

The small GTPase Rab27B regulates amylase release from rat parotid acinar cells

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Summary

Small GTPase Rab is a large family of putative membrane trafficking proteins, and each member is thought to regulate a specific type(s) of membrane trafficking. However, little is known about the involvement of Rab protein(s) in secretory granule exocytosis in exocrine cells or the molecular mechanism underlying this process. We show that Rab27B, a closely related isoform of Rab27A that regulates lysosome-related granule exocytosis in cytotoxic T lymphocytes, is abundantly expressed on amylase-containing secretory granules in rat parotid gland acinar cells. We also identify the putative Rab27B effector protein, Slac2-c (Slp homologue lacking C2 domains-c)/MyRIP, which was originally described as a myosin Va/VIIa and actin binding protein, in rat parotid glands. The results of subcellular fractionation, immunoprecipitation and

immunohistochemical studies indicate that the Rab27B–Slac2-c complex is formed on secretory granules in vivo. The introduction of either a specific Rab27 binding domain (i.e. a recombinant Slp homology domain of Slac2-b that specifically binds Rab27A/B but not other Rabs) or functionally blocking antibodies that specifically disrupt Rab27B–Slac2-c complex in vitro strongly inhibited isoproterenol-stimulated amylase release from streptolysin O-permeabilized parotid acinar cells. Our results indicate that the Rab27B–Slac2-c complex is an important constituent of secretory granule exocytosis in parotid acinar cells.

Key words: Rab27B, Slac2-c/MyRIP, Parotid gland, Secretory granule exocytosis, Griscelli syndrome

Introduction

Parotid gland acinar cells are typical exocrine cells that secrete serous saliva containing amylase. The cells have many secretory granules containing proteins such as amylase, and the exocytosis of these proteins is induced by both the sympathetic and the parasympathetic nervous stimulation systems. In particular, a β -adrenergic stimulant, a sympathetic agonist, dramatically evokes amylase release (Butcher et al., 1975; Suzuki and Ohshika, 1985; Castle and Castle, 1998). Amylase release is thought to require several steps; the budding of secretory granules at the ER and *cis*-Golgi, maturation, transport to the apical surface, docking, priming and fusion between the secretory granule membrane and the apical plasma membrane (Castle and Castle, 1998; Dohke et al., 1998; Fujita-Yoshigaki, 1998). However, the full details of secretory granule exocytosis in the parotid gland have yet to be elucidated.

Small GTPase Rab is believed to be an essential component in the control of intracellular membrane trafficking in eukaryotic cells (Zerial and McBride, 2001; Segev, 2001; Pfeffer, 2001). More than 60 distinct Rab proteins have been identified in humans (Bock et al., 2001; Pereira-Leal and Seabra, 2001), and each member is thought to regulate a specific type of membrane trafficking (Zerial and McBride, 2001; Segev, 2001; Pfeffer, 2001). In the parotid gland, the expression of four Rab proteins, Rab3D (Ohnishi et al., 1996; Ohnishi et al., 1997; Raffaniello et al., 1999), Rab4 (Nashida et al., 2003), Rab11 (Castle et al., 2002) and Rab26 (Yoshie et

al., 2000) have been reported thus far. Among them, Rab3D has previously been proposed as a critical factor in amylase release in exocrine cells (Ohnishi et al., 1996; Ohnishi et al., 1997; Raffaniello et al., 1999). Recently, however, normal amylase release kinetics has been observed in response to secretory stimulation even in Rab3D-deficient mice; Rab3D seems to control the size of the secretory granules, rather than exocytosis itself (Riedel et al., 2002). This finding strongly indicates the presence of additional Rab(s) that regulate secretory granule exocytosis in the parotid gland.

More recently, much attention has been focused on the roles of Rab27A/Ram (Nagata et al., 1990; Chen et al., 1997; Tolmachova et al., 1999) and Rab27B/c25KG (Nagata et al., 1989; Chen et al., 1997; Ramalho et al., 2001), the closest homologues of the Rab3 subfamily (Pereira-Leal and Seabra, 2001; Fukuda, 2003a), in the control of granule exocytosis, since Rab27A proteins have been shown to regulate the late step of granule exocytosis in cytotoxic T lymphocytes (Haddad et al., 2001; Stinchcombe et al., 2001), dense-core vesicle exocytosis in some endocrine cells (Yi et al., 2002; Fukuda et al., 2002c) and dense granule secretion in platelets (Novak et al., 2002). The physiological importance of the Rab27A protein in granule exocytosis is evident, because mutations in the *RAB27A* gene cause defects in granule exocytosis in cytotoxic T lymphocytes in human hemophagocytic syndrome (Griscelli syndrome) (Ménasché et al., 2000) and *ashen* mice (Wilson et al., 2000). Patients with

Griscelli syndrome and *ashen* mice also exhibit partial albinism because of defects in melanosome transport (Bahadoran et al., 2001; Hume et al., 2001; Wu et al., 2001; Ménasché et al., 2003; Bahadoran et al., 2003). The recent discovery of Rab27 effector families (reviewed by Fukuda, 2002a), the Slp (synaptotagmin-like protein) family (Slp1/Jfc1, Slp2-a, Slp3-a, Slp4/granuphilin and Slp5) (Fukuda and Mikoshiba, 2001; Fukuda et al., 2001; Kuroda et al., 2002a; Kuroda et al., 2002b) and the Slac2 (Slp homologue lacking C2 domains) family (Slac2-a/melanophilin, Slac2-b, and Slac2-c/MyRIP) (Fukuda et al., 2001; Kuroda et al., 2002a; Matesic et al., 2001; El-Amraoui et al., 2002; Fukuda et al., 2002b; Fukuda and Kuroda, 2002; Fukuda, 2002b) has led to new insights into the mechanism of melanosome transport in melanocytes. Slac2-a directly and simultaneously interacts with Rab27A via the N-terminal Slp homology domain (SHD=Rab27A/B effector domain) (Kuroda et al., 2002a; Fukuda et al., 2002b) and with myosin Va, an actin-based motor protein, via the C-terminal domain, and the formation of a tripartite protein complex (Rab27A–Slac2-a–myosin Va) is essential for melanosome transport (Fukuda et al., 2002b; Wu et al., 2002; Strom et al., 2002; Provance et al., 2002; Kuroda et al., 2003; Fukuda and Kuroda, 2003). By contrast, little is known about the mechanism of granule exocytosis mediated by Rab27A (especially regarding the positive regulator(s) of Rab27A), although Slp family members with tandem C2 domains may be involved in this process (Fukuda et al., 2002c; Fukuda, 2002c; Fukuda, 2003b; Coppola et al., 2002; Torii et al., 2002; Zhao et al., 2002). In addition, the physiological role(s) of Rab27B, the closest isoform of Rab27A, has not been determined, although Rab27B may have functions similar to those of Rab27A (Barral et al., 2002).

