Regulation of Platelet Dense Granule Secretion by the Ral GTPase-Exocyst Pathway*

Mitsunori Kawato*†, Ryutarou Shirakawa‡, Hirokazu Kondo *, Tomohito Higashi*, Tomoyuki Ikeda, Katsuya Okawa*, Shuya Fukai*, Osamu Nureki†, Toru Kita‡, and Hisanori Horiuchi*†

From the *Department of Cardiovascular Medicine and ‡Frontier Technology Center, Graduate School of Medicine, Kyoto University, Kyoto, 606-8507, Japan, †Department of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, 226-8501, Japan, and §Life Science Division, Synchrotron Radiation Research Organization, University of Tokyo, Tokyo, 113-0032, Japan

Non-hydrolyzable GTP analogues, such as guanosine 5’-(β,γ-imido)triphosphate (GppNHp), induce granule secretion from permeabilized platelets in the absence of increased intracellular Ca2+. Here, we show that the GppNHp-induced dense granule secretion from permeabilized platelets occurred concomitantly with the activation of small GTPase Ral. This secretion was inhibited by the addition of GTP-Ral-binding domain (RBD) of Sec5, which is a component of the exocyst complex known to function as a tethering factor at the plasma membrane for vesicles. We generated an antibody against Sec5-RBD, which abolished the interaction between GTP-Ral and the exocyst complex in vitro. The addition of this antibody inhibited the GppNHp-induced secretion. These data indicate that Ral mediates the GppNHp-induced dense granule secretion from permeabilized platelets through interaction with its effector, the exocyst complex. Furthermore, GppNHp enhanced the Ca2+ sensitivity of dense granule secretion from permeabilized platelets, and this enhancement was inhibited by Sec5-RBD. In intact platelets, the association between Ral and the exocyst complex was induced by thrombin stimulation with a time course similar to that of dense granule secretion and Ral activation. Taken together, our results suggest that the Ral-exocyst pathway participates in the regulation of platelet dense granule secretion by enhancing the Ca2+ sensitivity of the secretion.

Platelet activation occurs in response to vascular injury and plays an essential role in thrombus formation and hemostasis. Several agonists, including thrombin, adenosine diphosphate (ADP), and thromboxane A2, can activate platelets at sites of vascular injury (1). The activation of platelets by these agonists involves the elevation of intracellular Ca2+ concentration, which leads to cell shape change, granule secretion, and platelet aggregation (1, 2). In activated platelets, however, it has been demonstrated that levels of intracellular Ca2+ vary considerably, ranging from 0.2 to 2 μM (3).

Platelets store self-agonists, such as ADP and serotonin, in dense granules (2, 4). Release of these contents plays an important role in further platelet activation and recruitment of circulating platelets to the site of vascular injury. Although an increase in intracellular Ca2+ concentration is the trigger for regulated exocytosis in platelets (5, 6), it has long been known that non-hydrolyzable GTP analogues, such as GTPγS and GppNHp, induce granule secretion from permeabilized platelets even at low concentrations of Ca2+ (7–11). Non-hydrolyzable GTP analogues have also been shown to shift the Ca2+ concentration-response curve for dense granule secretion to the left in electropерmeabilized platelets, indicating that GTPγS and GppNHp increase the Ca2+ sensitivity of platelet secretion (7). These observations suggest that some GTPases are involved in the process, although the molecular basis remains unclear.

Members of the Ras superfamily of small GTPases are classified into several groups such as Ras subfamily that is implicated in cell proliferation, Rho subfamily involved in cytoskeletal regulation, and Rab subfamily involved in vesicle transport (12, 13). Small GTPases cycle between inactive GDP-bound form and active GTP-bound form that exert their function through specific interaction with effector proteins. The activation process is performed by GDP/GTP exchange reaction that is mediated by GDP/GTP exchange factors. The inactivation process is performed by GTP hydrolysis that is mediated by GTPase-activating proteins.

