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The small GTPase Rab33A participates in regulation of amylase release from parotid acinar cells

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ABSTRACT

Amylase is released from exocrine parotid acinar cells via typical exocytosis. Exocytosis of amylase-containing granules occurs through several steps, including formation, maturation, and transport of granules. These steps are thought to be regulated by members of the small GTPase Rab family. We previously demonstrated that Rab27 and its effectors mediate amylase release from parotid acinar cells, but the functional involvement of other Rab proteins in exocrine granule exocytosis remains largely unknown. Here, we studied isoproterenol (IPR)-induced amylase release from parotid acinar cells to investigate the possible involvement of Rab33A, which was recently suggested to regulate exocytosis in hippocampal neurons and PC12 cells. Rab33A was endogenously expressed in parotid acinar cells and present in secretory granules and the Golgi body. Functional ablation of Rab33A with anti-Rab33A antibody or a dominant-negative Rab33A-T50N mutant significantly reduced IPR-induced amylase release. Our results indicated that Rab33A is a novel component of IPR-stimulated amylase secretion from parotid acinar cells.

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1. Introduction

Small GTPase Rab proteins are believed to be essential components in the control of intracellular membrane trafficking in eukaryotic cells [1–4]. More than 60 distinct Rab proteins have been identified in mice and humans, and they make up the largest subfamily among the Ras superfamily of small GTPases [3,5,6]. The membrane trafficking system plays a crucial role not only in general housekeeping of intracellular events in all cell types, but also in regulated exocytosis in secretory cells.

Parotid gland acinar cells are typical exocrine cells that secrete serous saliva containing amylase. Amylase and many other proteins are synthesized and stored in secretory granules in these cells and are released by exocytosis, which is dramatically enhanced by β-adrenergic stimulation with isoproterenol (IPR) [7–9]. The process of amylase release occurs via several steps, including budding of secretory granules at the endoplasmic reticulum and *cis*-Golgi, followed by granule maturation, transport to the luminal surface, docking, priming, and fusion with the apical plasma membrane (APM) [10,11]. We previously reported that Rab27 and its effectors regulate secretory granule exocytosis in parotid acinar cells and that proper GDP/GTP cycling of Rab27 is required for IPR-induced amylase release [12,13]. In addition, several other Rab proteins, including Rab3D, Rab4, Rab11, and Rab26, are involved in exocytosis and/or endocytosis in parotid acinar cells [14–19].

Rab33 is not as well characterized as other Rab proteins and may be involved in membrane trafficking on autophagosomes or in the Golgi body [20–22]. Two isoforms, Rab33A and Rab33B, are present in mice and humans. In contrast to the ubiquitous expression of Rab33B [23,24], Rab33A expression is more limited and is found in

Abbreviations: APM, apical plasma membrane; BLM, basolateral plasma membrane; Cyto, cytosol; DAPI, 6-diamidino-2-phenylindole; EGTA, ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GST, glutathione *S*-transferase; ICM, intracellular membrane; IPR, isoproterenol; LAMP1, lysosome-associated membrane protein 1; RT-PCR, reverse transcription-PCR; SGM, secretory granule membrane; SLO, streptolysin O; VAMP2, vesicle-associated membrane protein 2.

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the brain, lymphocytes, melanocytes, and neuroendocrine PC12 cells [24–26]. Rab33A mediates anterograde vesicular transport for membrane exocytosis and axon outgrowth in cultured rat hippocampal neurons [26]. However, whether Rab33A is endogenously expressed in parotid acinar cells and whether it mediates exocrine amylase release are unknown.

In this study, we investigated the expression and subcellular distribution of Rab33A in rat parotid acinar cells as a model of exocrine exocytosis. We examined the effect of functional blocking of Rab33A on IPR-induced amylase release from the parotid acinar cells.

2. Materials and methods

2.1. Antibodies and materials

Two anti-Rab33A antibodies were used in this study. A rabbit polyclonal antibody that specifically recognizes Rab33A, but not Rab33B, was prepared as described [24,27]. The other anti-Rab33A antibody was obtained commercially from Proteintech (Chicago, IL). Unless otherwise specified, throughout this study, "anti-Rab33A antibody" refers to the former antibody synthesized by Tsuboi and Fukuda [27]. Purified mouse anti-γ-adaptin and anti-GM130 antibodies were purchased from BD Biosciences (San Jose, CA). Anti-lysosome-associated membrane protein 1 (LAMP1), anti-Rab27, and anti-vesicle-associated membrane protein 2 (VAMP2) antibodies were from Calbiochem (San Diego, CA), Immuno-Biological Laboratories (Takasaki, Japan), and Synaptic Systems (Göttingen, Germany), respectively. QUICK-clone of rat brain was purchased from TaKaRa Bio (Kyoto, Japan). Recombinant GST fusion proteins of Rab33A were prepared using the standard method as described [22,28].

