Rab3GEP Is the Non-redundant Guanine Nucleotide Exchange Factor for Rab27a in Melanocytes*

Received for publication, May 29, 2008 Published, JBC Papers in Press, June 17, 2008, DOI 10.1074/jbc.M804134200

Ana C. Figueiredo†1, Christina Wasmeier†, Abul K. Tarafder‡, José S. Ramalho§, Rudi A. Baron‡, and Miguel C. Seabra†§

From 1Molecular Medicine, National Heart and Lung Institute, Imperial College London, London SW7 2AZ, United Kingdom and the 2Centre of Ophthalmology, Biomedical Institute for Research in Light and Image, University of Coimbra, 3004-548 Coimbra, Portugal

Rab GTPases regulate discrete steps in vesicular transport pathways. Rabs require activation by specific guanine nucleotide exchange factors (GEFs) that stimulate the exchange of GDP for GTP. Rab27a controls motility and regulated exocytosis of secretory granules and related organelles. In melanocytes, Rab27a regulates peripheral transport of mature melanosomes by recruiting melanophilin and myosin Va. Here, we studied the activation of Rab27a in melanocytes. We identify Rab3GEP, previously isolated as a GEF for Rab3a, as the non-redundant Rab27a GEF. Similar to Rab27a-deficient ashen melanocytes, Rab3GEP-depleted cells show both clustering of melanosomes in the perinuclear area and loss of the Rab27a effector Mlph. Consistent with a role as an activator, levels of Rab27a-GTP are decreased in cells lacking Rab3GEP. Recombinant Rab3GEP exhibits guanine nucleotide exchange activity against Rab27a and Rab27b in vitro, in addition to its previously documented activity against Rab3. Our results indicate promiscuity in Rab GEF action and suggest that members of related but functionally distinct Rab subfamilies can be controlled by common activators.

Rab proteins belong to the Ras superfamily of small GTPases and regulate discrete steps in vesicular transport pathways (1). More than 60 Rab family members are known in mammalian cells. Different Rab s are localized to specific cellular compartments where they are primary determinants of membrane identity and exert multiple local activities (2–4). Rab proteins are posttranslationally modified by geranylgeranylation, which is required for membrane association and proper function (5). After prenylation, Rabs form a complex with Rab escort protein, which is dissociated upon membrane association of the newly prenylated Rabs.

Regulation of Rab function is determined by the ability to cycle between a GDP-bound inactive form and a GTP-bound active form (6). Rabs are activated by specific guanine nucleotide exchange factors (GEFs)3 that stimulate the release of GDP and the binding of GTP. Activated Rab-GTP binds effector proteins, which perform critical functions in the organization of membrane traffic. GTPase-activating proteins return Rabs to their inactive state by accelerating intrinsic Rab GTP hydrolysis. Once back in the GDP-bound form, Rabs are extracted from membranes by Rab guanine dissociation inhibitor (7).

The regulation of Rab activation plays a crucial role in determining when and where a given Rab exerts its cellular activity. In contrast to the large number of GEFs known for the Ras, Rho, and Arf families, few Rab GEFs have been identified to date (8). These include Sec2, the GEF for Sec4 (9); the Vps9 domain-containing proteins Rabex-5, Varp, and the Rin family, which catalyze nucleotide exchange on Rab5 and closely related Rab family members (10–12); Rabin-3, a GEF specific for Rab8 (13); and Rab3GEP; and GRAB as activators for Rab3 (14, 15).

To gain insight into the mechanisms of Rab activation, we have studied these processes using Rab27a as a model Rab. Rab27a controls organelle transport in melanocytes and cytotoxic T lymphocytes (4, 16) as well as regulated exocytosis in secretory cells such as pancreatic β cells (17). It regulates these diverse functions largely by interacting with a family of effector proteins containing a conserved N-terminal Rab27 binding domain (18, 19). In melanocytes, Rab27a recruits melanophilin (Mlph) and myosin Va to the melanosome membrane, allowing tethering of melanosomes to the actin cytoskeleton at the cell periphery (4). The loss of any component of this tripartite complex results in disruption of peripheral tethering and clustering of melanosomes in the perinuclear area of the cell.

