EPI64 Protein Functions as a Physiological GTPase-activating Protein for Rab27 Protein and Regulates Amylase Release in Rat Parotid Acinar Cells

Akane Imai, Sumio Yoshie, Koutaro Ishibashi, Maiko Haga-Tsujimura, Tomoko Nashida, Hiromi Shimomura, and Mitsunori Fukuda

From the Departments of Biochemistry and Histology, The Nippon Dental University, School of Life Dentistry at Niigata, 1-8 Hamaura-cho, Chuo-ku, Niigata 951-8580, Japan and the Laboratory of Membrane Trafficking Mechanisms, Department of Developmental Biology and Neurosciences, Graduate School of Life Sciences, Tohoku University, Aobayama, Aoba-ku, Sendai, Miyagi 980-8578, Japan

Rab27, a small GTPase, is generally recognized as an important regulator of secretion that interacts with Rab27-specific effectors to regulate events in a wide variety of cells, including endocrine and exocrine cells. However, the mechanisms governing the spatio-temporal regulation of GTPase activity of Rab27 are not firmly established, and no GTPase-activating protein (GAP) specific for Rab27 has been identified in secretory cells. Previously, we identified a TBC protein, EPI64, as a candidate GAP specific for Rab27 in response to IPR stimulation, and that this activity is required for IPR-induced amylase release.

Small GTPase Rab27 proteins are believed to play important roles in intracellular membrane trafficking (reviewed in Refs. 1–4). More than 60 distinct Rab isoforms have been identified in humans and mice, and these proteins form a large subfamily of the small GTPase superfamily (5–7). Rab activity is regulated by the GDP/GTP cycle (1–4, 8), with GTP-Rab and GDP-Rab representing active and inactive forms, respectively. Distinct individual Rab interact with a specific effector/regulator, and these complexes regulate different steps of membrane trafficking in cells, and they function as either an accelerator or a brake (9, 10). GAP bound to a particular Rab is hydrolyzed by intrinsic GTPase activity, and this activity is enhanced by a specific Rab-GAP-activating protein (GAP) (11).

The TBC (Tre-2/Bub2/Cdc16) domain is a conserved protein motif with ~200 amino acids and is known to be present in a variety of molecules in eukaryotic organisms (12). Because the TBC domain of yeast, Gypsy (GAP for Ypt proteins), has been shown to function as a GAP domain for small GTPase Ypt/Rab, TBC domain-containing proteins (referred to as TBC proteins hereafter) in other species are also expected to function as specific Rab-GAPs. Humans and mice each have more than 40 TBC proteins, and substantial evidence has accumulated recently indicating that some mammalian TBC domains function as a GAP domain for the Rab family small GTPases (11, 12) (see also Table in Ref. 13). However, since the GAP activity of most of the mammalian TBC proteins has been determined by in vitro GAP assays or by forced overexpression of TBC proteins in cultured cells, little is known about the Rab-GAP function of “endogenous” TBC proteins in mammalian cells.

Previously, we identified a TBC protein, EPI64, as a candidate GAP specific for Rab27; Rab27 regulates a variety of secretion

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[2] To whom correspondence may be addressed: Dept. of Biochemistry, School of Life Dentistry at Niigata, The Nippon Dental University, 1-8 Hamaura-cho, Chuo-ku, Niigata 951-8580, Japan. Tel.: 81-25-267-1500; Fax: 81-25-267-1134; E-mail: imaiaki@ntg.ndu.ac.jp.

[3] The abbreviations used are: GAP, GTPase-activating protein; AP, apical plasma membrane; AQ5, aquaporin 5; BLM, basolateral plasma membrane; EPI64, EBSP50-PDZ interactor of 64 kDa; IgG, immunoglobulin G; IPR, isoprotolenol; LNA, locked nucleic acid; RBD35, Rab-binding domain specific for Rab35; RT, reverse transcription; SGM, secretory granule membrane; SHD, synaptotagmin-like protein homology domain; SLO, streptolysin O; TBC, Tre-2/Bub2/Cdc16.

[4] § The JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 39, pp. 33854 –33862, SEPTEMBER 30, 2011 © 2011 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
events in secretory cells and melanosome transport in melanocytes (reviewed in Ref. 14). EPI64 (also called TBC1D10A/Rab27A-GAPα) was originally described as an EB50-binding protein (EBP50-PDZ interactor of 64 kDa) involved in microvillar formation (15, 16). We showed that overexpression of EPI64 in cultured melanocytes caused dissociation of endogenous Rab27A from mature melanosomes and induced peri-nuclear aggregation of melanosomes as a result of the Rab27A inactivation (17). However, we have not determined whether endogenous EPI64 protein functions as a Rab27-GAP in melanocytes or secretory cells under physiological conditions.