2.2. Preparation of parotid acinar cells and RT-PCR

All animal protocols were designed and performed in accordance with the Guidelines of The Nippon Dental University for Care and Use of Laboratory Animals. Parotid acinar cells were prepared from the parotid glands of male Wistar rats (approximately 10 weeks of age) as described [29]. Total RNA from rat parotid acinar cells was prepared using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was performed as described [30] using specific primers corresponding to *Rab33a* (forward: 5′-TGCATGCAGTGGTCTTTGTC-3′; and reverse: 5′-TTCAGGGCTAAGTTGGAGGG-3′) and *Gapdh* (forward: 5′-AACATCATCCCTGCATCCAC-3′; and reverse: 5′-GACAACCTGGTCCTCAGTGT-3′).

2.3. Preparation of cell membranes and cytosolic fractions and western blotting

Subcellular fractions were prepared as described [31–34]. Specifically, parotid acinar cells were homogenized using a glass homogenizer with a Teflon pestle. Five subcellular fractions were prepared from the homogenate by differential centrifugation: the basolateral plasma membrane (BLM), the APM, the intracellular membrane (ICM), the secretory granule membrane (SGM), and the cytosol (Cyto) [12]. A protein assay was performed using a protein assay kit (BioRad, Hercules, CA). SDS-PAGE and western blot analysis were performed as described [12]. Proteins were separated by electrophoresis on 10% or 12.5% SDS-polyacrylamide gels and transferred to PVDF membranes using the iBlot® dry blotting system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Membranes were probed with anti-Rab33A (1:1000), anti-γ-adaptin (1:5000), anti-GM130 (1:1000), anti-LAMP1 (1:1000), or anti-Rab27 (1:200) antibodies and washed. The membranes were incubated with anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich) (1:3000) or peroxidase-conjugated rabbit anti-mouse IgG + A + M (Zymed, South San Francisco, CA) (1:5000) and washed again. Immunoreactive bands were visualized using Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific Inc., Rockford, IL) according to the manufacturer's instructions.

2.4. Immunohistochemistry

Parotid glands were fixed with 4% (w/v) paraformaldehyde dissolved in 0.07 M phosphate buffer (pH 7.3) for 8 h at 25 °C. Fixed specimens were then rapidly frozen in isopentane that was precooled to −35 °C. Frozen sections were cut at 6-μm thickness using a cryostat, mounted on amino silane-coated glass slides (Matsunami Glass, Osaka, Japan), and immunostained using the indirect fluorescence method. Individual sections were incubated overnight at 4 °C with a mixture of monoclonal antibody against VAMP2 (1:1000) or GM130 (1:200) and polyclonal antibody against Rab33A (1:100). Sections were washed and incubated with a mixture of Alexa Fluor 594-labeled anti-mouse IgG (1:100) and Alexa Fluor 488-labeled anti-rabbit IgG (1:100) for 2 h at room temperature. DAPI was used for nuclear counterstaining. Stained sections were examined and photographed using a confocal laser-scanning microscope (LSM 710; Carl Zeiss, Göttingen, Germany).

2.5. Amylase release from streptolysin O (SLO)-permeabilized parotid acinar cells

Amylase release from SLO-permeabilized acinar cells was measured as described [13]. Parotid acinar cells were washed twice with incubation medium (20 mM HEPES-NaOH, pH 7.2, 140 mM KCl, 1 mM $MgSO_4$, 1 mM Mg-ATP, 0.1 mg/ml trypsin inhibitor, and 0.1% bovine serum albumin). The effect of anti-Rab33A antibody (normal rabbit IgG served as a control) or purified recombinant GST fusion proteins on amylase release was investigated as follows. The cell suspension (100 μl) was pipetted into a tube containing 2 μl of 2500 U/ml SLO (diluted in 10 mM PBS, pH 7.0, 10 mM dithiothreitol) and either the effectors or control effectors and incubated at 37 °C for 5 min. The suspension was then stimulated with 1 μM IPR for 20 min, followed by addition of 900 μl incubation medium and immediate filtration through glass filter paper to remove acinar cells. The filtrate, which contained released amylase, was used for the amylase assay. On the other hand, the maximum total amylase activity of the system was assayed from a centrifuged supernatant after acinar cells were homogenized in 0.1% Triton X-100. Amylase activity was measured as described by Bernfeld [35]. Activity was measured as the amount of maltose that was produced from starch. The difference between amylase activity in the presence and absence of IPR in control conditions was considered 100% amylase release.