In this study, we took a candidate gene approach to identify Rab27 GEFs and studied Rab27a activation using melanosome transport in melanocytes as a model system. We identify Rab3GEP, previously isolated as a GEF for Rab3 (14), as the non-redundant GEF required for the activation of Rab27a in melanocytes.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse wild-type melanocytes (melan-ink4a) and Rab27a-deficient ashen melanocytes (melan-ash2) (20) were maintained in RPMI 1640 supplemented with 10% fetal

*This work was supported by the Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a Ph.D. studentship from the Foundation for Science and Technology, Portugal.

‡ To whom correspondence should be addressed. E-mail: m.seabra@imperial.ac.uk.

3 The abbreviations used are: GEF, guanine nucleotide exchange factor; GRAB, guanine nucleotide exchange factor for Rab3a; GFP, green fluorescent protein; siRNA, small interfering RNA; PBS, phosphate-buffered saline; GST, glutathione S-transferase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; GTPyS, guanosine 5’-3-O-(thio)triphosphate or guanosine 5’-O-(thiotriphosphate).
calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, 200 mM phorbol 12-myristate 13-acetate, and 200 µM cholera toxin at 37 °C under a humidified atmosphere containing 10% CO₂. HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin.

Antibodies—The following antibodies were used at the dilutions indicated: anti-Rab3GEP rabbit polyclonal antibody (a kind gift of J. Miyoshi and Y. Takai), 1:5000; anti-actin mouse monoclonal antibody (MP Biomedicals), 1:10,000; anti-V5 mouse monoclonal (Invitrogen), 1:200; anti-GFP rabbit polyclonal (Abcam), 1:1000; anti-Rab3 (a/b/c/d) mouse monoclonal (Synaptic Systems), 1:1000; anti-Rab27a mouse monoclonal 4B12 (described previously), 1:10,000; anti-calnexin rabbit polyclonal (Stressgen), 1:10,000; and anti-melanophilin goat polyclonal (Everest Biotech), 1:10,000 for immunoblotting and 1:200 for immunofluorescence.

Small Interfering RNAs (siRNAs)—siRNA oligonucleotides were purchased from Dharmacon. Sense sequences for individual duplexes targeting mouse Rab3GEP were as follows: Rab3GEP-1, gcgaagaggccagccauuu; Rab3GEP-2, ggccaggccaccaauuu; Rab3GEP-3, gcagacaggccaccaauuu; Rab3GEP-4, uucaagcauucgccgauu; and targeting mouse agacccacuacuauauu; Rab3GEP-3, gcacacacguacccugcgauu; Rab3GEP-4, uucaagcauucgccgauu; and targeting mouse M1ph, ggccaaauaaaccaagggau. GRAB-specific and Rab3A-b-, c-, or d-specific siRNAs were pools of four oligonucleotides (siGENOME SMARTpool). A pool of four non-targeting oligonucleotides was used as a negative control.

Plasmid Constructs—Human Rab3GEP was amplified by PCR from expressed sequence tag BC63386 using flanking primers containing BglII and SacII restriction sites and was subcloned into pENTR vectors modified as described in Ref. 21 and additionally containing an N-terminal GFP or V5 tag to generate pENTR/GFP-human Rab3GEP and pENTR/V5-human Rab3GEP. To generate pFastBacHT-B-Rab3GEP, the 5’ 4.3-kb coding sequence of rat Rab3GEP was amplified by PCR using flanking primers containing Sall and Psp1406I restriction sites and pCMV-Rab3GEP4.3 (a kind gift of J. Miyoshi and Y. Takai) as a template, and blunt end-ligated into pCR-BluntII-TOPO-Rab3GEP4.3. The resulting full-length 4.8-kb Rab3GEP cDNA (in pCR-Rab3GEP) was digested using Sall and NotI and ligated into pCR-BluntII-TOPO-Rab3GEP4.3. The resulting full-length 4.8-kb Rab3GEP cDNA (in pCR-Rab3GEP) was digested using Sall and NotI and subcloned into pFastBacHT-B (Invitrogen).

Adenovirus Production and Infection of Melanocytes—Expression cassettes containing cDNA sequences assembled in pENTR were incorporated into the adenovirus packaging vector pAd/BLOCK-it-DEST (Invitrogen) through recombination between pENTR attL sites and pAd/BLOCK-it-DEST attR sites by using LR cloning II (Invitrogen) according to the supplier’s protocol. pAd plasmids were linearized with PacI restriction enzyme and transfected into HEK-293 producer cells using FuGENE6. Viral particles were harvested after lysis of infected cells 11 days later and were subjected to one round of amplification in HEK-293 cells. Cell lysates were prepared by three rounds of freeze-thawing, cleared by centrifugation at 2000 × g for 15 min, and used to transduce melanocytes seeded the day before infection.