In the present study, we investigated the subcellular localization and Rab27-GAP function of EPI64 in regulated secretion using isoproterenol (IPR)-induced amylase release from rat parotid acinar cells as a secretion model. We showed that EPI64 enhanced the GTPase activity of Rab27 in response to IPR stimulation, and that Rab27-GAP activity is required for IPR-induced amylase release. We further found that both EPI64 mRNA and EPI64 protein levels increased following IPR stimulation. Based on these findings, we discuss the possible regulatory mechanism of EPI64 during IPR-induced amylase release from parotid acinar cells.

**EXPERIMENTAL PROCEDURES**

Materials—Anti-EPI64-C and anti-EPI64-TBC antibodies were generated by immunizing New Zealand White rabbits with a C-terminal peptide (CAHHR5QESLTSQESEDTYL; an artificial Cys residue is added to the N terminus for conjugation of the peptide with keyhole limpet hemocyanin) and a TBC-domain peptide (CGKVKLQQNPFGKDE), respectively, of human EPI64. Each antibody was partially purified by ammonium sulfate fractionation as described previously (18) and then affinity-purified by exposure to antigenic peptide bound to FMP-activated-cellulofine beads according to the manufacturer’s instructions (Seikagaku Co., Tokyo, Japan). Anti-Rab27A/B and anti-aquaporin 5 (AQPS) rabbit polyclonal antibodies were purchased from Immuno-Biological Laboratories Ltd. (Takasaki, Japan) and Millipore (Billerica, MA), respectively. Anti-Rab27A and anti-vesicle-associated membrane protein-2 (VAMP-2) mouse monoclonal antibodies were obtained from Abcam (Cambridge, UK) and Synaptic Systems (Göttingen, Germany), respectively. Anti-GDI rabbit polyclonal antibody was obtained from Zymed Laboratories Inc. (San Francisco, CA). Anti-Noc2 rabbit polyclonal antibody was prepared as described previously (19). Horseradish peroxidase-conjugated anti-T7 tag mouse monoclonal antibody was purchased from Merck Biosciences Novagen (Darmstadt, Germany). Actinomycin D, PCR primers, and other chemical products were purchased from Sigma-Aldrich. Protein kinase A inhibitor 5–24 was from Calbiochem Merck KGaA (Darmstadt, Germany). Rat tissue cDNA QUICK-clones were from TAKARA Bio Inc. (Kyoto, Japan). Locked nucleic acid (LNA) antisense oligonucleotides (20), corresponding to rat EPI64 (5'-TCTCTGGGAG-CAAGAGCATA-3') (EPI64 antisense LNA), were provided by GeneDesign Inc. (Osaka, Japan).

Preparation of Total RNA and Subcellular Fractionation of Parotid Acinar Cells—All animal protocols were devised and performed in accordance with the Guidelines of the Nippon Dental University for the Care and Use of Laboratory Animals. Parotid acinar cells were prepared from parotid glands of male Wistar rats (~10 weeks old) by enzyme digestion using trypsin (Sigma-Aldrich) and collagenase (CLSPA; Worthington Biochemical Co., Lakewood, NJ) as described previously (21). Total RNA from parotid acinar cells was prepared with an RNasy Plus Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. Subcellular fractions were prepared from the homogenate of the acinar cells as described previously (22). Specifically, parotid acinar cells were homogenized in a 20-fold volume of buffer A (5 mM HEPES-NaOH buffer (pH 7.2) containing 50 mM mannitol, 0.25 mM MgCl₂, 25 mM β-mercaptoethanol, 0.1 mM EGTA, 2 µM leupeptin, 2.5 µg/ml trypsin inhibitor, 0.1 mM 4-aminophenylmethanesulfonyl flavide hydrochloride (p-APMSF), 5 mM benzamidine, and 2 µg/ml aprotinin) using a glass homogenizer with a Teflon pestle. Homogenate was centrifuged at 9,750 x g for 10 min at 4 °C. Supernatant was then re-centrifuged at 35,000 x g for 30 min at 4 °C, and further centrifuged at 100,000 x g for 1 h. The precipitate was the intracellular membrane (ICM) fraction, and the supernatant was the cytosolic (Cyto) fraction. The 35,000 x g pellet was suspended in buffer A containing 10 mM MgCl₂ and left on ice for 30 min. The suspension was centrifuged at 3,000 x g for 15 min, and the resultant precipitate was obtained as the basolateral plasma membrane (BLM) fraction. The resultant supernatant was re-centrifuged at 100,000 x g for 1 h, and the resultant precipitate was recovered as the apical plasma membrane (APM) fraction. The APM and BLM fractions were characterized by specific enzyme activities, γ-glutamyl transpeptidase activity for the APM, and K⁺-stimulated p-nitrophe- nyl-phosphatase activity for the BLM (23, 24). A secretory granule membrane (SGM) fraction was prepared by centrifugation in 40% Percoll gradient (25). Protein assays were performed using a protein assay kit (Bio-Rad).

