited the secretion in a concentration-dependent manner (Fig. 1B), supporting our

![FIGURE 1. Specific binding of SHD with GTP-Rab27 and its inhibitory effect on dense granule secretion in permeabilized platelets.](image)
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**FIGURE 2. A GTP-Rab27 pull-down assay using SHD revealed that GTP-Rab27 was predominant in unstimulated platelets.**

A. GTP-loaded-His<sub>6</sub>-Rab27A purified from E. coli (50 ng) (lane 1) was incubated at 4 °C for 30 min with glutathione beads coated with GST (lane 2), 10 μg of GST-SHD (lane 3), and 20 μg of GST-SHD (lane 4) followed by washing, and the bead-associated His<sub>6</sub>-Rab27A was detected by immunoblotting with anti-Rab27 antibody as described under “Experimental Procedures.” Platelet lysate isolated from 1 ml of blood was also analyzed (lane 5). The data shown are representative of three independent experiments with similar results. B. GTP-Rab27 in platelet lysate isolated from 1 ml of whole blood (lane 2) was incubated with glutathione beads coated with 10 μg of GST-SHD at 4 °C for 30 min, and bead-associated Rab27 was detected by immunoblotting with anti-Rab27 antibody as described under “Experimental Procedures.” As controls, the same amounts of platelet lysates loaded in vitro with GDP (lane 1) or GppNHp (lane 3) were also analyzed in the same method. The data shown are representative of five independent experiments with similar results.

previous observation that Rab27 regulates dense granule secretion in platelets (28).

**Establishment of GTP-Rab27 Pull-down Assay with SHD of Slac2-b**—Most of Rab27 was present in the membrane fraction in platelets (34). To solubilize Rab27 from the membrane fraction in platelets, we used 0.5% Triton X-100, under which condition the interaction of the SHD with GTP-Rab27 was not affected. As shown in Fig. 2A, platelets (∼5 × 10<sup>7</sup>) isolated from 1 ml of blood contained ∼50 ng of Rab27, determined using purified recombinant His<sub>6</sub>-Rab27A as a reference. When the same amount (50 ng) of the recombinant His<sub>6</sub>-Rab27A protein was used for a pull-down assay, almost all of the GTP-loaded form was bound to beads coated with 10 or 20 μg of GST-SHD but not to GST beads (Fig. 2A). Thus, this GST-SHD pull-down assay was established, and we used this method to evaluate the GTP-Rab27 levels in platelets.

**Evaluation of GTP-Rab27 Level in Platelets**—Using this pull-down assay, we evaluated the ratio of GTP-bound form of Rab27 in platelets. The results were calibrated as described previously (13), where the GDP/GTP-bound status of Rab3D in pancreatic acini was evaluated by their pull-down assay using a Rab3-effector RIM-1 (13). They solubilized pancreatic acinar cells, and the cell lysates were loaded with GTPγS or GDP. The amount of GST-RIM-1-associated Rab3D loaded with GTPγS was defined as 100%, and that loaded with GDP was defined as 0% (13). Here, we analyzed the GDP/GTP status of Rab27 in platelets and found that more than 70% (74.3 ± 3.0%, means ± S.E. of five individuals) of Rab27 was the GTP-bound form in unstimulated platelets (Fig. 2B).

We also evaluated the GDP/GTP-bound status of Rab27 by the thin layer chromatography method. Isolated platelets were first incubated at 37 °C for 3 h with [32P]phosphorus that would be incorporated into GTP and GDP in platelets. As shown below (see Fig. 4B), nucleotide bound to immunoprecipitated Rab27 was predominantly GTP (62.7 ± 4.7%, means ± S.E. of three individuals). Thus, we obtained consistent results both by the pull-down and by the thin layer chromatography methods. We concluded that Rab27 was predominantly in the GTP-bound form in unstimulated platelets.