Microscopy—Cells grown on coverslips were rinsed with phosphate-buffered saline (PBS), fixed with 3% (w/v) paraformaldehyde in PBS for 20 min, and rinsed with PBS. For immunofluorescence, fixed cells were permeabilized with 0.05% saponin in PBS with 0.5% bovine serum albumin for 15 min. Coverslips were incubated with primary antibody, washed, incubated with Alexa Fluor 488- or Alexa Fluor 568-conjugated secondary antibody (Molecular Probes), and washed again in the same buffer. Coverslips were mounted with Moviol mounting medium (Calbiochem) and subjected to phase contrast and fluorescence microscopy using a DM-IRBE confocal microscope (Leica) fitted with 40 × 1.0 NA oil immersion Fluotar objective lens. All images are single sections in the z-plane. Images were processed with Leica TCS-NT and Adobe Photoshop software.

S. frugiperda Virus Production—pFastBacHT-B-Rab3GEP and pFastBacHT-B-GRAB were used to transform DH10Bac Escherichia coli (Invitrogen) for transposition into bacmid according to the manufacturer’s protocol. The production of recombinant baculovirus was performed according to the manufacturer’s instructions (Invitrogen). Briefly, 2 µg of bacmid diluted in 100 µl of unsupplemented Grace’s medium were mixed with 10 µl of Cellfectin diluted in 100 µl of unsupplemented Grace’s medium. The reaction mixture was used to transfect Spodoptera frugiperda cells (9 × 10⁵ cells/well in 6-well plates) and incubated for 96 h at 27 °C to isolate a PF viral stock. Viral stocks were amplified and diluted at 1:100 into 1 liter of S. frugiperda cell culture (2 × 10⁶ cells/ml). After 96 h, the cells were pelleted at 800 × g, snap frozen in liquid nitrogen, and stored at −80 °C.

Recombinant Proteins—His₆-tagged Rab1a, Rab3a, Rab5a, Rab27a, Rab27b, and NusA-His₆-Rab8a were expressed in E. coli BL21-codon plus (DE3) RIPL (Stratagene). His₆-Rab3GEP and His₆-GRAB were prepared from S. frugiperda cell pellets. Recombinant proteins were purified on a nickel-Sepharose column by fast protein liquid chromatography as described previously (22). Recombinant GST-Rab27a, GST, and GST-Slp1₁-₁₀₀ (amino acids 1–200) were expressed in E. coli BL21 and purified on glutathione-Sepharose beads.

Subcellular Fractionation—Cells were washed with PBS and disrupted by sonication in 50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, and protease inhibitor mixture (Roche Diagnostics). Homogenates were cleared by centrifugation at 800 × g for 10 min at 4 °C and used for immunoblot analysis. Alternatively, samples were subjected to ultracentrifugation at 100,000 × g for 1 h at 4 °C. Equivalent volumes are determined by the absorbance at 280 nm.
of the cytosolic soluble fraction and the resuspended pellet were analyzed by immunoblotting.

**Immunoblotting**—Protein samples were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride membrane (Millipore). Membranes were blocked in 5% nonfat dried milk and 0.1% Tween 20 in PBS (PBS-T), incubated with primary antibody in PBS-T, washed three times with PBS-T, incubated with horseradish peroxidase-labeled secondary antibodies (Dako, 1:10,000), and washed as above. Bound antibody was detected using the ECL Plus Western blotting detection system (Amersham Biosciences).

**GST Pulldown Assay**—Melanocytes treated with siRNAs were lysed 72 h after transfection in buffer containing 50 mM Tris-HCl, pH 7.2, 1 mM dithiothreitol, 150 mM NaCl, 1% CHAPS, and protease inhibitor mixture (Roche Diagnostics). Cell lysates were clarified by centrifugation at 12,000 × g for 15 min at 4 °C. 20 μg of total protein was incubated with 2 μM GST or GST-Slp1 and 20 μl of glutathione-Sepharose 4B beads for 45 min at room temperature in a reaction volume of 100 μl. Beads were washed three times in lysis buffer, resuspended in SDS-PAGE sample buffer, and boiled for 5 min. Samples were subjected to immunoblot analysis with anti-Rab27a monoclonal antibody. The results were quantified by densitometry using Fuji Film Intelligent Dark Box LAS-3000 and Aida Image Analyze 3.52 software. To visualize GST and GST-Slp1, polyvinylidene difluoride membranes were incubated for 1 min in Amido Black staining solution (Sigma).