IPR Stimulation of Parotid Acinar Cells—Suspensions of parotid acinar cells or slices <1 mm³ in volume were equally divided into three tubes. Preincubation was performed at 37 °C for 10 min in 5 ml of Hank’s balanced salt solution containing 0.5% bovine serum albumin. The cells or slices were then stimulated with 1 µM IPR for 0, 5, or 30 min. After incubation, the medium was immediately removed by centrifugation at 500 x g for 1 min at 4 °C. Acinar cells were washed twice with ice-cold homogenization buffer A and subjected to preparation of total RNA and subcellular fractions. Parotid slices were used for immunohistochemistry as described below.

Immunoblotting—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis were performed as described previously (26). Samples were separated by 10% SDS-PAGE. Proteins in the gel were transferred to Immun-Blot PVDF membranes (Bio-Rad) using a semi-dry blotter. Membranes were probed with anti-EPI64-C polyclonal antibody (1/500 dilution) or anti-Rab27 polyclonal antibody (1/250 dilution). Membranes were washed and then incubated with anti-rabbit immunoglobulin G (IgG) horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich) (1/3000 dilution), followed again by washing. Immunoreactive bands were visualized using an ECL or ECL plus detection system (GE Healthcare Biosciences, Piscataway, NJ) according to the man-
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PCR was normalized to expression of with Universal Probe Library (Roche) was used to evaluate gene expression by real-time PCR. PCR was performed with KOD-plus DNA polymerase (TOYOBO, Osaka, Japan) and specific primers corresponding to rat EPI64 (forward: 5'-CAGAATCT-CCTGAAAAATTGTAC-3'; and reverse: 5'-TGGATGCTC-CAGTCTCCTC-3') and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: 5'-AACATCATCCCTGCATCC-AC-3'; and reverse: 5'-GACAACCTGGTCTCAGTGT-3'). Real-time PCR was carried out using a LightCycler Carousel-Based System (Roche). A TaqMan Master kit (Roche) with Universal Probe Library (Roche) was used to evaluate gene expression of EPI64, as a target, and TaqMan Gene Expression Assays (Assay ID, Rn99999916_s1, Applied Biosystems) was used to evaluate gene expression of GAPDH, as a reference, in parotid acinar cells. PCR primers and probe (#65) for EPI64 (Tbc1d10a: NM_001015022.3) were designed with the Probe Library Assay Design Center. EPI64 expression by real-time PCR was normalized to GAPDH, an endogenous control.

Amylase Release from Permeabilized Parotid Acinar Cells—Amylase release from streptolysin O (SLO)-permeabilized acinar cells was measured as described previously (26). Lyophilized SLO powder (Sigma-Aldrich) was dissolved in 10 mM PBS (pH 7.0) and was activated with 10 mM dithiothreitol for 1 h at 0 °C. 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 the anti-EPI64-TBC IgG on IPR-induced amylase release was investigated as follows. The cell suspension (100 μl) was pipetted into a tube containing 2 μl of 2500 units/ml SLO together with either anti-EPI64-TBC IgG, normal rabbit IgG, or EPI64 antisense LNA, and incubated at 37 °C for 5 min (for IgG) or 30 min (for antisense LNA). The suspension was then stimulated with 1 μM IPR for 20 min. The reaction mixture was added to 900 μl of incubation medium, and was immediately filtered through glass filter paper to remove acinar cells, and the filtrate, containing released amylase, was used for the amylase assay. Total amylase activity was measured as described by Bernfeld (27).

Immunohistochemistry—IPR-stimulated parotid slices were immediately fixed with 4% (w/v) paraformaldehyde dissolved in 0.07 M phosphate buffer (pH 7.3) for 8 h at room temperature. Fixed specimens were then rapidly frozen in isopentane precooled to −35 °C. Frozen sections (6 μm) were cut using a cryostat, mounted on AP5-coated glass slides (Matsunami Glass, Osaka, Japan), and processed for the following immunostaining procedures. Since both anti-EPI64-TBC and anti-AQP5 polyclonal antibodies were prepared from rabbits, we used a Zenon Rabbit IgG Labeling kit (Molecular Probes, Eugene, OR) for double immunostaining. The sections were incubated for 1–2 h at room temperature with diluted Zenon labeling complexes (Alexa Fluor 488-labeled anti-EPI64-TBC rabbit IgG: 1/200 dilution; Alexa Fluor 594-labeled anti-AQP5 rabbit IgG: 1/200 dilution) and treated as described in the manufacturer’s instructions. The other sections were co-immunostained with anti-EPI64-TBC rabbit antibody and anti-Rab27A (1/100 dilution) mouse monoclonal antibody followed by exposure to Alexa Fluor 488- and 594-labeled secondary antibodies (each 1/100 dilution), respectively. Stained sections were examined

Manufacturer’s instructions. The intensity of immunoreactive bands on a film was quantified using Image Gauge (LAS-1000 software; Fuji Film, Tokyo, Japan).