Ral GTPases are members of the Ras subfamily, consisting of RalA and RalB. Most of the Ral-GDP/GTP exchange factors so far identified, such as Ras-GDP dissociation stimulator, Rgl, and Rlf, are downstream effector molecules of Ras, indicating that Ras controls Ral activation (14, 15). On the other hand, the elevation of intracellular Ca2+ levels also induces Ral activation independently of Ras activation (16, 17). Ral GTPases have several effector molecules and exert multiple functions, including

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1 Recipient of Japan Society for the Promotion of Science (JSPS) Research Fellowship for Young Scientists.
2 To whom correspondence should be addressed: Dept. of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, 606-8507, Japan. Tel.: 81-75-751-3778; Fax: 81-75-751-3203; E-mail: horiuchi@kuhp.kyoto-u.ac.jp.

3 The abbreviations used are: GTPγS, guanosine 5’-(γ-thio)triphosphate; GppNHp, guanosine 5’-(β,γ-imido)triphosphate; GDP, guanosine diphosphate; RBD, Ral-binding domain; GST, glutathione S-transferase; SLO, streptolysin-O; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; GLUT4, glucose transporter 4; PKCa, protein kinase Ca.
endocytosis through Ral-binding protein 1 (RalBP1), exocytosis through the exocyst complex, cytoskeletal organization through filamin, and tumorigenesis through yet unknown factors (18, 19). In platelets, both RalA and RalB are abundantly expressed (20, 21) and localized on dense granules (22). Ral is rapidly activated after stimulation with various agonists, and its activation is mediated by an increase in intracellular Ca$^{2+}$ levels (17). However, the function of Ral in platelets remains unclear.

The exocyst complex is composed of eight proteins (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84) and has been implicated in tethering of vesicles to specific regions on the plasma membrane in some cell types (23). The exocyst complex is one of the Ral effectors, and Ral binds to Sec5 in a GTP-dependent manner (24–26). Exo84 has also been shown to interact with active Ral (27), and its binding is competitive with Sec5 (28). In budding yeast, the exocyst complex plays an essential role in vesicle transport required for the growth of daughter cells under control of the Rab family small GTPase Sec4p (29, 30). In mammals, the exocyst complex is implicated in the formation of cell polarity by targeting vesicles to the basolateral membrane in epithelial cells (31) through interaction with GTP-Ral (25, 32). Exo70 has been reported to bind the Rho family GTPase TC10 and play a role in the targeting of the glucose transporter 4 (GLUT4) to the plasma membrane in response to insulin stimulation in adipocytes (33, 34).

In this study, we examined the role of Ral GTPase and its effector, the exocyst complex, in platelet dense granule secretion. We demonstrate that Ral regulates the GppNHp-induced dense granule secretion through interaction with the exocyst complex, by showing that the addition of a Ral-binding domain (RBD) of Sec5 or anti-Sec5-RBD antibody inhibits the GppNHp-induced secretion from permeabilized platelets. We further show that GTP-Ral enhanced the Ca$^{2+}$ sensitivity of dense granule secretion from permeabilized platelets and propose that the Ral-exocyst pathway could play a role in dense granule secretion in intact platelets.

**EXPERIMENTAL PROCEDURES**

**Constructs, Antibodies, Materials, and Other Methods**—cDNA encoding full-length RalA, RalB, and Sec5-RBD (amino acids 1–120) were cloned from the Marathon-Ready human brain cDNA (Clontech) by PCR. Truncated RalA (amino acids 9–183) was prepared as described (35). Sec5-RBD T11A mutant was produced by PCR mutagenesis. All of the sequences of PCR products were confirmed by sequencing using a 3100 Genetic Analyzer (Applied Biosystems). These cDNAs were subcloned into the prokaryotic expression vector pRSET A (Invitrogen) to produce His$_6$-tagged proteins and pGEX-2T (GE Healthcare) to produce glutathione S-transferase (GST) fusion proteins. These His$_6$-tagged and GST fusion proteins were produced in *Escherichia coli* strain BL21 (DE3) and purified according to the manufacturer’s instructions. 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. Protein concentrations were determined by Bradford’s method (Bio-Rad) or from the intensities of the bands on Coomassie Blue-stained SDS-polyacrylamide gels using bovine serum albumin as a standard.