**High Level of GTP-bound Rab27 Was Maintained by Constitutive GDP/GTP Exchange Activity in Platelets**—We analyzed how the high level of GTP-Rab27 was maintained in unstimulated platelets. To examine this issue, permeabilized platelets were incubated in Buffer A (calculated free Ca<sup>2+</sup> concentration was ∼20 nm) (36) without the addition of ATP or platelet cytosol, where GTP would also be depleted by diffusion. In the platelets, the GTP-Rab27 level drastically decreased in a time-dependent manner measured by the Rab27 pull-down assay (Fig. 3, A and C), indicating that Rab27 in platelets was not fixed to be in the GTP-bound form. On the other hand, the same treatment of permeabilized platelets at 4 °C did not decrease GTP-Rab27 (Fig. 3A), indicating that this reaction is temperature-dependent. Importantly, this decrease was rescued by the addition of GTP in the system (Fig. 3, B and C), indicating that the high level of GTP-bound Rab27 was maintained most likely by GDP/GTP exchange reaction.

We examined whether the GDP/GTP exchange reaction indeed occurred in unstimulated platelets. We incubated permeabilized platelets with 10 μM [α-<sup>32</sup>P]GTP at 30 °C in the absence of platelet cytosol. As shown in Fig. 3D, GDP-Rab27-associated radioactivity increased in a time-dependent manner, whereas amounts of GTP-Rab27 pulled down by the SHD were constant at any time point (data not shown), indicating that GDP/GTP exchange continuously took place to maintain the high level of GTP-Rab27 in unstimulated platelets. The rate of [α-<sup>32</sup>P]GTP binding to endogenous Rab27 in permeabilized platelets was 0.032/min. Recombinant Rab27A protein purified from E. coli in vitro was examined in the same condition. The rate of [α-<sup>32</sup>P]GTP binding to recombinant Rab27A was 0.0035/min (data not shown). Therefore, the GDP/GTP exchange rate for Rab27 in platelets was ∼9 times faster than that of recombinant Rab27A. Since the exchange was so efficient in permeabilized platelets, in which the cytosol was extensively depleted due to diffusion, it is likely that the Rab27 GDP/GTP exchange factor, if any, is primarily membrane-associated.

**GTP-bound Rab27 Decreased upon Granule Secretion through Enhanced GTP Hydrolysis Activity**—We next examined the effect of granule secretion induced by an agonist on the
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FIGURE 3. Time-dependent decrease of GTP-Rab27 in permeabilized platelets, which was rescued by addition of GTP. A and B, permeabilized platelets were incubated without the exogenous addition of ATP or platelet cytosol for the indicated periods at 30 °C or 4 °C, in the absence (A) or presence (B) of 1 mM GTP. GTP-Rab27 (upper panels) was measured by the GTP-Rab27 pull-down assay as described under “Experimental Procedures.” The total Rab27 in the samples was shown in the lower panels. The data shown are representative of three independent experiments with similar results. C, time-dependent relative changes of GTP-Rab27 shown in A and B were presented using the GTP-Rab27 ratio at time 0 as a standard (1.0). The results shown are expressed as means ± S.E. of three independent experiments. D, permeabilized platelets were incubated with [α-32P]GTP at 10 μM (10,000 cpm/pmol) for the indicated periods at 30 °C or 4 °C, and then GTP-Rab27 was pulled down by GST-SHD beads followed by quantification of [α-32P]GTP as described under “Experimental Procedures.” The results shown are representative of two independent experiments with similar results.

GDP/GTP status of Rab27. Upon 0.5 units/ml thrombin stimulation at 30 °C, GTP-Rab27 decreased from 74.3 ± 3.0 to 27.1 ± 6.3% at 3 min in intact platelets quantified by the pull-down assay (Fig. 4A). Similar results were obtained by the thin layer chromatography (Fig. 4B). After the thrombin stimulation for 3 min, GTP-Rab27 decreased from 62.7 ± 4.7 to 31.4 ± 1.4%, whereas GDP-Rab27 increased from 37.3 ± 4.7 to 68.6 ± 1.4%. Thus, Rab27-bound GTP drastically decreased with concomitant increase of Rab27-bound GDP upon stimulation, indicating that GDP hydrolysis of Rab27 was enhanced upon stimulation. The rate of GDP hydrolysis on Rab27 in thrombin-stimulated intact platelets in the thin layer chromatography method was 0.104/min. The rate of [γ-32P]GTP hydrolysis on recombinant Rab27A purified from E. coli was 0.0056/min (data not shown). Therefore, the GDP hydrolysis velocity on Rab27 in stimulated platelets was ~18 times faster than that of recombinant Rab27A.