Reverse Transcription (RT)-PCR and Real-time PCR—Parotid cDNA was prepared from total RNA of rat parotid acinar cells, and cDNA from other tissue was made using the QUICK-clone system. Reverse transcription of total RNA from parotid acinar cells was performed using a Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland). Synthesized cDNA was made from 1 μg of the total RNA preparation, and 1/20 of the reverse transcriptase reaction mix was used for PCR or quantitative real-time PCR. PCR was performed with KOD-plus DNA polymerase (TOYOBO, Osaka, Japan) and specific primers corresponding to rat EPI64 (forward: 5'-CAGAATCT-CCTGAAAAATTGTAC-3'; and reverse: 5'-TGGATGCTC-CAGTCTCCTC-3') and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: 5'-AACATCATCCCTGCATCC-AC-3'; and reverse: 5'-GACAACCTGGTCTCAGTGT-3'). Real-time PCR was carried out using a LightCycler Carousel-Based System (Roche). A TaqMan Master kit (Roche) with Universal Probe Library (Roche) was used to evaluate gene expression of EPI64, as a target, and TaqMan Gene Expression Assays (Assay ID, Rn99999916_s1, Applied Biosystems) was used to evaluate gene expression of GAPDH, as a reference, in parotid acinar cells. PCR primers and probe (#65) for EPI64 (Tbc1d10a: NM_001015022.3) were designed with the Probe Library Assay Design Center. EPI64 expression by real-time PCR was normalized to GAPDH, an endogenous control.

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Immunoprecipitation—Homogenate (500 μg of protein) of parotid acinar cells was solubilized with 1% Triton X-100 at 4 °C for 1 h. Insoluble materials were removed by centrifugation at 15,000 × g for 10 min. Supernatant was incubated with anti-EPI64-C IgG (10 μg/ml) at 4 °C for 1 h and with Dynabeads M-280 sheep anti-rabbit IgG (Dynal Biotech, Oslo, Norway) for 1 h at 4 °C. After washing the beads five times with 50 mM HEPES-KOH (pH 7.2), 150 mM NaCl, and 0.5% Triton X-100, the beads were boiled in Laemmli SDS-sample buffer. Immunoblotting was performed as described above.

Immunohistochemistry—IPR-stimulated parotid slices were immediately fixed with 4% (w/v) paraformaldehyde dissolved in 0.07 M phosphate buffer (pH 7.3) for 8 h at room temperature. Fixed specimens were then rapidly frozen in isopentane precooled to −35 °C. Frozen sections (6 μm) were cut using a cryostat, mounted on AP5-coated glass slides (Matsunami Glass, Osaka, Japan), and processed for the following immunostaining procedures. Since both anti-EPI64-TBC and anti-AQP5 polyclonal antibodies were prepared from rabbits, we used a Zenon Rabbit IgG Labeling kit (Molecular Probes, Eugene, OR) for double immunostaining. The sections were incubated for 1–2 h at room temperature with diluted Zenon labeling complexes (Alexa Fluor 488-labeled anti-EPI64-TBC rabbit IgG: 1/200 dilution; Alexa Fluor 594-labeled anti-AQP5 rabbit IgG: 1/200 dilution) and treated as described in the manufacturer’s instructions. The other sections were co-immunostained with anti-EPI64-TBC rabbit antibody and anti-Rab27A (1/100 dilution) mouse monoclonal antibody followed by exposure to Alexa Fluor 488- and 594-labeled secondary antibodies (each 1/100 dilution), respectively. Stained sections were examined...
and photographed under a confocal laser scanning microscope (LSM 710; Carl Zeiss, Göttingen, Germany).

Treatment of Parotid Acinar Cells with Actinomycin D or EPI64 Antisense LNA—Parotid acinar cells were washed twice with incubation medium (20 mM HEPES-NaOH (pH 7.2), 140 mM KCl, 1 mM MgSO₄, 1 mM Mg-ATP, 0.1 mg/ml trypsin inhibitor, and 0.1% bovine serum albumin). The effects of actinomycin D or EPI64 antisense LNA on IPR-induced up-regulation of EPI64 mRNA and EPI64 protein were investigated as follows. The cell suspension (100 μl) was pipetted into a tube containing 2 μl of 2500 units/ml SLO together with EPI64 antisense LNA (final concentration; 50 nM), or 2 μl of 1 mg/ml saponin together with actinomycin D (final concentration; 50 μg/ml) and incubated at 37 °C for 20 min. The cell suspensions were then stimulated with 1 μM IPR for 30 min. After the stimulation, the cells were collected by centrifugation at 90 g for 10 min, resuspended in 100 μl of RNAlater (Ambion, Austin, TX) (or homogenization buffer A) on ice, and subjected to preparation of total RNA for RT-PCR analysis (or homogenate for SDS-PAGE) as described above.