Antibodies against Sec5-N terminus and anti-Sec8-C terminus polyclonal antibodies were raised in rabbits against the N-terminal peptide of human Sec5 (MQSRQPLVTGISPNEGIP, corresponding to amino acids 1–20) and the C-terminal peptide of human Sec8 (EQAAIKQATDKKITT, corresponding to amino acids 958–974), respectively. Anti-Sec5-RBD and anti-GST antibodies were purchased from BD Transduction Laboratories. Anti-RalB mouse monoclonal antibody was from Millipore. Anti-His$_6$ mouse monoclonal antibody and rabbit IgG were from Sigma. Horseradish peroxidase-labeled anti-rabbit and anti-mouse IgG polyclonal antibodies were from GE Healthcare; these were used as secondary antibodies for Western blot analysis and visualized by the enhanced chemiluminescence method (GE Healthcare). Streptolysin-O (SLO) was provided by Dr. S. Bhakdi (Mainz University, Mainz, Germany) (36). [3H]Serotonin (20.3 Ci/mmol) was purchased from PerkinElmer Life Sciences. Unless otherwise specified, all of the other chemicals including nucleotides and thrombin were purchased from Sigma.

**Platelet Dense Granule Secretion Assays**—The assay method for the Ca$^{2+}$-induced dense granule secretion using permeabilized platelets was described previously (37–41). The assay for the GppNHp-induced dense granule secretion was developed by modification of this method. Briefly, [3H]serotonin-loaded platelets (~20,000 cpm/assay, 1 × 10$^8$ platelets/assay) were permeabilized with SLO in Buffer A, where the calculated free Ca$^{2+}$ concentration was ~20 nM (42). The permeabilized platelets were incubated with the ATP regeneration system, 100 μM GppNHp, and various tested materials at 4 °C for 15 to 30 min. Then, unless otherwise specified, permeabilized platelets were stimulated at 30 °C for 15 min, and the reaction was stopped by the addition of ice-cold Buffer A (1:4, v/v). Finally, released [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. In the secretion assay using intact platelets, [3H]serotonin-loaded platelets were stimulated with 0.5 unit/ml thrombin at 30 °C for the indicated periods in modified Hepes-Tyrode’s buffer (50 mM Hepes/KOH, pH 7.4, 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), and released [3H]serotonin was measured as described above.

**Assay Analyzing Specific Binding of GST-Sec5-RBD with Active Ral**—GDP- or GppNHp-bound His$_6$-RalA and RalB (10 μg each), prepared as described (40, 43), were incubated with glutathione-Sepharose beads (GE Healthcare) coated with wild-type or mutant GST-Sec5-RBD (20 μg each) at 4 °C for 1 h in Buffer A. After washing the beads, bead-associated His$_6$-Ral proteins were analyzed by immunoblotting with anti-His$_6$ antibody.

**GTP (GppNHp)-Ral Pulldown Assay Using GST-Sec5-RBD**—Platelet samples were lysed (1:4, v/v) in ice-cold Buffer A con-


**Results**

GppNHp, but Not GTP, Induced Dense Granule Secretion from Permeabilized Platelets—We have previously established a semi-intact dense granule secretion assay in platelets permeabilized with SLO, using calcium chloride as a stimulus (37–41). In this assay, the secretion is dependent on the exogenous addition of ATP and cytosol, and more than 50% of the serotonin stored in dense granules is released within 1 min upon stimulation with 20 μM Ca\(^{2+}\).