Decrease of GTP-Rab27 was also observed in permeabilized platelets undergoing the Ca2+-induced granule secretion (Fig. 5A), and the degree of decrease was similar to that in thrombin-stimulated intact platelets (Fig. 4A). To examine whether GDP/GTP exchange activity was altered during the Ca2+-induced granule secretion, permeabilized platelets were first incubated with [α-32P]GTP, which would be loaded to small GTPases including Rab27 followed by the addition of excess non-labeled GTP. These platelets were incubated with ATP and platelet cytosol with or without Ca2+ stimulation. Then, time-dependent change of [α-32P]GTP-Rab27 was compared. As shown in Fig. 5B, [α-32P]GTP levels associated with immunoprecipitated Rab27 decreased over time similarly with or without Ca2+ stimulation. Since [α-32P]GTP level associated with immunoprecipitated Rab27 would be affected by GDP/GTP exchange but not GTP hydrolysis, these results indicated that the GDP/GTP exchange activity was not affected upon stimulation.

Granule secretion is triggered by elevation of Ca2+ concentration (38). Since calcium ions regulate many cellular functions, we tried to address which triggered the decrease of GTP-Rab27, elevated Ca2+ concentration or secretion itself. We addressed this issue by using NEM, which is an inhibitor of an essential regulator of the SNARE system, NEM-sensitive factor. NEM inhibited the Ca2+-induced dense granule secretion in a concentration-dependent manner in permeabilized platelets (Fig. 6A) as shown previously (39). Under these conditions, NEM treatment inhibited the decrease of the GTP-Rab27 level, although permeabilized platelets were stimulated with high concentration of Ca2+ (Fig. 6B). These data indicated that the fusion of the granules with plasma membrane, rather than the elevation of Ca2+ concentration, is required for enhancement of Rab27-associated GTP hydrolysis.

GTP Hydrolysis of Rab27 Did Not Appear Essential for the Dense Granule Secretion—To find out the implication of the high level of GTP-bound Rab27 and its secretion-dependent GTP hydrolysis, we tried to address whether GTP hydrolysis was required for granule secretion. It is well known that non-hydrolyzable GTP analogue at 100 μM promotes the Ca2+-independent granule secretion in permeabilized platelets (40). However, in permeabilized platelets preincubated with a lower concentration of GppNHp at 10 μM, we observed a significant increase of the granule secretion upon Ca2+ stimulation (in Fig. 7A, compare lane 4 and lane 5) (p < 0.01), although the secretion level without Ca2+ stimulation was rather high (Fig. 7A, lane 4). Importantly, the Ca2+-dependent secretion was reduced by the addition of the SHD (Fig. 7A, compare lane 5 and lane 6) (p < 0.05), indicating that Rab27 was involved in this secretion. Under this condition, in contrast to GTP-bound Rab27 (Fig. 7B, lanes 1–3), the GppNHp-bound form of Rab27 did not decrease upon granule secretion measured by the pull-down assay, indicating that the majority of Rab27 remained bound to GppNHp in the permeabilized platelets (Fig. 7B, lanes 4–6). Taken together, GTP hydrolysis of Rab27 did not appear necessary for the induction of granule secretion in platelets.
DISCUSSION

In this study, we have analyzed the GDP/GTP cycle of endogenous Rab27 in platelets during regulated exocytosis and demonstrated that Rab27 was predominantly present in the GTP-bound form in unstimulated platelets and GTP-bound Rab27 decreased upon granule secretion. The activation status of Rab27 was regulated by constitutive GDP/GTP exchange activity and secretion-dependent GTP hydrolysis activity. Furthermore, we showed that GTP hydrolysis of Rab27 would not be essential for inducing the secretion. With these experimental results, we propose that the function of Rab27 in the platelet secretion is to maintain the granules in a preparative status for the secretion rather than to mediate the secretion signal.