RESULTS

Expression and Subcellular Localization of EPI64 in Parotid Acinar Cells—We previously showed that EPI64 is a candidate Rab27-GAP, because its overexpression in melanocytes caused inactivation of endogenous Rab27A molecules (17). To determine whether EPI64 actually functions as a physiological Rab27-GAP, we chose amylase release from parotid acinar cells as a secretion model for the following reasons. First, parotid acinar cells are typical exocrine cells that contain many secretory granules to which both Rab27A and Rab27B are localized (31). These secretory granules undergo exocytosis in response to IPR stimulation (31), and some EPI64 signal was also present in the cytosolic fraction (lanes 2 and 5 in Fig. 1D).

Inhibition of Amylase Release by Anti-EPI64-TBC IgG—To investigate the involvement of EPI64 in IPR-induced amylase release from parotid acinar cells, we tested the effect of antibody against the TBC/Rab-GAP domain (anti-EPI64-TBC) on IPR-induced amylase release from SLO-permeabilized parotid acinar cells (Fig. 2). Interestingly, introduction of the anti-EPI64-TBC IgG into SLO-permeabilized parotid acinar cells caused a reduction in IPR-induced amylase release in a dose-dependent manner (up to ~50% of control IPR-induced amylase release) (closed diamonds in Fig. 2). By contrast, control rabbit IgG had no effect on IPR-induced amylase release under the same experimental conditions (open squares in Fig. 2).

Effect of Anti-EPI64-TBC IgG on the GTP Binding Status of Rab27—If EPI64 actually functions as Rab27-GAP during IPR-induced amylase release under physiological conditions, anti-EPI64-TBC IgG should affect the GTP binding status of Rab27. Therefore, we measured the amount of endogenous GDP-Rab27 in SLO-permeabilized parotid acinar cells with or without anti-EPI64-TBC IgG treatment using a GDP-Rab27 pull-down assay with a GST-SHD bait (29, 35) (Fig. 3A). The results of the pull-down assay clearly indicated that the amount of endogenous GDP-Rab27 was significantly decreased in response to IPR stimulation under control conditions (~50% of the unstimulated conditions; compare lane 1 with lanes 3/5 in Fig. 3B), whereas anti-EPI64-TBC IgG completely inhibited the reduction of GDP-Rab27 to the level before the stimulation (lane 4 in Fig. 3B). Based on this result, we concluded that the anti-EPI64-TBC IgG inhibited the GAP activity of endogenous EPI64 molecules, which in turn inhibited the reduction of GDP-Rab27 following IPR stimulation.

Furthermore, EPI64 and its substrate Rab27 were co-localized at the luminal sites of parotid acinar cells before and after IPR stimulation (Fig. 4B), and the EPI64/Rab27A complex was also detected before and after IPR stimulation (Fig. 4A and data
not shown) by co-immunoprecipitation assays. These results also supported our conclusion that EPI64 functions as a Rab27-GAP in parotid acinar cells.

Redistribution and Increased Protein Expression of EPI64 in Parotid Acinar Cells after IPR Stimulation—Although the EPI64 protein was predominantly localized at the APM of parotid acinar cells under resting conditions, the subcellular localization of EPI64 may change following IPR stimulation, as does Rab27 distribution (from secretory granules to the APM, and then to the cytosol) in response to stimuli (22). To investigate this possibility, we performed an immunohistochemical analysis with the anti-EPI64-TBC antibody. Parotid slices were incubated with 1 μM IPR for 5 min or 30 min, and each section was immunostained with anti-EPI64-TBC antibody or anti-AQP5 antibody (a marker for the APM (41)) (Fig. 5A, panels a–i). Although EPI64 was observed at the APM before IPR stimulation, consistent with the result of the subcellular fractionation analysis (Fig. 1D), it appeared to translocate from the APM to the cytosol after 30 min of stimulation. In addition, immunostaining signals of EPI64 unexpectedly increased in the cells after IPR stimulation (compare Fig. 5A, panels a and g). To confirm whether increased EPI64 signals were attributable to increased protein expression, a quantitative immunoblotting analysis was performed. As anticipated, up-regulation of EPI64 protein expression was clearly observed after IPR stimulation for 5–60 min (Fig. 5B, panels a–i). The results of the subcellular fractionation analyses clearly showed that the amount of EPI64 protein at the APM and the cytosol was decreased and increased, respectively,
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FIGURE 2. Inhibition of amylase release by anti-EPI64-TBC IgG. The effect of purified antibody against the TBC/Rab-GAP domain (anti-EPI64-TBC IgG) on IPR-induced amylase release from SLO-permeabilized parotid acinar cells was investigated as described under "Experimental Procedures." Amylase release is represented as the percentage of that without the antibody (means ± S.E. of four independent experiments, performed in triplicate). 100% amylase release corresponds to 5.80 ± 0.46% of the total amylase activity (100.1 ± 11.8 mg maltose/0.1 ml cell suspension). Data were analyzed by two-way ANOVA, followed by Williams' post hoc test. *, p < 0.025; **, p < 0.005. Note that the anti-EPI64-TBC IgG (closed diamonds) inhibited IPR-induced amylase release from SLO-permeabilized parotid acinar cells in a dose-dependent manner, whereas control rabbit IgG (open squares) had no effect.