3. Results

3.1. Endogenous Rab33A expression in parotid acinar cells

To investigate the possible involvement of Rab33A in amylase release from parotid acinar cells, we first investigated its mRNA expression in rat parotid acinar cells with RT-PCR. As shown in Fig. 1A, bands of the expected size (175 bp for *Rab33a* and 237 bp for *Gapdh*) were detected in both parotid cells and brain. We next investigated the expression of Rab33A protein with western blotting using two different anti-Rab33A antibodies. Consistent with the results of previous studies [24,26], a single immunoreactive

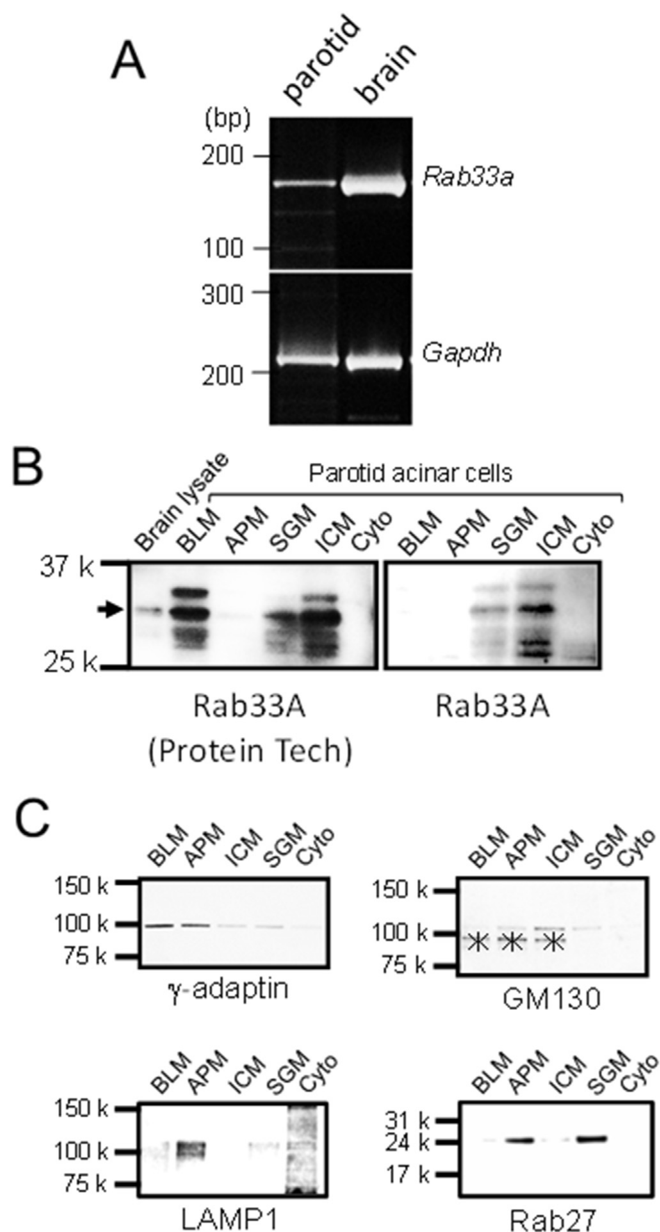

Fig. 1. Expression of Rab33A in rat parotid acinar cells. (A) Expression of mRNA for *Rab33a* in rat parotid acinar cells was analyzed with RT-PCR and compared with expression in the rat brain. *Gapdh* was used as a loading control. Molecular weight markers (in base pairs) are shown on the left. (B) Expression of Rab33A protein in subcellular fractions of rat parotid acinar cells was analyzed with western blotting with anti-Rab33A antibodies. BLM, APM, SGM, ICM, and Cyto indicate fractions enriched for basolateral plasma membranes, apical plasma membranes, secretory granule membranes, intracellular membranes, and the cytosol, respectively. Molecular mass markers (in kDa) are shown on the left. The arrow indicates the position of the Rab33A band. (C) Western blot analysis of γ-adaptin, GM130, LAMP1, and Rab27 in subcellular fractions of rat parotid gland acinar cells. The asterisk corresponds to presumably non-specific bands of the anti-GM130 antibody.

band was detected in the brain lysate, and similar immunoreactive bands were also observed in the parotid acinar cell lysate (Fig. 1B).