By modification of this method, we first confirmed the previous finding that dense granules were secreted by stimulation with non-hydrolyzable GTP analogue in permeabilized platelets (7, 11). GppNHp induced the dense granule secretion in time- and concentration-dependent manners (Fig. 1, A and B) in a condition where the calculated free Ca\(^{2+}\) concentration was ~20 nM (42). On the other hand, GTP did not induce the secretion of dense granules at 100 μM (data not shown) or even at 1 mM (Fig. 1A). Interestingly, although the addition of ATP was required for the GppNHp-induced secretion (data not shown), exogenous addition of platelet cytosol was not required, unlike for the Ca\(^{2+}\)-induced secretion (38). These results indicated that GppNHp and Ca\(^{2+}\) stimuli utilized distinct pathways in permeabilized platelets.

Involvement of Ral in the GppNHp-induced Dense Granule Secretion from Permeabilized Platelets—The GppNHp dependence in the secretion suggested that some GTPases were involved in the process. Among numerous GTP-binding proteins, we focused on Ral because Ral preferentially localizes on dense granules in platelets (22) and is rapidly activated in response to agonist stimulations (17). To test whether Ral is involved in the regulation of the GppNHp-induced dense granule secretion from permeabilized platelets, we first characterized the RBD of Sec5, a Ral-binding component of the exocyst complex (24–26, 35). The interaction between Sec5-RBD and Ral is specific because it has been demonstrated that Sec5 does not bind to other small GTPases, such as RhoA, Rac1, Cdc42, Rab8, K-Ras, or H-Ras (26). We produced and purified wild-type Sec5-RBD and the Sec5-RBD T11A mutant lacking the Ral binding activity (35) as GST fusion proteins. Sec5-RBD interacted with the GppNHp-bound forms of RalA and RabB but not with GDP-bound forms (Fig. 2A). On the other hand, Sec5-RBD T11A did not bind either the GppNHp- or GDP-bound forms (Fig. 2A).

Next, we examined the state of activation of Ral in permeabilized platelets by a GTP-Ral pulldown assay using GST–Sec5-RBD. When permeabilized platelets were incubated with 100 μM GppNHp, both RalA and RabB were converted to the active forms in a time-dependent manner (Fig. 2, B and C). About 17% of total RalA or RabB were activated after a 15-min incubation with GppNHp (Fig. 2C). In contrast, incubation of permeabilized platelets with 1 mM GTP did not activate RalA (Fig. 2B). Importantly, the time course of the Ral activation was well correlated with that of the GppNHp-induced dense granule secretion (compare Figs. 1A and 2C).

We then analyzed the effects of Sec5-RBD on the GppNHp-induced secretion. The addition of Sec5-RBD in permeabilized platelets inhibited the GppNHp-induced dense granule secretion (Fig. 3A) in a concentration-dependent manner (Fig. 3B). This inhibition was presumably due to the blocking of endogenous active Ral by Sec5-RBD. On the other hand, the addition of Sec5-RBD T11A or GST did not inhibit the secretion (Fig. 3B). These results indicated that Ral was involved in the regulation of the GppNHp-induced dense granule secretion.

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**Table 1**

| Treatment       | Dense Granule Secretion |
|-----------------|-------------------------|
| None            | 0%                      |
| 10 μM GTP       | 50%                     |
| 100 μM GTP      | 75%                     |
| 1 mM ATP        | 90%                     |

**Figure 1**

A. Time course of dense granule secretion in SLO-permeabilized platelets incubated with 1 mM GTP. B. Time course of dense granule secretion in SLO-permeabilized platelets incubated with 100 μM GppNHp. C. Time course of dense granule secretion in SLO-permeabilized platelets incubated with 100 μM GppNHp and 10 μM ATP.

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**Figure 2**

A. Western blot analysis of RalA and RabB activation in GppNHp-treated SLO-permeabilized platelets. B. Western blot analysis of RalA and RabB activation in GppNHp-treated SLO-permeabilized platelets after incubation with 1 mM GTP. C. Western blot analysis of RalA and RabB activation in GppNHp-treated SLO-permeabilized platelets after incubation with 100 μM GppNHp and 10 μM ATP.