FIGURE 3. Effect of anti-EPI64-TBC IgG on the GTP-binding status of Rab27. The amount of GTP-Rab27 in SLO-permeabilized parotid acinar cells with or without anti-EPI64-TBC IgG treatment was measured using a GTP-Rab27 pull-down assay with GST-SHD. A, GTP-Rab27 trapped by GST-SHD was detected by immunoblotting using anti-Rab27 antibody. Typical data from five independent experiments are shown. Bars indicate means ± S.E. of five independent experiments. *, p < 0.01 (Student's unpaired t test). Note that the amount of endogenous GTP-Rab27 was significantly decreased after IPR stimulation under control conditions (lanes 3 and 5), whereas such reduction was not observed in the presence of anti-EPI64-TBC IgG (lane 4). These results indicated that the anti-EPI64-TBC antibody inhibited the GTPase activity of Rab27, possibly through inhibition of Rab27-GAP activity of EPI64.

FIGURE 4. Interaction between EPI64 and Rab27 in parotid acinar cells. A, endogenous interaction between EPI64 and Rab27 as revealed by co-immunoprecipitation assays. Cell homogenate of 1 µg of protein was applied to lane 1. Immunoprecipitates with control rabbit IgG and anti-EPI64-C IgG were loaded on lanes 2 and 3, respectively. B, co-localization between EPI64 and Rab27A at the luminal sites of parotid acinar cells. Parotid gland slices were incubated without (a–c) and with 1 µM IPR for 5 min (d–f). Each section was co-immunostained with anti-EPI64-TBC antibody (a and d; green) and anti-Rab27A antibody (b and e; red). Merged images are shown in c and f. Arrowheads represent acinar luminal sites. The insets show magnified views of the boxed area. Scale bars, 5 µm.

EPI64 protein expression described above (Fig. 5C, total panel). Therefore, we concluded that IPR stimulation rapidly induced EPI64 mRNA levels and consequently EPI64 protein levels.

Effects of Actinomycin D Treatment and Antisense Oligonucleotides on Expression of EPI64 and Amylase Release—Finally, we assessed whether up-regulation of EPI64 protein expression was essential for IPR-induced amylase release. Parotid acinar cells were treated with actinomycin D, an RNA synthesis inhibitor. In the presence of actinomycin D (50 µg/ml for 50 min), up-regulation of the level of both EPI64 mRNA and EPI64 protein was completely abolished (Fig. 7, A and B, respectively). Interestingly, treatment of parotid acinar cells with actinomycin D inhibited IPR-induced amylase release to ~60% of that of the control cells (Fig. 7C). To rule out the possibility that actinomycin D suppressed the expression of genes other than EPI64 that were essential for amylase release, we further tested the effect of EPI64 antisense LNA on IPR-induced amylase release from SLO-permeabilized parotid acinar cells. The results showed that the antisense LNA significantly inhibited both up-regulation of EPI64 mRNA/EPI64 protein (Fig. 7, D and E) and IPR-induced amylase release (Fig. 7F), suggesting that increased EPI64 protein after IPR stimulation also contributes to amylase release.
DISCUSSION

In a previous study, we identified EPI64 as a candidate Rab27-GAP because overexpression of EPI64, but not a catalytically inactive EPI64 mutant, in cultured melanocytes induced inactivation of endogenous Rab27A molecules, and EPI64 exhibited Rab27A-GAP activity in vitro (17). However, whether EPI64 actually functions as a physiological Rab27-GAP in secretory cells was not investigated. In the present study, we investigated the biological function of EPI64 and its regulatory mechanism in secretory vesicle exocytosis using rat parotid acinar cells as a secretion model, because these parotid acinar cells express both Rab27A/B and their effectors, Slac2-c, Slp4-a, and Noc2, and because they have been shown to participate in amylase release (22, 31, 37). We found that EPI64 was expressed endogenously in rat parotid acinar cells, and that it was enriched in the APM and less abundant in the cytosol of parotid acinar cells at rest (Fig. 1). We noted that EPI64 was not localized on the secretory granule membranes, where Rab27 is localized under resting conditions (31). Such APM-specific localization of EPI64, together with our previous observations of IPR stimulation-dependent redistribution of Rab27 (from secretory granules to the APM, and to the cytosol) (22), prompted us to hypothesize that EPI64 on the APM promotes the GTPase activity of Rab27, which is translocated from secretory granules to the APM after exocytosis. Moreover, we suggested that the inactivated GDP-Rab27 is released from the APM due to the function of a GDP dissociation inhibitor (22). This hypothesis was strongly supported by our finding that the introduction of an antibody against the TBC/Rab-GAP domain of EPI64 in SLO-permeabilized parotid acinar cells completely inhibited the stimulation-dependent reduction in GTP-Rab27 (Fig. 3). In addition, the redistribution of Rab27 in saponin-permeabilized parotid acinar cells (particularly the move from the APM to the...