In this study, we first investigated the expression of Rab27 proteins and their effectors in rat parotid glands and found that Rab27B–Slac2-c and Rab27B–Slp4-a complexes are formed on secretory granules in parotid acinar cells. We also discovered that the specific Rab27 binding domain (i.e. SHD of Slac2-b) or functionally blocking antibodies that specifically disrupt the Rab27B–Slac2-c complex, but not the Rab27B–Slp4-a complex, strongly inhibit amylase release from parotid acinar cells. Based on our findings, we propose that Slac2-c functions as a positive regulator of Rab27 protein during the exocytosis of secretory granules and that Rab27 proteins may play pivotal roles in lysosome-related granule exocytosis in some immune cells and in endocrine and exocrine exocytosis.

Materials and Methods

Antibodies

Anti-Rab27A mouse monoclonal antibody and anti-myosin VIIa rabbit polyclonal antibody were purchased from Transduction Laboratories (Lexington, KY, USA) and Alexis Biochemicals (Lausen, Switzerland), respectively. Anti-VAMP-2 (vesicle-associated membrane protein-2)/synaptobrevin-2 mouse monoclonal antibody was obtained from Synaptic Systems (Göttingen, Germany). Fluorescent dye-conjugated secondary antibodies (Alexa Fluor 594-labeled anti-mouse and Alexa Fluor 488-labeled anti-rabbit IgGs) were from Molecular Probes Inc. (Eugene, OR, USA). The anti-myosin Va rabbit polyclonal antibody was prepared as described previously (Fukuda et al., 2002b). The anti-synaptotagmin I (Syt I) rabbit polyclonal and mouse monoclonal (SYA148) antibodies were

also prepared as described previously (Fukuda et al., 2002a). New Zealand White rabbits were immunized with the purified GST fusion proteins (GST-Slp1-SHD, GST-Slp2-a-SHD, GST-Slp3-a-SHD, GST-Slp4-a-C2B, GST-Slp5-C2A, GST-Slac2-a-SHD, GST-Slac2-b-SHD, GST-Slac2-c-SHD, and GST-Rab27B) (Fukuda et al., 2002b; Fukuda et al., 2002c; Kuroda et al., 2002a; Fukuda and Kuroda, 2002) and specific antibodies were affinity-purified by exposure to antigen-bound Affi-Gel 10 beads (Bio-Rad Laboratories; Hercules, CA, USA) as described previously (Fukuda and Mikoshiba, 1999). The specificity of each antibody was checked by immunoblotting and/or immunocytochemistry with recombinant T7-tagged Slp1-5 and Slac2-a/b/c (or FLAG-tagged Rab27A/B) expressed in COS-7 cells (Fukuda et al., 2002a; Fukuda et al., 2002c) (and data not shown).

Immunoprecipitation and immunoblotting

Rat parotid glands were homogenized in a buffer containing 5 mM Hepes-NaOH, pH 7.2, 50 mM mannitol, 0.25 mM MgCl₂, 25 mM β -mercaptoethanol, 0.1 mM EGTA, 2 μ M leupeptin, 2.5 μ g/ml trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidin and 2 μ g/ml aprotinin, and the protein concentrations were adjusted to 10 mg/ml. After solubilization with 1% Triton X-100 at 4°C for 1 hour, the insoluble materials were removed by centrifugation at 17,400 g for 10 minutes. The supernatant obtained was incubated with anti-Slp4-a IgG, anti-Slac2-c IgG or anti-Syt I-C2A IgG (10 μ g/ml) for 1 hour at 4°C and then incubated with protein A-Sepharose beads (Amersham Biosciences, Buckinghamshire, UK) for 1 hour at 4°C. After washing the beads five times with 50 mM Hepes-KOH, pH 7.2, 150 mM NaCl and 0.2% Triton X-100, the proteins bound to the beads were analyzed by 7.5% (for Slp and Slac2) or 12.5% SDS-PAGE (for Rab27A/B) and then immunoblotted with anti-Rab27A mouse monoclonal antibody (1/250 dilution), anti-Rab27B (1 μ g/ml), anti-myosin Va (2 μ g/ml), anti-myosin VIIa (1/100 dilution), anti-Slp4-a (4 μ g/ml), anti-Slac2-c rabbit polyclonal antibodies (4 μ g/ml) and anti-Syt I (SYA148) mouse monoclonal antibody (1/250 dilution). SDS-PAGE and the immunoblot analysis were performed as described previously (Fukuda et al., 1999). Unless otherwise noted, SDS samples were boiled for 3 minutes with β -mercaptoethanol (i.e. reducing condition). The immunoreactive bands were visualized using enhanced chemiluminescence (Amersham Biosciences). The intensity of some immunoreactive bands on the x-ray film was quantified with a Lane Analyzer (version 3.0) (ATTO Corp., Tokyo, Japan).