We have established two methods for quantifying GTP-Rab27. One is the pull-down method in which we use the GTP-Rab27-binding domain, SHD, of a Rab27-specific effector molecule, Slac2-b (29). Since this SHD specifically interacted with GTP-Rab27 (Fig. 1A) and the interaction was unaffected by the detergent to solubilize Rab27, we could obtain reproducible results by this method. Another method is the thin layer chromatography method in which radioactive nucleotide bound to immunoprecipitated Rab27 was analyzed. The results obtained by these two independent methods were coincided. In contrast to the thin layer chromatography, which utilizes high levels of radioactivity and much primary antibodies, the pull-down assay is a preferred method to obtain equivalent results. Therefore, most of the experiments were performed with the pull-down assay, and key experiments for critical confirmation were performed with both methods.

FIGURE 4. GTP-Rab27 decreased upon granule secretion through enhanced GTP hydrolysis activity. A, isolated platelets were stimulated with 0.5 units/ml thrombin for the indicated periods at 30 °C. Then, GTP-Rab27 (upper panel) was measured by the GTP-Rab27 pull-down assay as described under "Experimental Procedures." The total Rab27 in the samples was shown in the lower panel. Relative changes of GTP-Rab27 were presented using the GTP-Rab27 ratio at time 0 as a standard (1.0). The data shown are expressed as means ± S.E. of three independent experiments. B, isolated platelets were labeled with [32P]phosphorus and then stimulated with 0.5 units/ml thrombin at 30 °C for 3 min. Rab27 was immunoprecipitated, and the bound nucleotides were analyzed by thin layer chromatography as described under "Experimental Procedures." The results shown are representative of three independent experiments with similar results.

FIGURE 5. The decrease of the GTP-Rab27 ratios upon the Ca2⁺-induced secretion was not dependent on alteration of GDP/GTP exchange activity. A, permeabilized platelets were stimulated in the presence of platelet cytosol and ATP by 20 μM Ca2⁺ for the indicated periods at 30 °C, and GTP-Rab27 (upper panel) was measured by the GTP-Rab27 pull-down assay as described under "Experimental Procedures." The total Rab27 in the samples was shown in the lower panel. Relative changes of GTP-Rab27 were presented using the GTP-Rab27 ratio at time 0 as a standard (1.0). The data shown are expressed as means ± S.E. of three independent experiments. B, permeabilized platelets were incubated with 10 μM [α-32P]GTP at 30 °C for 30 min followed by the addition of cold excess GTP at 1 mM. These platelets were then incubated with or without 20 μM Ca2⁺ at 30 °C for the indicated periods, and the radioactivity associated with immunoprecipitated Rab27 was measured as described under "Experimental Procedures." The results shown are representative of two independent experiments with similar results.
We investigated the mechanism by which GTP-Rab27 is maintained at such a high level. Two possibilities were conceivable; GTP bound to Rab27 is statically kept without entering the GDP/GTP cycle, or the GTP-bound form is maintained in a dynamic equilibrium between GTP hydrolysis and GDP/GTP exchange activities. Here, the data demonstrated the high rates of nucleotide exchange for Rab27 in unstimulated platelets. First, GTP-Rab27 levels decreased in permeabilized platelets without the addition of GTP in the milieu. Second, this decrease was rescued by the addition of GTP in the system. Third, \[^{32}P\]GTP added in the system was incorporated to Rab27 in a time-dependent manner, whereas total GTP-Rab27 levels were not changed. Thus, the high level of GTP-bound Rab27 in unstimulated platelets was maintained by constitutive GDP/GTP exchange activity. Since we detected GDP/GTP exchange activity for Rab27 in permeabilized platelets, where the cytosol was extensively depleted, the majority of the GDP/GTP exchange activity could be membrane-associated.

Rybin et al. (41) have examined the GDP/GTP cycle of Rab5 on purified early endosomal membrane in the steady states by experiments analyzing xanthosine 5'-triphosphate bound to mutant Rab5, which preferentially binds xanthosine 5'-triphosphate instead of GTP. They demonstrated that Rab5 activity is regulated in a dynamic equilibrium by constitutive GTP hydrolysis activity on purified early endosome membrane. Then, they speculated that Rab5 undergoes multiple cycles of nucleotide binding and hydrolysis on the early endosome membrane (41).

Since GDP-bound Rab GTPases could be present in cytosol by forming a complex with RabGDI, the data that most of Rab27 localizes in the membrane fraction (33) could reflect the predominant existence of Rab27 in the GDP-bound form in platelets. Since GDP/GTP exchange occurred in Rab27 in permeabilized platelets without the addition of platelet cytosol containing RabGDI (Fig. 3, A, B, and D), Rab27 appears to undergo multiple GDP/GTP cycle on the membrane, similar to Rab5, without recycling between cytosol and membrane.