FIGURE 5. Redistribution and increased protein expression of EPI64 in parotid acinar cells after IPR stimulation. A, redistribution of EPI64 protein was observed after IPR stimulation by immunohistochemistry. Parotid gland slices were incubated with 1 μM IPR for 5 min or 30 min. Each section was co-immunostained with anti-EPI64-TBC antibody (a, d, and g; green) and anti-aquaporin 5 antibody (AQP5, an APM marker; b, e, and h; red). Merged images are shown in c, f, and i. Arrowheads represent acinar luminal sites. Scale bars, 5 μm. B, increased expression of EPI64 protein in parotid acinar cells after IPR stimulation as revealed by immunoblotting using anti-EPI64-C antibody (Total panel). Alteration of EPI64 protein in the APM fraction (APM panel) and cytosolic fraction (Cyto panel) was also demonstrated by using quantitative immunoblotting. Typical data from four independent experiments are shown. C, intensity of EPI64 bands in B was measured. Bars indicate means ± S.E. of four independent experiments. Data were analyzed by two-way ANOVA, followed by Williams’ post hoc test. *, p < 0.025; **, p < 0.005.

FIGURE 6. Effect of IPR stimulation on the mRNA expression of EPI64 in parotid acinar cells. IPR stimulation-dependent increase in the level of EPI64 mRNA as revealed by RT-PCR analysis (A) and real-time PCR analysis (B). The acinar cells were stimulated with 1 μM IPR for 5 or 30 min, and PCR analyses were performed as described under “Experimental Procedures.” Typical data from four independent experiments are shown in A. The size of the molecular weight markers (bp, base pair) is shown at the right in A. Bars in B indicate means ± S.E. of four independent experiments. *, p < 0.01 (Student’s unpaired t test). Note that EPI64 mRNA expression was dramatically increased by IPR stimulation.
EPI64 Functions as a Rab27-GAP in Rat Parotid Acinar Cells

A, effect of actinomycin D, an RNA synthesis inhibitor, on mRNA expression of EPI64. Actinomycin D treatment and real-time PCR analyses were performed as described under "Experimental Procedures." The control is represented by EPI64 mRNA expression of untreated parotid acinar cells. Bars indicate means ± S.E. of four independent experiments. *, p < 0.05; **, p < 0.01 (Student’s unpaired t test).

B, effect of actinomycin D on the protein expression of EPI64. Actinomycin D was investigated by immunoblotting with anti-EPI64-C antibody. Note that IPR stimulation-dependent up-regulation of EPI64 protein was completely inhibited by treatment with actinomycin D (lane 4). C, effect of actinomycin D on IPR-induced amylase release from parotid acinar cells was investigated. Parotid acinar cells were incubated with actinomycin D for 30 min and then stimulated with 1 μM IPR for 20 min. Amylase release is represented as the percentage of that without actinomycin D (means ± S.E. of four independent experiments, performed in duplicate). 100% amylase release corresponds to 100% amylase release of 5.45 ± 0.41% of the total amylase activity (60.7 ± 4.6 mg maltose/0.1 ml cell suspension). Data were analyzed by Student’s unpaired t test. *, p < 0.01. Actinomycin D inhibited IPR-induced amylase release from parotid acinar cells. D, effect of EPI64 antisense LNA on EPI64 mRNA expression in parotid acinar cells. Antisense LNA treatment and real-time PCR analyses were performed as described under "Experimental Procedures." SLO-permeabilized parotid acinar cells were incubated with 50 nM antisense LNA for 30 min and then stimulated with 1 μM IPR for 20 min. The control is represented by EPI64 mRNA expression of untreated parotid acinar cells. Bars indicate means ± S.E. of three independent experiments. Data were analyzed by Student’s unpaired t test. *, p < 0.05; **, p < 0.01. E, effect of EPI64 antisense LNA on the protein expression of EPI64. EPI64 antisense LNA was investigated by immunoblotting with anti-EPI64-C antibody. Note that IPR stimulation-dependent up-regulation of EPI64 protein was also completely inhibited by treatment with EPI64 antisense LNA (lane 4). F, effect of EPI64 antisense LNA on IPR-induced amylase release from parotid acinar cells. Parotid acinar cells were incubated with 50 nM antisense LNA for 30 min and then stimulated with 1 μM IPR for 20 min. 100% amylase release corresponds to 3.44 ± 0.66% of the total amylase activity (52.6 ± 10.0 mg maltose/0.1 ml cell suspension). Data were analyzed by Student’s unpaired t test. *, p < 0.05. Note that both EPI64 mRNA/EPI64 protein expression and IPR-induced amylase release from the acinar cells were inhibited by EPI64 antisense LNA.