Subcellular fractionation of the rat parotid glands was performed as described previously (Imai et al., 2003). Equal proportions (25 μ g) of total homogenates, the apical plasma membranes, and the secretory granule membranes of parotid acinar cells were subjected to SDS-PAGE and then immunoblotted with anti-Rab27A, anti-Rab27B, anti-Slp4-a, and anti-Slac2-c antibodies as described above.

Immunohistochemistry

Parotid glands were perfusion-fixed via the carotid artery with 4% paraformaldehyde in 0.07 M phosphate buffer (pH 7.3) and subsequently immersed in the fixative for 8 hours at room temperature. The fixed specimens were then rapidly frozen in isopentane precooled to –35°C. Frozen sections were cut at a thickness of 6 μ m using a cryostat, mounted on APS-coated glass slides and processed for the following immunostaining procedures using the indirect fluorescence method. Individual sections were incubated overnight at 4°C with a mixture of the monoclonal antibody against VAMP-2 (1/400 dilution) and a polyclonal antibody against Rab27B (1/200 dilution), Slp4-a (1/200 dilution), or Slac2-c (1/100 dilution). After incubation, the sections were exposed to a mixture of Alexa Fluor 594-labeled anti-mouse IgG (1/100 dilution) and Alexa Fluor 488-labeled anti-rabbit IgG (1/100 dilution) for 2 hours at room temperature. The stained sections were examined and photographed under a confocal fluorescence microscope (LSM 510; Carl Zeiss Co.,

Ltd., Germany). Immunoreaction specificity was checked using two negative controls: omission of the first step of the immunoreaction and incubation of tissue sections with a normal mouse or rabbit serum instead of each primary antibody. The sections subjected to the control procedures were completely immunoreaction negative, which confirmed the specificity of the immunohistochemical findings (data not shown).

Inhibition of the Rab27B–Slac2-c interaction by specific antibodies in vitro

FLAG-tagged Rab27B proteins were expressed in COS-7 cells and affinity-purified by anti-FLAG M2 Affinity Gel (Sigma Chemical Co., St Louis, MO, USA) as described previously (Kuroda et al., 2002a). The FLAG-Rab27B beads were preincubated for 30 minutes at 4°C with purified anti-Rab27B IgG or control rabbit IgG (5 µg) in 100 µl of 50 mM Hepes-KOH, pH 7.2, 150 mM NaCl, 1 mM MgCl₂, 0.2% Triton X-100 and protease inhibitors (buffer 1). COS-7 cell lysates containing either T7-Slac2-c or T7-Slp4-a were added to the beads and incubated for 1 hour at 4°C. After washing the beads five times with buffer 1, the proteins bound to the beads were analyzed by 10% SDS-PAGE followed by immunoblotting with horseradish peroxidase (HRP)-conjugated anti-T7 tag antibody (1/10,000 dilution; Novagen, Madison, WI, USA) and HRP-conjugated anti-FLAG M2 antibody (1/10,000 dilution; Sigma Chemical Co.) as described previously (Fukuda et al., 1999). The effect of the anti-Slac2-c antibody on the interaction between T7-Slac2-c and FLAG-Rab27B was similarly examined using T7-Slac2-c beads (Fukuda and Kuroda, 2002).

Preparation of parotid acinar cells and measurement of amylase release from streptolysin O (SLO)-permeabilized parotid acinar cells

The Institutional Animal Care and Use Committee of our university approved the animal protocol. Rat parotid acinar cells were prepared according to the method described by Dohke et al. (Dohke et al., 2002). We referred to Takuma and Ichida (Takuma and Ichida, 1994) and Fujita-Yoshigaki et al. (Fujita-Yoshigaki et al., 1996) for methods regarding amylase release from SLO-permeabilized acinar cells. Lyophilized powder of SLO (Sigma Chemical Co.) was dissolved in 10 mM PBS, pH 7.0, and after activation with 10 mM dithiothreitol for 1 hour at 37°C it was placed on ice until used. The parotid acinar cells were then washed twice with the incubation medium (140 mM KCl, 20 mM Hepes-NaOH, pH 7.2, 1 mM MgSO₄, 1 mM Mg-ATP, 0.1 mg/ml trypsin inhibitor, and 0.1% BSA). The cell suspension (100 µl) was pipetted into a tube containing 2 µl of 2500 Units/ml SLO with or without anti-Rab27B IgG, anti-Slac2-c-SHD IgG, control rabbit IgG, GST-Slac2-b-SHD, GST-Slac2-c-ABD (actin binding domain; amino acid residues 672–856 of mouse Slac2-c), GST-Slac2-c-ABD(RA) (Kuroda et al., 2003; Waselle et al., 2003) or GST alone and incubated at 37°C for 5 minutes. Thereafter, the suspension was stimulated by 1 µM isoproterenol for 20 minutes. The reaction was stopped by the addition of 900 µl of incubation medium, and the reaction medium was immediately collected by passage through a glass filter paper. To measure the total amylase activity, the acinar cells were homogenized in 0.1% Triton X-100. The amylase activity was then measured according to the method described by Bernfeld (Bernfeld, 1955).

Results

Expression of Rab27A, Rab27B, Slp4-a and Slac2-c in rat parotid glands

To investigate the possible involvement of small GTPase Rab27A/B and its effectors (Slp and Slac2) in amylase release from parotid acinar cells, we first investigated their expression