**FIGURE 6.** The Ca\(^{2+}\) stimulation did not induce the decrease of GTP-Rab27 ratios when the secretion was inhibited by NEM in permeabilized platelets. A, permeabilized platelets were incubated in the presence of platelet cytosol and ATP with various concentration of NEM at 4 °C for 30 min followed by the Ca\(^{2+}\) stimulation at 30 °C for 1 min. The Ca\(^{2+}\)-induced dense granule secretion was analyzed as described under "Experimental Procedures." The data shown are expressed as means ± S.E. of three independent experiments. B, GTP-Rab27 (upper panel) in these platelets was measured by the GTP-Rab27 pull-down assay as described under "Experimental Procedures." The total Rab27 in the samples was shown in the lower panel. Relative changes of GTP-Rab27 were presented using the GTP-Rab27 ratio at time 0 as a standard (1.0). The data shown are expressed as means ± S.E. of three independent experiments.

**FIGURE 7.** GTP hydrolysis of Rab27 did not appear essential for the dense granule secretion. A, permeabilized platelets were incubated with 10 μM GTP (lanes 1–3) or GppNHp (lanes 4–6) in the absence of platelet cytosol and ATP at 30 °C for 15 min followed by the addition of platelet cytosol and ATP. These permeabilized platelets were incubated with (lanes 3 and 6) or without (lanes 1, 2, 4, and 5) 1 μM GST-SHD at 4 °C for 15 min. These permeabilized platelets were incubated with or without Ca\(^{2+}\) for 1 min, and secreted \[^{3}H\]serotonin was analyzed as described under "Experimental Procedures." The results shown are expressed as means ± S.E. of five independent experiments. *, p < 0.01, lane 1 versus lane 2, lane 2 versus lane 3, and lane 4 versus lane 5. #, p < 0.05, lane 5 versus lane 6. B, GTP- (or GppNHp-) Rab27 (upper panel) in these platelets was measured by the GTP-Rab27 pull-down assay as described under "Experimental Procedures." The total Rab27 in the samples was shown in the lower panel. The results shown are representative of three independent experiments with similar results.
GDP/GTP Cycle of Rab27 in Exocytosis

Here, we demonstrated that the high level of GTP-Rab27 markedly decreased upon stimulation in platelets due to enhanced Rab27 GTP hydrolysis activity. Since the GDP/GTP exchange activity for Rab27 appeared unchanged by granule secretion (Fig. 5B), this decrease of GTP-Rab27 would be exclusively due to the enhanced GTP hydrolysis activity upon stimulation. The granule secretion is triggered by increased Ca\(^{2+}\) concentration. However, the Rab27 GTP hydrolysis activity was not enhanced by increased Ca\(^{2+}\) concentration alone. It was coupled with granule secretion per se since increased Ca\(^{2+}\) concentration did not decrease the GTP-Rab27 level when the secretion was inhibited by NEM.

What is the implication of the high active status of Rab27 in unstimulated platelets and GTP hydrolysis upon granule secretion? If GTP hydrolysis of Rab27 is not required for the secretion, the predominant presence of Rab27 in its active form would imply that Rab27 does not mediate the secretion signal directly but rather plays a role to maintain vesicles in the preparative state for secretion. If GTP hydrolysis of Rab27 is required for fusion, GTP-Rab27 would function as a negative regulator, where the secretion signal induces GTP hydrolysis of Rab27 to trigger the secretion. To date, two types of functional mechanisms of GTPases are known. Some require GTP hydrolysis for the function, and the others do not require it. For example, elongation factor EF-Tu requires GTP hydrolysis to perform the proofreading of elongation of the correct amino acid at the end of peptide under production (42). On the other hand, the GTP-bound form of Rab5 is the active form, which induces membrane docking/fusion of early endosomes without requiring GTP hydrolysis (11, 41).

Therefore, elucidation of the implication of GTP hydrolysis of Rab27 is essential to understand the functional mechanism of Rab27 in the regulation of the secretion.