**GDP/GTP Exchange Assay**—Rab3GEP activity was assayed by measuring the association of [35S]GTPγS with His-tagged Rab1a, Rab3a, Rab5a, Rab8a, Rab27a, and Rab27b. For time course exchange assays, 480 pmol of His-Rab was incubated at 30 °C with 0 or 60 pmol of recombinant Rab3GEP in a 300-μl reaction volume containing 50 mM Tris-HCl, pH 7.5, 12 mM MgCl2, 2 mM EDTA, 0.2 mg/ml bovine serum albumin, 0.6 pmol of [35S]GTPγS (Amersham Biosciences), and 959.4 pmol of GTPγS (Fluka) (1562.5 dpm/pmol). At each time point, a 50-μl aliquot was removed, and the reaction was stopped by snap freezing in liquid nitrogen. Protein samples were applied to nitrocellulose filters (Whatman) pre-equilibrated in ice-cold wash buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.2 mg/ml bovine serum albumin). Filters were washed twice with 2 ml of ice-cold wash buffer. Protein-bound radioactivity was determined by scintillation counting.

**RESULTS**

**Down-regulation of Rab3GEP in Melanocytes Results in Melanosome Aggregation**—To identify the GEF for Rab27 in mammalian cells, we took a candidate gene approach. We
Rab3GEP Is the Nucleotide Exchange Factor for Rab27a

Rab3GEP Is the Nucleotide Exchange Factor for Rab27a

hypothesized that GEFs for the closely related Rab3 proteins could function as Rab27 GEFs. We examined two previously identified Rab3 GEFs, Rab3GEP (14) and GRAB (15). To gain insight into the function of these GEF proteins in melanocytes and investigate their putative roles as Rab27a GEFs, we used siRNA. Immunoblotting revealed that endogenous Rab3GEP was efficiently depleted by mouse Rab3GEP-specific siRNAs but not by non-targeting siRNAs in mouse melanocytes (Fig. 1J). Depletion of Rab3GEP with four different siRNA oligonucleotides resulted in clustering of melanosomes in the perinuclear area as opposed to the even distribution observed in control cells transfected with non-targeting siRNAs (Fig. 1, A–D and F). All four duplexes were similarly efficient at inducing aggregation of melanosomes (Rab3GEP-1, 81%; Rab3GEP-2, 83%; Rab3GEP-3, 87%; and Rab3GEP-4, 82% of cells 72 h after transfection) (Fig. 1H). The number and morphology of melanosomes were similar to control cells. The melanosome aggregation phenotype resembles that observed in mouse melanocytes lacking functional Rab27a (ashen) (Fig. 1G), Mlph (leaden), or myosin Va (dilute) (4) and in cells treated with Mlph-specific siRNA (Fig. 1E).

Melanosome clustering was induced by depleting cells of Rab3GEP using Rab3GEP-1 siRNA, and Rab3GEP activity was subsequently reintroduced using adenoviruses expressing tagged human Rab3GEP or GFP as a control (Fig. 2). Human Rab3GEP contains five nucleotides that differ from the corresponding mouse sequence in the target region for Rab3GEP-1 and is thus resistant to siRNA-mediated knockdown. In cells transduced with virus carrying GFP alone, melanosome distribution was not altered (Fig. 2, C and D), with melanosome clustering seen in 65% of Rab3GEP siRNA-treated cells. However, expression of exogenous GFP-tagged or V5-tagged human Rab3GEP rescued the observed phenotype (Fig. 2, E–H), dramatically reducing the number of cells with clustered melanosomes to 8 or 9%, respectively. As expected, expression of human Rab3GEP did not reverse the clustering of melanosomes induced by depletion of Mlph (Fig. 2, J and F).

This suggests that Rab3GEP plays a role in melanosome transport and functions in the same pathway as Rab27a/Mlph/myosin Va. A putative role for Rab3GEP as a GEF for Rab27a in melanocytes would be consistent with these results. Furthermore, our data suggest that Rab3GEP activity is not redundant in melanocytes.