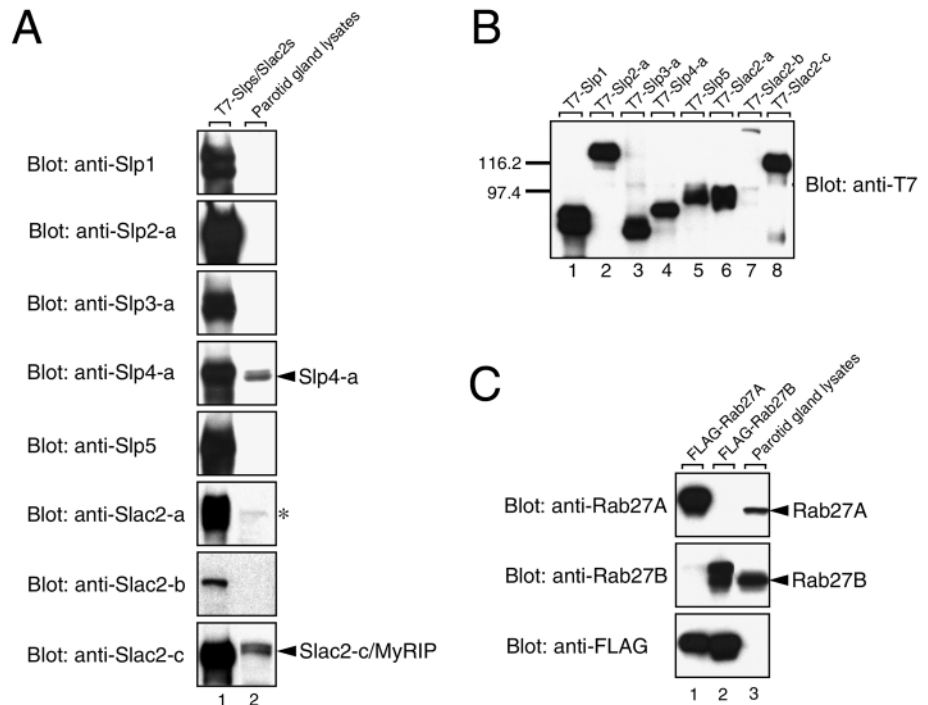
in rat parotid glands by immunoblot analyses using specific antibodies against Rab27A, Rab27B, Slp1-5 or Slac2-a/b/c. The cross-reactivity of each antibody with the other Rab27 effectors was checked by probing with recombinant T7-tagged Slp1-5 and Slac2-a/b/c (so-called ‘Rab27 effector panels’; Fig. 1B) (Fukuda et al., 2002c) and the specificity of the antibodies was confirmed (data not shown). As shown in Fig. 1C (lane 3 in the top and middle panels), rat parotid glands expressed both isoforms of Rab27, although expression of Rab27B was dominant according to the results of the quantitative analysis. Expression of two Rab27 effectors, Slp4-a (also called granuphilin) (Yi et al., 2002; Torii et al., 2002; Fukuda et al., 2002c; Fukuda, 2003b) and Slac2-c (also called MyRIP) (El-Amraoui et al., 2002; Fukuda and Kuroda, 2002), was observed in the rat parotid glands, but the other Rab27 effectors were not detected (Fig. 1A, arrowheads). Although Slac2-c was originally described as a myosin Va/VIIa binding protein in vitro (El-Amraoui et al., 2002; Fukuda and Kuroda, 2002), no expression of potential binding partners, myosin Va and VIIa, was detected by either immunoblotting or co-immunoprecipitation assays (data not shown).

Immunoprecipitation experiments were performed to determine whether the two Rab27 effectors identified actually interact with both isoforms of Rab27 under physiological conditions (Fig. 2A). Consistent with our previous in vitro binding experiments in COS-7 cells (Kuroda et al., 2002a; Fukuda and Kuroda, 2002), Slp4-a–Rab27A/B and Slac2-c–Rab27A/B complexes were easily detected in the immunoprecipitates (solid arrowheads in Fig. 2A, lanes 2 and 3), and the anti-Slp4-a antibody always immunoprecipitated Rab27A/B more efficiently than the anti-Slac2-c antibody. This difference may be explained by the fact that Slp4-a can interact with both the GTP- and GDP-bound forms of Rab27A/B (Fukuda, 2003b), whereas Slac2-c only interacts with the GTP-bound form of Rab27A/B (El-Amraoui et al., 2002; Fukuda and Kuroda, 2002). By contrast, the control anti-Syt I antibody did not immunoprecipitate Rab27A/B (Fig. 2A, lane 4), a finding consistent with the fact that Syt I is not endogenously expressed in rat parotid glands (Imai et al., 2001) and lacks an N-terminal Rab27 binding domain.

Localization of Rab27–Slac2-c and Rab27–Slp4-a complexes on the secretory granule membranes of rat parotid glands

Since the Rab27A protein is known to be present on lysosome-related secretory granules in cytotoxic T lymphocytes (Haddad et al., 2001; Stinchcombe et al., 2001) and on dense-core vesicles in some endocrine cells (Yi et al., 2002; Fukuda et al., 2002c), we next sought to determine whether Rab27A and Rab27B proteins are localized on secretory granules in exocrine cells (i.e. rat parotid acinar cells). Subcellular fractionation studies clearly indicated that both Rab27A and Rab27B proteins are highly enriched on the secretory granule membranes (Fig. 2B, lane 3 in top and second panels) of rat parotid glands. Smaller amounts of Rab27A/B proteins were also found on the apical plasma membranes (lane 2), but they were not found in the cytosolic fraction (data not shown). By contrast, Rab27 effector proteins (Slp4-a and Slac2-c) were found on both the secretory granules and the apical plasma membranes (Fig. 2B, third and bottom panels).