Intracellular localization of Rab33A in parotid acinar cells was investigated using subcellular fractions. Rab33A was enriched in the ICM fraction and also found in the SGM fraction to a lesser extent. In contrast, no Rab33A signal was detected in the APM or Cyto fractions. Unexpectedly, an immunoreactive band was observed in the BLM fraction only when the commercial antibody against Rab33A was used. Because the specificity of the commercial antibody has not yet been determined in detail, the observed band in the BLM fraction presumably corresponds to other Rab isoforms such as Rab33B or to unrelated proteins.

To determine the organelle localization of Rab33A, the same subcellular fractions were probed with antibodies against several organelle markers, including γ-adaptin as an early endosome and a late Golgi/*trans*-Golgi network marker, GM130 as a Golgi marker, LAMP1 as a lysosome marker, and Rab27 as a secretory granule and luminal plasma membrane marker [13,30] (Fig. 1C). Early endosomes and the trans-Golgi network were included in the BLM and APM fractions, the Golgi body was included mainly in the ICM fraction, lysosomes were contained in the APM fraction, and Rab27 was contained in the APM and SGM fractions. These results suggested that the Rab33A protein is present in secretory granules and the Golgi body.

3.2. Intracellular localization of Rab33A in parotid acinar cells

To confirm the intracellular localization of Rab33A in parotid acinar cells, we performed immunohistochemistry with antibodies against Rab33A, VAMP2 (a secretory granule marker) [36,37], and GM130 (a Golgi marker). As shown in Fig. 2A, Rab33A and VAMP2 were extensively colocalized in secretory granules in parotid acinar cells. In addition, Rab33A and GM130 were partially colocalized in the Golgi body of acinar cells (Fig. 2B).

3.3. Functional involvement of Rab33A in IPR-induced amylase release from SLO-permeabilized parotid acinar cells

Finally, we used two independent approaches to investigate the involvement of Rab33A in IPR-induced amylase release from parotid acinar cells. First, we added the anti-Rab33A antibody to SLO-permeabilized parotid acinar cells and evaluated its effect on IPR-induced amylase release. The anti-Rab33A antibody inhibited IPR-induced amylase release in a dose-dependent manner (up to approximately 60% of control IPR-induced amylase release). The control rabbit IgG had no effect under the same experimental conditions (Fig. 3).

Second, we synthesized two purified GST-Rab33A mutants, a dominant-negative GST-Rab33A T50N (TN) mutant, which mimics GDP-Rab33A, and a constitutively active GST-Rab33A Q95L (QL) mutant, which mimics GTP-Rab33A. These mutants (0, 10, 25 and 50 μg/ml) were incubated with SLO-permeabilized parotid acinar cells at 37 °C for 5 min, and their effect on amylase release induced by 1 μM IPR was evaluated (Fig. 4). Intriguingly, GST-Rab33A TN inhibited IPR-induced amylase release in a dose-dependent manner (up to approximately 70% of the control with 50 μg/ml GST-Rab33A TN). In contrast, no significant effect was observed for GST-Rab33A QL (Fig. 4) or GST alone (data not shown). Taken together, these results indicated that Rab33A is a novel component of the IPR-induced amylase release machinery in parotid acinar cells.