To address this issue, we used non-hydrolyzable GTP analogue, GppNHp, in the assay system with permeabilized platelets. It is well known that non-hydrolyzable GTP analogue at 100 \(\mu\)M promotes the Ca\(^{2+}\)-independent granule secretion in permeabilized platelets (40). However, in permeabilized platelets preincubated with a lower concentration of GppNHp at 10 \(\mu\)M, we observed a significant increase of the granule secretion upon Ca\(^{2+}\) stimulation. In this experiment, the GTP-Rab27 level did not decrease by the granule secretion, suggesting that almost all Rab27 bound GppNHp. Furthermore, the Ca\(^{2+}\)-induced secretion in the presence of 10 \(\mu\)M GppNHp was inhibited by the addition of the GTP-Rab27-binding domain, SHD (Fig. 7A, lane 6), indicating that Rab27 played a role for inducing the secretion. Therefore, we would conclude that GTP-Rab27 is the active form and GTP hydrolysis of Rab27 does not appear necessary for dense granule secretion in platelets.

Since Rab27 in platelets is predominantly present in its GTP-bound form and GTP hydrolysis appears unnecessary for the secretion, it is likely that GTP-Rab27 would not mediate extracellular signals but rather keep the vesicles in a preparative state for the secretion. Since increased Ca\(^{2+}\) concentration is the trigger of the secretion (38), the calcium ion signal would be mediated by a calcium-binding protein such as a Rab27 effector molecule Munc13-4 containing Ca\(^{2+}\)-binding C2 domains (28), and protein kinase C (33).

Nevertheless, we cannot completely exclude the possibility that GTP hydrolysis of Rab27 is required for the granule secretion due to some limitations in the experiments shown in Fig. 7 to address the requirement of GTP hydrolysis of Rab27 for the secretion. First, other GTPases as well as Rab27 would bind GppNHp in permeabilized platelets. Some of these GTPases might bypass the need for GTP hydrolysis of Rab27. Second, the spans of the Ca\(^{2+}\)-induced secretion and the inhibition by the SHD were relatively small in the assay due to rather high levels of secretion without Ca\(^{2+}\) stimulation in the presence of GppNHp. Although the differences are statistically significant, additional experiments would be required for the definite conclusion.

At this time, the biological implication of the secretion-dependent GTP hydrolysis of Rab27 remains unclear. It might play a role in recycling of Rab27 from the plasma membrane after one cycle of the secretion is completed.

Platelets contain many small GTPases, such as Ras, RhoA, Rap1, and Ral, that are predominantly present in their GDP-bound forms and transiently become GTP-bound forms upon stimulation in platelets (43–46). In contrast to these small GTPases, the GDP/GTP cycle of Rab27 appears unique. Given that Rab27 in platelets was predominantly in the GTP-bound form, the GDP/GTP exchange activity would be more dominant than the GTP hydrolysis activity in unstimulated platelets. Furthermore, this GDP/GTP exchange activity for Rab27 was not changed by the elevation of Ca\(^{2+}\) concentration (Fig. 5B), unlike that for other small GTPases (12, 46). Very recently, the GDP/GTP cycle of Arf6 has been demonstrated to be similar to that of Rab27 (46), where most of Arf6 is present in its GTP-bound form and GTP-Arf6 is decreased by agonist stimulation to regulate Rho family GTPases in platelets, although it has not been addressed whether GTP hydrolysis plays a role or not in its functions (46).

In pancreatic acini, most of Rab3D has been demonstrated to be in the GTP-bound form (13), although the GDP/GTP status of Rab3D in response to cellular stimulus has not been addressed. Since the Rab3 family is implicated in regulated exocytosis and related to Rab27 in the primary structure (16), the predominant GTP-bound form in the resting cells might be common in Rab GTPTases implicated in regulated exocytosis.

Here, we showed that Rab27 in unstimulated platelets was predominantly present in the GTP-bound active form, which was regulated by its constitutive GDP/GTP exchange activity in a dynamic equilibrium and that this high level of GTP-Rab27 drastically decreased upon granule secretion due to enhanced GTP hydrolysis activity. To elucidate the molecular mechanism of the regulation in the secretion, it would be crucial to identify GTPase-activating protein and GDP/GTP exchange factor for Rab27 in platelets and characterize their functions in the platelet secretion.

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