Fig. 1. Expression of small GTPase Rab27 and its effectors (Slp and Slac2) in rat parotid glands. (A) Expression of the Slp and Slac2 family members in rat parotid glands. Similar amounts of recombinant T7-tagged Slp1-5 and Slac2-a/b/c expressed in COS-7 cells (lane 1; see B) and total homogenates of rat parotid glands (50 μ g; lane 2) were loaded on 7.5% SDS-PAGE and immunoblotted with anti-Slp1, anti-Slp2-a, anti-Slp3-a, anti-Slp4-a, anti-Slp5, anti-Slac2-a, anti-Slac2-b, or anti-Slac2-c specific antibody. Note that the Slp4-a and Slac2-c proteins, but not the other Rab27 effectors, were easily detected in rat parotid glands (arrowheads). The asterisk indicates non-specific interaction of the anti-Slac2-a antibody. (B) Recombinant T7-Slp1-5 and T7-Slac2-a/b/c were used as positive controls in A. Similar amounts of the T7-tagged proteins except for T7-Slac2-b were loaded into each lane. The positions of the molecular mass markers (in kDa) are shown on the left. (C) Expression of Rab27A and Rab27B in rat parotid glands. The same amounts of FLAG-tagged Rab27A (lane 1) and Rab27B (lane 2), and total homogenates of rat parotid glands (50 μ g; lane 3) were loaded on 12.5% SDS-PAGE and immunoblotted with anti-Rab27A (top panel), anti-Rab27B (middle panel), or anti-FLAG tag antibody (bottom panel). Judging from the intensity of the Rab27A and Rab27B bands calibrated with the FLAG-tagged recombinant proteins, expression of Rab27B was dominant in parotid glands. Under our experimental conditions, Rab27A, Rab27B, Slp4-a, and Slac2-c were detected as single immunoreactive bands.



To further confirm the localization of Rab27B, Slac2-c and Slp4-a on the secretory granules of rat parotid glands at an immunohistochemical level, we stained endogenous Rab27B, Slac2-c and Slp4-a in the parotid acini using the specific antibodies. As shown in Fig. 3A-C, all three proteins were predominantly localized in the apical regions of the acinar cells, where secretory granules were enriched. We then compared their distribution with that of a known secretory granule marker, VAMP-2, in the single acinar cell (red in Fig. 3E,H,K) (Fujita-Yoshigaki et al., 1996). As expected, Rab27B was predominantly colocalized with VAMP-2 (green in Fig. 3D) on the secretory granules in the apical regions of the cells (yellow in Fig. 3F). Interestingly, colocalization of Slp4-a or Slac2-c with VAMP-2 was limited to the region very close to the apical plasma membrane (yellow in Fig. 3I and L, respectively). These findings, together with the results of the co-immunoprecipitation and subcellular fractionation studies described above, strongly indicate that Rab27–Slac2-c and Rab27–Slp4-a complexes are indeed formed on secretory granules in the apical regions of the parotid acinar cells.

Rab27B–Slac2-c complex controls amylase release in SLO-permeabilized parotid acinar cells

Since we and others have previously shown that Slp4-a negatively controls dense-core vesicle exocytosis in pancreatic β -cells and PC12 cells, possibly through an interaction with the GDP-bound form of Rab27A (Coppola et al., 2002; Torii et al., 2002; Fukuda et al., 2002c; Fukuda, 2003b), Slp4-a was also expected to negatively regulate amylase release in the parotid gland. By contrast, nothing is known about the endogenous

Rab27 effector(s) that positively controls regulated secretion in endocrine cells, although exogenously expressed Slp3-a or Slp5 enhances high-KCl-dependent neuropeptide Y secretion in PC12 cells (Fukuda et al., 2002c; Fukuda, 2003b). Since Slac2-c is the only Rab27-binding protein expressed in rat parotid glands, with the exception of Slp4-a, and Slac2-c is also expressed in some endocrine cells [e.g. pituitary AtT20 cells, pancreatic β -cell lines and chromaffin cells (Waselle et al., 2003); data not shown], we hypothesized that Slac2-c is a common positive regulator for Rab27 in granule exocytosis both in endocrine and exocrine tissues. To test this hypothesis, we investigated whether the Rab27–Slac2-c complex positively controls isoproterenol (IPR)-stimulated amylase release from SLO-permeabilized parotid acinar cells (see Materials and Methods for details). We used two different approaches: trapping of endogenous Rab27A/B by a specific Rab27 binding domain (i.e. the Slac2-b SHD) (Kuroda et al., 2002a) and inhibition by functionally blocking antibodies in SLO-permeabilized parotid acinar cells.

We and others previously determined that the N-terminal SHD of Slps and Slac2s functions as a specific Rab27A/B binding domain (Fukuda et al., 2001; Kuroda et al., 2002a; El-Amraoui et al., 2002; Strom et al., 2002). Expression of the SHD of Slp1-4 or Slac2-a has been shown to inhibit both melanosome transport in melanocytes (Wu et al., 2002; Strom et al., 2002; Kuroda et al., 2003) and regulated exocytosis in endocrine cells (Waselle et al., 2003). Therefore, if Rab27A/B plays a pivotal role in amylase release (i.e. granule exocytosis) in parotid gland acinar cells, introduction of a SHD fragment should inhibit amylase release by masking endogenous Rab27A/B and blocking the formation of the Rab27–effector