4. Discussion

The pathway of IPR-induced amylase release from salivary acinar cells includes granule formation, maturation, transport, tethering, docking, and fusion. These cellular events include a variety of membrane trafficking processes. We have previously shown that Rab26 and Rab27 regulate amylase-containing granule exocytosis in parotid acinar cells [13,17], but whether other Rab proteins are also involved in exocrine amylase release remains unknown. In the present study, we focused on Rab33A, which has recently been reported to be involved in vesicle exocytosis in other

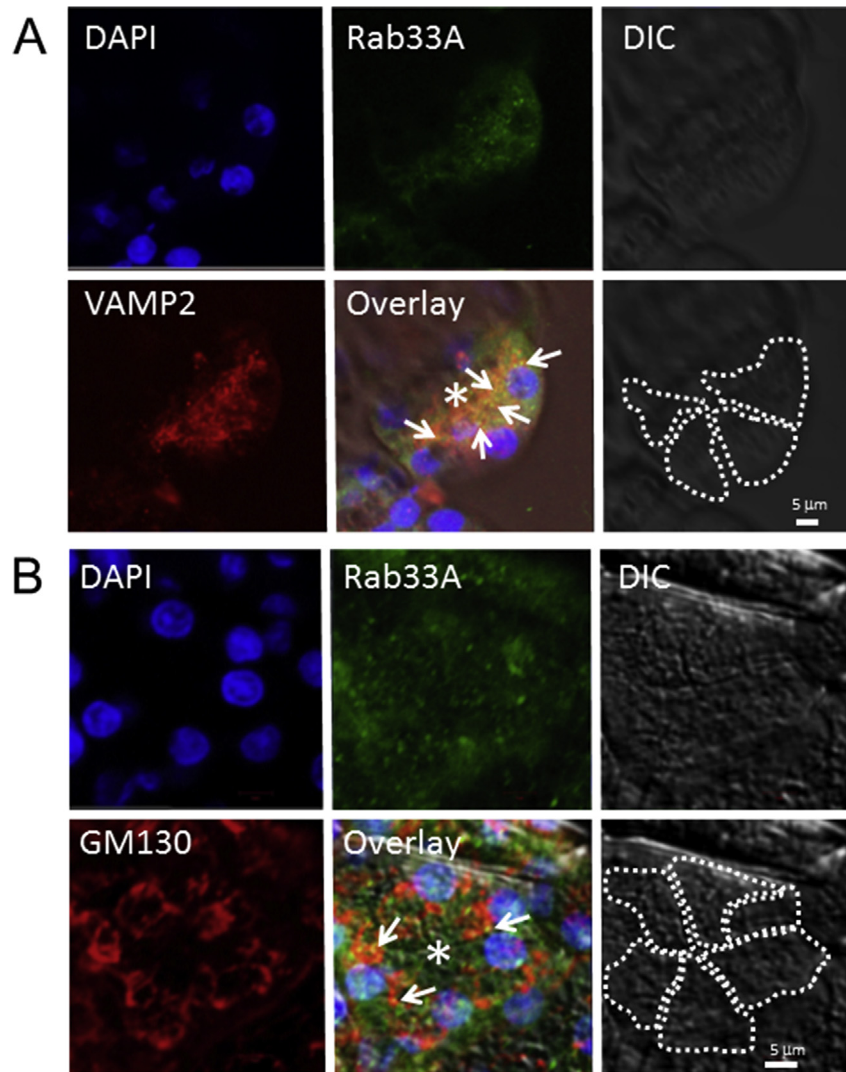

Fig. 2. Colocalization of VAMP2 or GM130 with Rab33A in parotid acinar cells was analyzed with immunohistochemistry. The sections were coimmunostained for Rab33A (green) and VAMP2 (red, A) or GM130 (red, B). The nuclei were stained with DAPI (blue). The asterisk indicates an acinar luminal site. The arrows indicate double-stained areas (yellow). Scale bars, 5 µm.

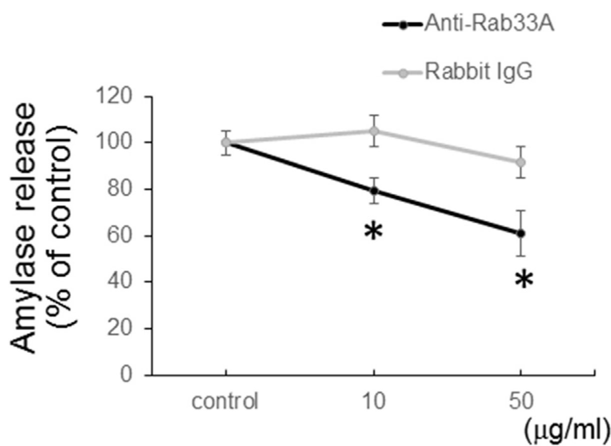

Fig. 3. Anti-Rab33A antibody inhibited amylase release in a dose-dependent manner. IPR-stimulated amylase release from SLO-permeabilized parotid acinar cells was expressed as a percentage of the IPR-stimulated release without rabbit IgG. Bars indicate the means ± SE of four independent experiments performed in triplicate. Data were analyzed with two-way ANOVA, followed by Williams' post-hoc test. $^{*}P < 0.01$.