Another important but unexpected finding was that both the EPI64 mRNA level and EPI64 protein level were increased by stimulation with IPR (Figs. 5 and 6), and that suppression of increases in EPI64 mRNA by actinomycin D or EPI64 antisense LNA inhibited IPR-induced amylase release (Fig. 7). Currently, the exact mechanism by which IPR induced transcription of EPI64 mRNA is unknown, but IPR stimulation is known to increase the cAMP level in parotid acinar cells as a result of activation of adenylate cyclase (28). Because a candidate CREB (cAMP response element-binding) protein binding site(s) (half site; TGACG) was identified in the human and mouse TBC1D10A/EPI64 genes by the CREB Target Gene Database, it is highly possible that the transcription of EPI64 mRNA was activated by CREB protein through protein kinase A (PKA) phosphorylation. In fact, EPI64 mRNA expression was suppressed by the protein kinase A inhibitor 5–24 (supplemental Fig. S2). Because treatment of parotid acinar cells with EPI64 antisense LNA significantly reduced the amount of amylase released, cytosolic EPI64 increase after IPR stimulation is also likely to regulate the biogenesis and/or recycling of secretory granules (e.g. transport of secretory granules to the apical region), in which Rab27 may be involved (42, 43). A proper GTP-GDP cycle of Rab27 is presumably required for these processes.

It has been very recently reported that members of the TBC1D10 family, including EPI64, function as GAPs for Rab35 (39, 40, 44), which is known to regulate a variety of cellular events, including recycling of T-cell receptor and oocyte yolk protein (40, 44, 45), cytokinesis (46), neurite outgrowth (47, 48), actin bundling (49, 50), and exosome secretion (39). However, Rab35 protein is expressed at very low levels in parotid acinar cells relative to those in the rat brain (supplemental Fig. S3A, lane 6), and we detected Rab35 in the SGM-rich fraction of parotid acinar cells (supplemental Fig. S3A, lane 8). The involvement of Rab35 in IPR-induced amylase release was also investigated by using a Rab35-specific trapper (GST-RBD35) that we have recently developed (30). However, GST-RBD35 had no effect on IPR-induced amylase release from SLO-permeabilized parotid acinar cells (supplemental Fig. S3B), in contrast to the inhibitory effect of GST-SHD, a Rab27-specific trapper, as previously reported (31), suggesting that Rab35 itself may not be directly involved in amylase secretion (51). Moreover, the level of endogenous GTP-Rab35 monitored by GST-RBD35 was increased by IPR stimulation (supplemental Fig. S3C, compare lanes 2 and 5), in contrast to the decreased level of cytosol) following IPR stimulation was inhibited by treatment with GTPγS, a non-hydrolyzable analog of GTP (supplemental Fig. S1). Therefore, the GTPase activity of Rab27 was likely to be required for dissociation of Rab27 from the APM. Although GAP is generally thought to be a negative regulator that terminates GTPase signaling, the anti-EPI64-TBC antibody attenuated IPR-induced amylase release in a dose-dependent manner (Fig. 2). This result may not be surprising in light of a recent report showing that the GTPase activity of Rab27 is not required for exocytosis itself in platelets, and that the GTPase activity of Rab27 occurs only after exocytosis (29). Therefore, we speculate that a Rab27 must undergo a proper GTP-GDP cycle for amylase release, and that inhibition of the cycle by the specific antibody, i.e. the accumulation of GTP-Rab27 on the APM, would inhibit amylase release by reducing the number of Rab27 molecules available to mediate the next round of exocytosis, preventing sustained amylase release.
of GTP-Rab27 (Fig. 3). However, since the addition of the anti-EPI64-TBC IgG into parotid acinar cells increased the amount of GTP-Rab35 regardless of the presence of IPR stimulation (supplemental Fig. 3C, lanes 3 and 4), EPI64 is also likely to be directly involved in amylase secretion. Rab35 may contribute to recycling of membrane proteins (e.g. β-adrenergic receptor) (40, 44, 45, 52) and/or biogenesis of new amylase-containing vesicles (53). Further extensive research is needed to determine the molecular mechanism by which EPI64 regulates two distinct small GTPases, Rab27 and Rab35, during amylase release from parotid acinar cells.

In summary, we have demonstrated that EPI64 protein enhanced the GTPase activity of Rab27 (and also Rab35) in parotid acinar cells, and that IPR-induced amylase release was regulated by EPI64 expression and Rab27-GAP activity. To our knowledge, this is the first report to demonstrate that endogenous EPI64 has Rab27-GAP activity under physiological conditions. EPI64 is expressed ubiquitously (15), and inactivation of Rab27 has been shown to occur after exocytosis in other secretory cells (29); therefore, we propose that EPI64 functions as a Rab27-GAP that regulates secretion events in a variety of secretory cells, similar to its role in parotid acinar cells. Our findings, together with previous reports on the function of EPI64 in microvillar formation (15) and in exosome secretion (39), indicate that EPI64 is a multifunctional protein that regulates Rab27/35 through its GAP activity and microvillar formation independent of its GAP activity (16).

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