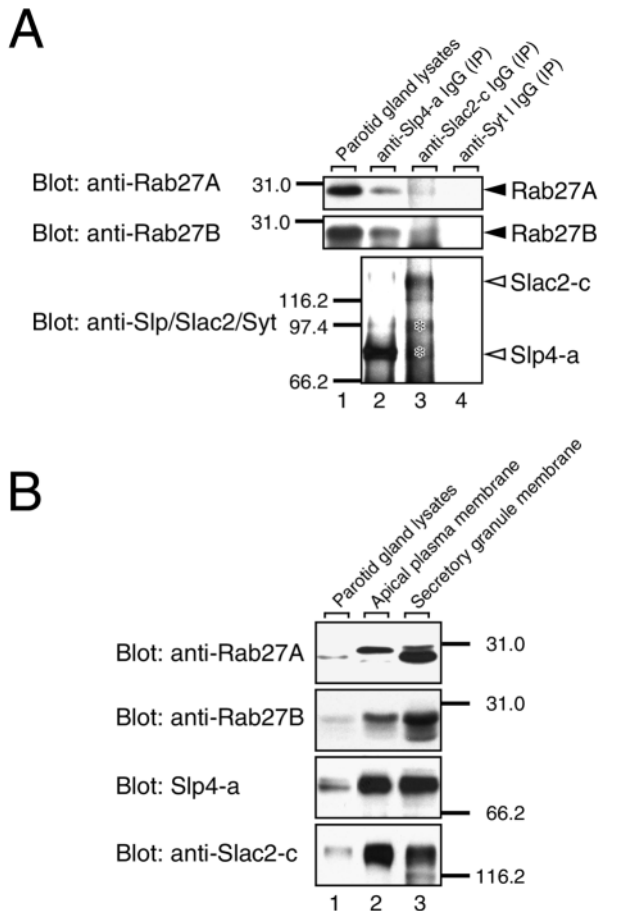


Fig. 2. Localization of Rab27–Slac2-c and Rab27–Slp4-a complexes on secretory granules in rat parotid glands. (A) In vivo formation of Rab27–Slac2-c and Rab27–Slp4-a complexes in rat parotid glands. Immunoprecipitation with anti-Slp4-a, anti-Slac2-c, or anti-Syt I IgG was performed as described in the Materials and Methods. Co-immunoprecipitated Rab27A/B was detected by immunoblotting with anti-Rab27A/B specific antibody (12.5% non-reduced gels; solid arrowheads in the top and middle panels). Immunoprecipitated Slp4-a, Slac2-c and Syt I were separately visualized by immunoblotting with specific antibodies (10% reduced gels; open arrowheads in bottom panel). The asterisks in lane 3 indicate the degradation products of Slac2-c. Note that both anti-Slp4-a and anti-Slac2-c IgGs, but not Syt I IgG, immunoprecipitated Rab27A and Rab27B (lanes 2 and 3 in the top and middle panels). The positions of the molecular mass markers (in kDa) are shown on the left. (B) Localization of Rab27A, Rab27B, Slac2-c and Slp4-a proteins in secretory-granule-enriched fractions. Subcellular fractionation of the rat parotid glands was performed as described previously (Imai et al., 2003). A 25 µg amount of total homogenate (lane 1), apical plasma membrane (lane 2), and secretory granule membrane (lane 3) from rat parotid glands was loaded on 7.5% (for Slp4-a and Slac2-c) or 12.5% SDS-PAGE (for Rab27A/B) and immunoblotted with anti-Rab27A (top panel), anti-Rab27B (second panel), anti-Slp4-a (third panel), or anti-Slac2-c antibody (bottom panel). Note that both the Rab27A and Rab27B proteins were highly enriched on the secretory granule membranes, whereas the Slp4-a and Slac2-c proteins were localized on both the apical plasma membrane and the secretory granule membrane. The positions of the molecular mass markers (in kDa) are shown on the right.

complex. To test this, we selected GST-Slac2-b-SHD as a specific Rab27 binding domain, because even after affinity-purification the GST fusion proteins of other SHDs were often

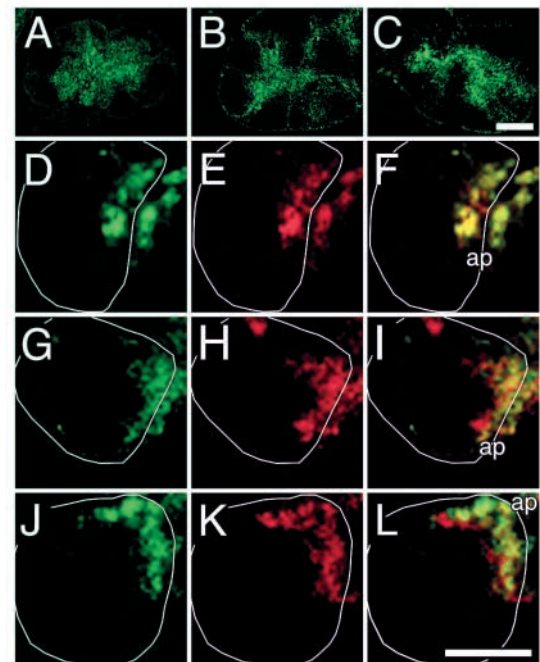


Fig. 3. Colocalization of Rab27B, Slp4-a and Slac2-c with VAMP-2, a secretory granule marker, in rat parotid acinar cells. Each section was immunostained for Rab27B (A,D), Slp4-a (B,G), or Slac2-c (C,J) and double-stained for VAMP-2 (E,H,K) to confirm the presence of secretory granules in the apical regions of the acinar cells. The merged images are shown in F, I and L. Note that Rab27B, Slp4-a and Slac2-c were mainly colocalized with VAMP-2 on secretory granules that are close to the apical plasma membrane of the acinar cells (F,I,L, respectively). The cells are outlined in white; ap, apical plasma membrane. Scale bars: 5 µm.

contaminated by degradation products and such GST-SHDs often interacted non-specifically with other Rabs (e.g. Rab3, Rab8 and Rab10) (Kuroda et al., 2002a). By contrast, GST-Slac2-b-SHD (hereafter simply designated as GST-SHD) was affinity-purified with little contamination by degradation products and specifically recognized Rab27A and Rab27B in vitro (Kuroda et al., 2002a). When GST-SHD was introduced into SLO-permeabilized parotid acinar cells, IPR-stimulated amylase release was inhibited in a dose-dependent manner (with a maximal inhibition value of around 40–50%), whereas GST alone had no effect (Fig. 4A, shaded and solid bars). Similarly, the actin binding domain of Slac2-c (GST-Slac2-c-ABD) attenuated IPR-stimulated amylase release in a dose-dependent manner, whereas the GST-Slac2-c-ABD(RA) mutant lacking actin binding capacity (Kuroda et al., 2003; Waselle et al., 2003) had no effect (Fig. 4B, shaded and solid bars).