cell types, including neurons, PC12 cells, and human umbilical vein endothelial cells [24,26,38]. We investigated the role of Rab33A on IPR-induced amylase release from rat parotid acinar cells. We found that Rab33A was endogenously expressed in parotid acinar cells (Fig. 1A and B) and was localized in both secretory granules and the Golgi body (Figs. 1B and 2). We then used two reagents, anti-Rab33A IgG and a dominant-negative Rab33A TN construct, and showed that Rab33A is functionally involved in IPR-induced amylase release from parotid acinar cells (Figs. 3 and 4).

How Rab33A regulates amylase release is an unanswered question that requires further study. Based on the results of subcellular analyses (Fig. 1B and C), we hypothesize that Rab33A functions upstream of Rab27A during amylase release. Rab27 is present in both secretory granules and the APM. Rab27 generally mediates docking of secretory granules to the plasma membrane through interaction with its effector molecule [39], and it moves to the APM from the granule membrane during exocytosis [37]. We have identified the Rab27 effectors, synaptotagmin-like protein 4-a (Slp4-a)/granuphilin-a, a Slp homologue lacking C2 domains (Slac2-c)/MyRIP, and Noc2, in secretory granules or the APM. These effectors are involved in the control of IPR-induced amylase release

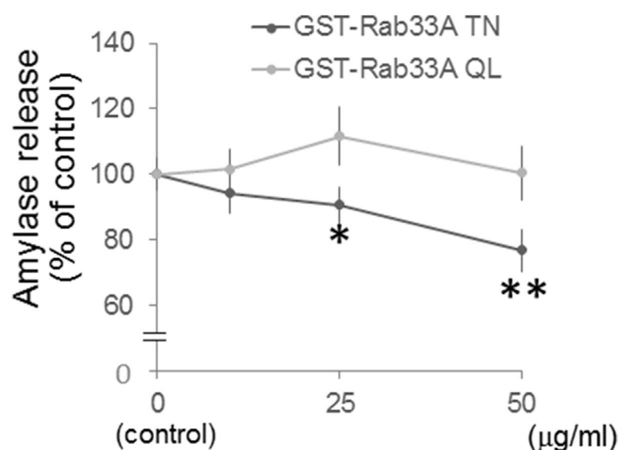

Fig. 4. Effect of GST-Rab33A T50N (TN) and GST-Rab33A Q95L (QL) on amylase release from SLO-permeabilized parotid acinar cells. GST-Rab33A TN inhibited amylase release in a dose-dependent manner, but GST-Rab33A QL did not. IPR-stimulated amylase release from SLO-permeabilized parotid acinar cells was expressed as a percentage of the IPR-stimulated release without GST fusion proteins. Bars indicate the means ± SE of four independent experiments performed in triplicate. Data were analyzed with two-way ANOVA, followed by Williams' post-hoc test. $^{*}P < 0.05$. $^{**}P < 0.01$.

from parotid acinar cells [13,16,30]. We have also identified a Tre-2/Bub2/Cdc16 domain-containing protein, EPI64, as a GTPase-activating protein for Rab27 at the APM [40]. In striking contrast, Rab33A was present in both secretory granules and the Golgi body, but was not found at the APM. The Golgi body is where immature secretory granules are synthesized, and Rab33 regulates transport between the Golgi body and endoplasmic reticulum [41]. Thus, Rab33A is likely involved in early steps of amylase release, e.g., in secretory granule formation and maturation rather than in exocytosis itself. Another important remaining question is which Rab33A effectors function during amylase release. Currently, at least three Rab33A-binding proteins, RUFY2, RUFY3, and Atg16L1, have been reported [24,42], but their involvement in exocrine amylase release has not been investigated. Future investigation is necessary to answer these two questions.

In summary, we show that Rab33A is present in secretory granules and the Golgi body in rat parotid acinar cells. This study is the first to demonstrate that Rab33A participates in regulation of amylase release from SLO-permeabilized exocrine parotid acinar cells.

Conflict of interest

None of the authors of this work has any conflict of interest.

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