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**Figure 3**

A. Western blot analysis of RalA and RabB activation in SLO-permeabilized platelets incubated with 100 μM GppNHp and 10 μM ATP. B. Western blot analysis of RalA and RabB activation in SLO-permeabilized platelets incubated with 100 μM GppNHp and 10 μM ATP and 1 μM Sec5-RBD T11A. C. Western blot analysis of RalA and RabB activation in SLO-permeabilized platelets incubated with 100 μM GppNHp and 10 μM ATP and 1 μM Sec5-RBD.
Involvement of the Exocyst Complex in the GppNHp-induced Dense Granule Secretion from Permeabilized Platelets—Among the effector molecules of Ral, exocyst complex could be a candidate for mediating the GppNHp-induced secretion from permeabilized platelets. To examine this possibility, we first confirmed the existence of the exocyst complex in platelet extracts by affinity chromatography. As shown in Fig. 4A, seven protein bands ranging in molecular mass from 70 to 110 kDa were specifically detected in the GTP/H9253S-RalA lane (lane 3). MALDI-TOF MS analysis of these proteins identified all the eight components of the human exocyst complex and another Ral effector, Ral-binding protein 1 (RalBP1). Human Sec15 protein is composed of two isoforms that share 66% identity and 75% similarity (24); we detected both Sec15-like 1 and -like 2 isoforms in human platelets.

Subcellular distribution analysis revealed that RalA was exclusively membrane-bound, whereas the exocyst complex...
was equally distributed in both cytosol and membrane fractions of intact platelets (left panel in Fig. 4B). In SLO-permeabilized platelets, most of the exocyst complex was retained in the cells, possibly because of its large size, in the condition where most of PKCα, a cytosolic protein, leaked out from the cells by diffusion (right panel in Fig. 4B). These results might explain why exogenous addition of platelet cytosol was dispensable for the GppNHp-induced secretion from permeabilized platelets but not for the Ca\(^{2+}\)-induced secretion (38).

To examine the involvement of the exocyst complex in the secretion, an anti-Sec5-RBD antiserum was raised in rabbits by injecting GST-Sec5-RBD (amino acids 1–120) as an antigen. We first affinity-purified the antibody against the GST part of the fusion protein from the antiserum and then used the anti-GST antibody as a control in the following experiments. Subsequently, anti-Sec5-RBD antibody was affinity-purified from serum depleted of anti-GST antibodies. In Western blot analysis, the anti-Sec5-RBD antibody recognized a single band at 100 kDa for Sec5 and Sec8 from human platelet cytosol (Fig. 5A). This antibody immunoprecipitated not only Sec5 but also Sec8 from human platelet cytosol (Fig. 5B), implying that the anti-Sec5-RBD antibody was capable of immunoprecipitating the entire exocyst complex. Further-
more, this antibody, but not the anti-GST antibody, concentration-dependently inhibited the interaction of Sec5 and Sec8 with GppNHp-RalA in vitro (Fig. 5C), indicating that the anti-Sec5-RBD antibody abrogated the interaction between Ral and the exocyst complex. Importantly, the addition of the anti-Sec5-RBD antibody inhibited the GppNHp-induced dense granule secretion in a concentration-dependent manner (Fig. 5D). In contrast, the anti-GST antibody or control rabbit IgG had no effect (Fig. 5D). These results indicated that the exocyst complex played an essential role for the GppNHp-induced dense granule secretion through interaction with active Ral.