In the second approach, we introduced functionally blocking antibodies that specifically disrupt the Slac2-c–Rab27B interaction. Interestingly, our anti-Rab27B antibody specifically inhibited the Slac2-c–Rab27B interaction in vitro (Fig. 5A, lane 2 in the middle panel), but had no effect on the Slp4-a–Rab27B interaction (Fig. 5A, lane 4 in the middle panel). This difference may be explained if the Slp4-a–Rab27B interaction is stronger than the Slac2-c–Rab27B interaction (Fukuda, 2003b). Similarly, the anti-Slac2-c antibody

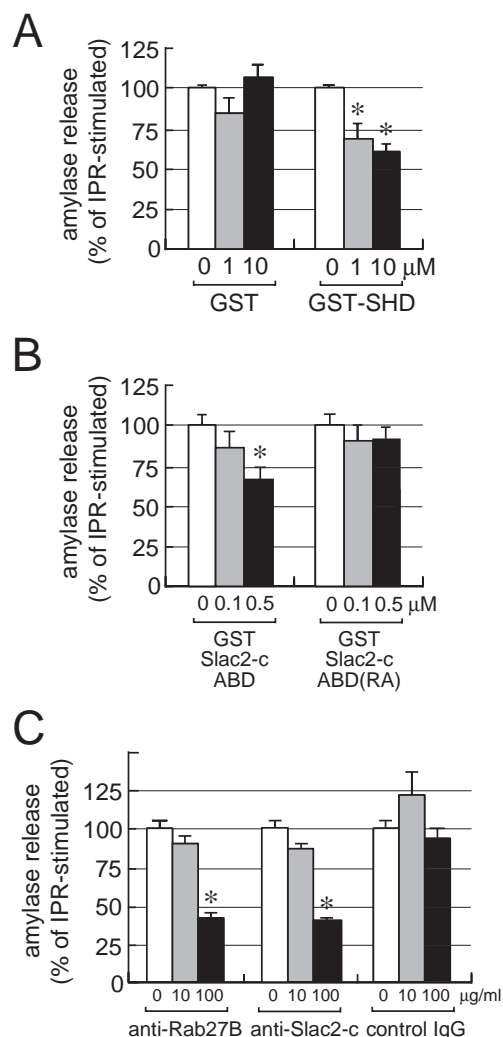


Fig. 4. Functional involvement of the Rab27B-Slac2-c complex in amylase release from SLO-permeabilized parotid acinar cells. (A) Inhibition of amylase release by SHD, a specific Rab27 binding domain, in SLO-permeabilized parotid acinar cells. GST-SHD, but not GST alone, inhibited amylase release in a dose-dependent manner. (B) Inhibition of amylase release by ABD, an actin binding domain of Slac2-c, in SLO-permeabilized parotid acinar cells. GST-ABD, but not GST-ABD(RA) lacking actin binding capacity, inhibited amylase release in a dose-dependent manner. (C) Inhibition of amylase release by anti-Rab27B and anti-Slac2-c specific antibodies. Both anti-Rab27B and Slac2-c IgGs inhibited amylase release in a dose-dependent manner. By contrast, control IgG (up to 100 μg/ml) had no significant effect on amylase release. IPR-stimulated amylase release from SLO-permeabilized parotid acinar cells was measured as described in the Materials and Methods. The released amylase activity is expressed as a percentage of the IPR-stimulated release without rabbit IgG or GST fusion proteins. Bars indicate the mean±s.e.m. of 3-5 independent experiments, performed in triplicate. * $P < 0.01$, Student's *t*-test.

specifically disrupted the Slac2-c-Rab27B interaction (Fig. 5B, lane 2 in middle panel), but not the Slp4-a-Rab27B interaction (data not shown). Thus, these two antibodies were useful tools for the specific disruption of the Slac2-c-Rab27B complex in vivo, without evaluating the involvement of the Slp4-a-Rab27B complex, a possible negative regulator of

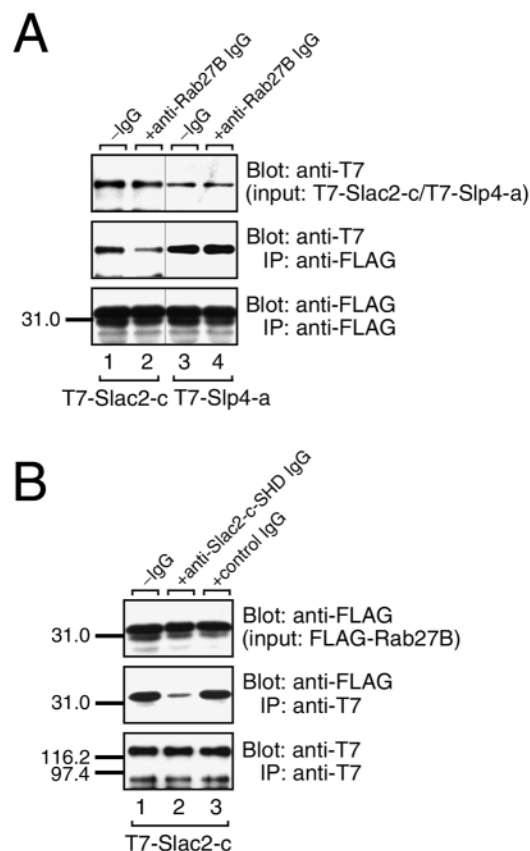


Fig. 5. Effect of anti-Rab27B and anti-Slac2-c-SHD antibodies on the interaction between Rab27B and Slac2-c in vitro. (A) FLAG-Rab27B binding activity of T7-Slac2-c and T7-Slp4-a in the presence or absence of anti-Rab27B IgG. The FLAG-Rab27B beads (bottom panel) were incubated with T7-Slac2-c (lanes 1 and 2) or T7-Slp4-a (lanes 3 and 4) in the presence or absence of the anti-Rab27B IgG as described in the Materials and Methods, and the T7-tagged proteins trapped by the beads were analyzed by immunoblotting with HRP-conjugated anti-T7 tag antibody (1/10,000 dilution) as described previously (Fukuda et al., 1999). Note that the anti-Rab27B IgG specifically disrupted the Rab27B-Slac2-c interaction (lane 2, middle panel) but had no effect on the Rab27B-Slp4-a interaction (lane 4, middle panel). (B) Inhibition of the Rab27B-Slac2-c interaction by the anti-Slac2-c antibody. The T7-Slac2-c beads (bottom panel) were incubated with either the anti-Slac2-c IgG or a control rabbit IgG, and the FLAG-Rab27B trapped by the beads was analyzed by immunoblotting with HRP-conjugated anti-FLAG tag antibody as described previously (Fukuda et al., 1999; Fukuda et al., 2002b). Note that the anti-Slac2-c IgG, but not control IgG, inhibited the interaction between Rab27B and Slac2-c (compare lanes 2 and 3 in the middle panel). Input means 1/80 volume of the reaction mixtures used for the immunoprecipitation studies (top panels in A and B). The positions of the molecular mass markers (in kDa) are shown on the left.