GppNHp-bound Ral Increased the Ca\textsuperscript{2+} Sensitivity of Dense Granule Secretion in Permeabilized Platelets—An increase in intracellular Ca\textsuperscript{2+} concentration is considered to be the trigger for the platelet secretion (6). To determine whether Ral was involved in the regulation of the Ca\textsuperscript{2+}-induced dense granule secretion, we analyzed the effects of GppNHp and Sec5-RBD on dense granule secretion induced by various concentrations of Ca\textsuperscript{2+} in permeabilized platelets. In the absence of GppNHp, the threshold for activation of [\textsuperscript{3}H]serotonin release by Ca\textsuperscript{2+} was \( \sim 600 \text{ nM} \), with maximal release occurring at 2 \( \mu \text{M} \) (closed squares in Fig. 6). The addition of 100 \( \mu \text{M} \) GppNHp shifted the Ca\textsuperscript{2+} concentration-response curve for [\textsuperscript{3}H]serotonin release to the left (closed circles in Fig. 6) as shown previously (7) and resulted in a decrease in the Ca\textsuperscript{2+} concentration required for half-maximal secretion from 1.2 \( \mu \text{M} \) to 90 nM. These data indicated that 100 \( \mu \text{M} \) GppNHp increased the Ca\textsuperscript{2+} sensitivity of dense granule secretion by \( \sim 13\text{-fold} \). Importantly, this increase in the Ca\textsuperscript{2+} sensitivity of the secretion was inhibited by the addition of Sec5-RBD (open circles in Fig. 6), indicating the involvement of GTP-Ral in increasing the Ca\textsuperscript{2+} sensitivity of dense granule secretion from permeabilized platelets. The effect of Sec5-RBD was specific because the addition of Sec5-RBD alone had no effect on Ca\textsuperscript{2+}-induced dense granule secretion (open squares in Fig. 6).

**Thrombin Induced the Association of Ral with the Exocyst Complex in Intact Platelets**—In the last set of experiments, we examined whether the Ral and the exocyst complex could function in intact platelets. Thrombin is a potent agonist for platelets. Thrombin rapidly induced the release of serotonin stored in dense granules in intact platelets (Fig. 7A). Moreover, thrombin immediately activated both RalA and RalB in a similar time course to that of the secretion (Fig. 7B and C). Both RalA and RalB were maximally activated at 30 s after thrombin stimulation, and the ratio of activated RalA to RalB to total was \( \sim 6\% \) (Fig. 7C). To examine the possible involvement of the Ral-exocyst pathway in intact platelets, we analyzed the association between Ral and the exocyst complex upon thrombin stimulation. As shown in Fig. 7D, anti-Sec8 antibody precipitated little RalA before thrombin stimulation, whereas RalA was clearly detected in the immunoprecipitates with anti-Sec8 antibody after 60 s of stimulation. Thus, thrombin induced the association of Ral with the exocyst complex in a similar time course to that of the secretion (Fig. 7A) and Ral activation (Fig. 7B and C), suggesting that the Ral-exocyst pathway could function in intact platelets.

**DISCUSSION**

It has long been known that non-hydrolyzable GTP analogues induce granule secretion from permeabilized platelets (7–11). Here we demonstrated that Ral GT-Pase and the exocyst complex mediated this GppNHp-induced dense granule secretion. We also showed that GTP-Ral enhanced the Ca\textsuperscript{2+} sensitivity of dense granule secretion from permeabilized platelets, and the Ral-exocyst pathway could play a role in intact platelets.

In this study, we demonstrated the involvement of Ral by showing that Sec5-RBD, which is a specific inhibitor of Ral, affected the secretion. The effect is specific because the Sec5-RBD T11A mutant that lacks the GTP-Ral binding activity (35) did not inhibit the secretion (Fig. 3, A and B). The time course of

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**FIGURE 5.** Anti-Sec5-RBD antibody blocked binding of Ral to the exocyst complex and inhibited the GppNHp-induced dense granule secretion. A, human platelet lysates (30 \( \mu \text{g} \) of proteins) were immunoblotted with preimmune serum and anti-Sec5-RBD antibody. B, human platelet cytosol was immunoprecipitated (IP) with preimmune serum and anti-Sec5-RBD antibody. Immunoprecipitated proteins were detected with anti-Sec5-RBD antibody or anti-Sec8 monoclonal antibody. C, glutathione-Sepharose beads coated with GST alone (lane 1), GDP-bound (lane 2), or GppNHp-bound GST-RalA (lanes 3–9) were incubated with the platelet cytosol in the presence of anti-GST antibody (Ab) (lane 4–6) or anti-Sec5-RBD antibody (lane 7–9) for 4 °C for 1 h. After washing the beads, bead-associated proteins were analyzed by immunoblotting with anti-Sec5-N-terminus polyclonal antibody and anti-Sec8 monoclonal antibody. The data shown are representative of three independent experiments with similar results. D, permeabilized platelets were first incubated with various concentrations of rabbit IgG (open squares), anti-GST antibody (open circles) and anti-Sec5-RBD antibody (closed circles) at 4 °C for 30 min and stimulated with 100 \( \mu \text{M} \) GppNHp at 30 °C for 15 min. Released [\textsuperscript{3}H]serotonin was measured as described under “Experimental Procedures.” The secretion without the GppNHp stimulation is also shown (●). Values are means ± S.E. from three independent experiments.
the GppNHp-induced secretion from permeabilized platelets is more than 10 times slower than that of the thrombin-induced secretion from intact platelets (compare Figs. 1A and 7A). This could be because of slow conversion of GDP to GppNHp on Ral in permeabilized platelets (Fig. 2, B and C). Furthermore, incubation of permeabilized platelets with GTP did not activate Ral (Fig. 2B) and did not induce appreciable secretion (Fig. 1A). This could be because of the strong GTPase-activating protein activity for Ral in permeabilized platelets. We consider that the decrease of GTP-Ral after the transient activation in thrombin-stimulated intact platelets is due to GTPase-activating protein activity in the cells, where GTP bound to Ral would be rapidly hydrolyzed to GDP (Fig. 7, B and C). On the other hand, in permeabilized platelets incubated with GppNHp, GppNHp would not be hydrolyzed once bound to Ral (Fig. 2). Therefore, we could observe the apparent difference in the time courses of the ratio of GTP-Ral between thrombin-stimulated intact platelets and GppNHp-incubated permeabilized platelets. GTPase-activating protein activity for Ral could also explain the reason why the maximal ratio of activated Ral to total in intact platelets (~6%) was lower than that in permeabilized platelets (~17%) (compare Figs. 7C and 2C).

To distinguish the role of RalA from that of RalB, we must, for example, knock down RalA or RalB in platelets or use platelets of either RalA or RalB knock-out mice. However, they are not technically available at the moment. Therefore, we could not distinguish their effects because Sec5-RBD, used as a Ral inhibitor, interacted with both RalA and RalB to a similar extent in vitro (Fig. 2A). Both RalA and RalB are present in platelets, highly similar (sharing 85% identity) (18, 19), and equally activated by agonist stimulation in intact platelets (Fig. 7, B and C). Hence, both RalA and RalB might play roles in the secretion in platelets.

Ral has several effectors (18, 19). Among them, the exocyst complex has been implicated in exocytosis. Wang et al. (46) showed that Sec5-RBD inhibits the GppNHp-induced granule secretion from permeabilized neuroendocrine PC12 cells, indicating that this secretion is Ral-dependent. They further showed that unprenylated wild-type RalA inhibits the GppNHp-induced secretion, but RalA E38R mutant lacking the Sec5 binding activity has no effect. They concluded that the exocyst complex is involved in the secretion (46). Strictly, however, if we assume a putative Ral effector that does not bind to RalA E38R, it is difficult to exclude the possibility that Ral mediates the secretion through such an effector but not the exocyst complex. Therefore, to demonstrate the involvement of the exocyst complex, we need, for example, to examine the effect of knockdown of an exocyst component or an inhibitory antibody against an exocyst component on the secretion. Here, we demonstrated the involvement of the exocyst complex by showing that an antibody against Sec5-RBD inhibited the GppNHp-induced secretion from permeabilized platelets (Fig. 5D).