amylase release. When either the anti-Rab27B or anti-Slac2-c antibody was introduced into SLO-permeabilized parotid acinar cells, IPR-stimulated amylase release was severely inhibited with a maximal inhibition value exceeding 60% (Fig. 4C, solid bars). It is important to note that these antibodies failed to inhibit basal amylase release (i.e. non IPR-stimulated release; data not shown), suggesting that Rab27B and Slac2-c

are involved in a late step of granule exocytosis rather than the granule biogenesis or granule translocation step. By contrast, control IgG had no significant effect on either IPR-stimulated or basal amylase release when applied at concentrations up to 100 µg/ml. Taken together, these results strongly indicate that the interaction of small GTPase Rab27B (and possibly Rab27A) with Slac2-c is a crucial process for regulating granule exocytosis (i.e. IPR-stimulated amylase release) in rat parotid acinar cells.

Discussion

We have demonstrated that Rab27B (and possibly Rab27A), a closely related homologue of the Rab3 isoforms (Rab3A/B/C/D) (Pereira-Leal and Seabra, 2001; Fukuda, 2003a) is present on amylase-containing secretory granules (Figs 2 and 3) and that this protein regulates amylase release from rat parotid acinar cells (Fig. 4). It should be noted that Rab27A is the only Rab associated with human disease (Ménasché et al., 2000; Seabra et al., 2002) and that patients with human Griscelli syndrome, and mice with the corresponding disease model, *ashen*, exhibit a defect in the late step of granule exocytosis in cytotoxic T lymphocytes (Ménasché et al., 2000; Haddad et al., 2001; Stinchcombe et al., 2001). Over-expression studies also indicate that in some endocrine cells Rab27A regulates dense-core vesicle exocytosis rather than vesicle biogenesis (Yi et al., 2001; Fukuda et al., 2002c). These observations together with the findings in our study on the functional blocking of Rab27B with specific antibodies clearly indicate that Rab27B regulates a secretory granule exocytosis step in parotid acinar cells rather than a granule biogenesis or granule translocation step, in contrast to the role of Rab3D in the regulation of secretory granule size (Riedel et al., 2002).

How does Rab27B (or Rab27A) activate granule exocytosis? By using functionally blocking antibodies we were able to show that the interaction of Rab27B with a Rab27 effector, Slac2-c/MyRIP (El-Amraoui et al., 2002; Fukuda and Kuroda, 2002), is a crucial step for amylase release (Fig. 4C). Slac2-c was originally described as a myosin Va/VIIa and actin binding protein and was thought to regulate retinal melanosome transport in retinal pigment epithelium cells (El-Amraoui et al., 2002) in a manner analogous to the function of Slac2-a in melanosome transport in melanocytes (Fukuda et al., 2002b; Wu et al., 2002; Strom et al., 2002; Provance et al., 2002; Kuroda et al., 2003). Interestingly, however, Slac2-c is also present at presynaptic areas in retinal photoreceptor cells (El-Amraoui et al., 2002) and on secretory granules in several endocrine cells (Waselle et al., 2003) as well as parotid acinar cells (this study), suggesting that Slac2-c may have an additional role(s) in granule exocytosis that is independent of myosins. Actually, neither myosin Va nor VIIa was expressed in rat parotid glands, and the Slac2-c–myosin complex was not detected by a co-immunoprecipitation assay. Since the ABD of Slac2-c, but not the ABD(RA) mutant, inhibits amylase release (Fig. 4B) and actin filaments are essential for amylase release (Tojyo et al., 1989), the most likely function of Slac2-c is as a bridge between the GTP-Rab27A/B on the secretory granules and the actin filaments near the apical plasma membrane (Waselle et al., 2003). Consistent with this explanation, Slac2-c was also enriched at the apical plasma membrane of rat

parotid acinar cells (Fig. 2B). We also identified Slp4-a, a possible negative regulator for Rab27A in endocrine cells (Coppola et al., 2002; Torii et al., 2002; Fukuda et al., 2002c; Fukuda, 2003b) on secretory granules in rat parotid glands. Based on these results, we propose that both exocrine and endocrine cells partially share common machinery for granule exocytosis (i.e. Rab27A/B–Slac2-c and Rab27A/B–Slp4-a complexes), although the two secretion systems are often triggered by different upstream signals (e.g. Ca^{2+} versus cAMP). Further work is needed to determine how different upstream signals can activate Rab27 proteins during granule exocytosis.

In summary, we have discovered that Rab27B is present on amylase-containing secretory granules in the rat parotid gland and that the Rab27B–Slac2-c complex is essential for amylase release. To our knowledge, Rab27B is the first Rab protein known to be directly involved in the exocytosis of secretory granules in parotid acinar cells. Our findings indicate that Rab27A/B proteins are not a specific regulator of the transport of lysosome-related organelles (e.g. melanosomes and lysosome-related granules in cytotoxic T lymphocytes) and that they may regulate a larger variety of granule exocytosis mechanisms, including endocrine (Yi et al., 2001; Fukuda et al., 2002c; Waselle et al., 2003; Desnos et al., 2003) and exocrine exocytosis, than previously thought (Tolmachova et al., 2003).

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