In addition to Sec5, Exo84, another component of the exocyst complex, also binds to active Ral (27, 28). However, the anti-Sec5-RBD antibody used here completely abolished the interaction between Ral and the exocyst complex in vitro (Fig. 5C), suggesting that the Ral-Sec5 interaction is dominant com-
pared with theRal-Exo84 interaction, although we cannot exclude the possibility that the steric hindrance by the anti-Sec5-RBD antibody abolished the interaction between Ral and Exo84.

An increase in intracellular Ca\textsuperscript{2+} concentration is the trigger for regulated exocytosis in many cell types, including platelets (1, 5, 6). Therefore, we examined the role of Ral on the Ca\textsuperscript{2+}-induced secretion from permeabilized platelets. The amount of secretion increased with increasing Ca\textsuperscript{2+} concentration (closed squares in Fig. 6), and the addition of Sec5-RBD alone did not inhibit the secretion (open squares in Fig. 6). However, this result did not imply that Ral was not involved in platelet dense granule secretion under physiological conditions because increased Ca\textsuperscript{2+} concentration alone did not induce Ral activation without the addition of non-hydrolyzable GTP analogues in permeabilized platelets (data not shown). GppNHp shifted the Ca\textsuperscript{2+} concentration-response curve for dense granule secretion to the left (closed circles in Fig. 6), indicating that GppNHp increased the Ca\textsuperscript{2+} sensitivity of the secretion as shown previously (7). The addition of Sec5-RBD inhibited this enhancement by GppNHp (open circles in Fig. 6). Thus, we conclude that Ral could regulate the Ca\textsuperscript{2+} sensitivity of platelet dense granule secretion. In other words, Ral could increase the efficiency of exocytosis in response to low levels of intracellular Ca\textsuperscript{2+} signals.

The intracellular Ca\textsuperscript{2+} concentration measured by fluorescence indicators is typically in the range of 40 to 80 nM in resting platelets (5). Real-time monitoring of intracellular Ca\textsuperscript{2+} concentrations of platelets by confocal imaging technique under flow condition revealed that intracellular Ca\textsuperscript{2+} concentrations vary considerably, ranging from 0.2 to 2 μM during thrombus formation, and the concentration is below 1 μM in many platelets (3). Thus, it is conceivable that the Ral-exocyst pathway could contribute to the regulation of dense granule secretion in such submaximally activated platelets. Ral is abundant in platelets (20, 21) and is rapidly activated by agonist stimulation in intact platelets (17). Furthermore, thrombin stimulation induced the association of Ral and the exocyst complex in intact platelets (Fig. 7D). These observations also support the idea that the Ral-exocyst pathway plays an important role in regulated exocytosis in platelets in certain physiological conditions.

The exocyst complex has been demonstrated to be involved in constitutive exocytosis, such as polarized delivery of membrane proteins to the basolateral surface in epithelial cells (31) and delivery of glutamate receptors to the postsynaptic membrane in neurons (47, 48). On the other hand, there have been only a few reports showing the involvement of the exocyst complex in regulated exocytosis. Inoue et al. (33, 34) have reported that Exo70, a component of the exocyst complex, binds the Rho family GTPase TC10 and plays a role in the targeting of glucose transporter 4 to the plasma membrane in insulin-stimulated adipocytes. Tsuboi et al. (49) have shown that the exocyst complex serves to regulate the docking of insulin-containing vesicles at sites of release in response to glucose stimulation in pancreatic β cells, although the involvement of a small GTPase regulating the exocyst complex remains to be determined. In this study, we provided the evidence that the exocyst complex and RalGTPase could control regulated exocytosis of platelet dense granules by increasing the Ca\textsuperscript{2+} sensitivity of exocytosis. It will be important to elucidate the mechanism of how the Ral-exocyst pathway functions in regulated exocytosis, especially in the regulation of the Ca\textsuperscript{2+} sensitivity of secretion.

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