Similar experiments using GRAB-specific siRNAs did not show any effect on melanosome distribution (Fig. 3, A–C). To exclude the possibility that the function of Rab3GEP in melanocytes could be mediated via one of the isoforms of Rab3, we also depleted cells of Rab3. Rab3a, b, c, and d were targeted with specific siRNAs, either separately (data not shown) or simultaneously (Fig. 3, D and E). Immunoblotting with an antibody reactive with all four isoforms demonstrated loss of Rab3 in the transfected melanocytes, but no effect on melanosome transport was observed.
Rab3GEP Is the Nucleotide Exchange Factor for Rab27a

To confirm that Rab3GEP does indeed possess Rab27a GEF activity, we performed in vitro GDP/GTP exchange assays with recombinant Rab3GEP produced in insect cells using the baculovirus expression system. Rab3GEP was assayed for the ability to stimulate [35S]GTP·S incorporation into bacterially expressed His₆-tagged Rab1a, Rab3a, Rab5a, Rab27a, and NusA-His₆-Rab8a. Purified Rab3GEP catalyzed the binding of [35S]GTP·S to Rab3a, Rab27a, and Rab27b in a time-dependent manner, but not to Rab1a, Rab5a, and Rab8a (Fig. 5A). This analysis suggests that Rab3GEP is a GEF specific for the related Rab3 and Rab27 subfamilies. We also performed in vitro GDP/GTP exchange assays with recombinant GRAB protein. GRAB showed exchange activity toward Rab3a but did not catalyze nucleotide exchange on Rab27a (Fig. 5B).

DISCUSSION

In this study we used a candidate gene approach to identify a nucleotide exchange factor for mammalian Rab27. We find that Rab3GEP, a protein described previously as a GEF for Rab3 proteins (14), acts as the non-redundant Rab27 GEF activity in melanocytes. We show that Rab3GEP has guanine nucleotide exchange activity for Rab27a and Rab27b as well as Rab3 proteins, but not for Rabs belonging to other subfamilies. Consistent with a key role for Rab3GEP as a Rab27 activator, siRNA-mediated knockdown of Rab3GEP expression in epidermal melanocytes results in the perinuclear clustering of melanosomes, a phenotype similar to that observed in melanocytes lacking Rab27, such as those isolated from the ashen (Rab27a-deficient) mouse. Depletion of Rab3 isoforms, on the other hand, does not affect melanosome distribution, suggesting that melanosome clustering induced by the loss of Rab3GEP is due to a reduction in Rab27 activation rather than Rab3 function.

Rab3GEP was initially purified from rat brain by Takai and co-workers (14) as a Rab3a GEF. It is a ubiquitously expressed protein of ~200 kDa that has been shown to be active on all four Rab3 isoforms (24). Rab3GEP plays an important role in neurotransmitter release and synaptic vesicle trafficking (25, 26) and affects stimulated secretion from neuroendocrine cells (27, 28).

The Caenorhabditis elegans Rab3GEP orthologue AEX-3 shows substantial levels of sequence homology over extended regions of the molecule. Studies in aex-3 mutants also implicated this protein in the regulation of synaptic transmission and linked its function to that of RAB-3 (29). However, aex-3

by immunoblot analysis for Rab27a (19). Depletion of Rab3GEP in melanocytes led to a substantial decrease (~65% of total) in the amount of endogenous Rab27a pulled down by GST-Slp1 compared with treatment with non-targeting or Mlph siRNA (Fig. 4A). This decrease supports a GDP/GTP exchange activity for Rab3GEP on Rab27a. The fraction of Rab27a still interacting with Slp1 (~35% of total) may result from non-transfected cells (~20%). Additionally, although the Rab27 binding domain of Slp1 shows preferential interaction with the GDP-bound form of Rab27a, a low level of binding to Rab27a-GDP has also been observed (19).

To further examine the activation state of Rab27a in Rab3GEP-depleted cells, we analyzed the expression and subcellular localization of the Rab27 effector Mlph by immunoblotting and immunofluorescence (Fig. 4, B–H). In Rab27a-deficient ashen melanocytes, a dramatic decrease in Mlph levels is observed in comparison with wild-type cells, suggesting that Mlph requires interaction with Rab27a-GTP for stability (23). Therefore, we hypothesized that loss of Rab27-GTP in Rab3GEP-depleted cells would result in Mlph destabilization. Consistent with this, Mlph was no longer detectable in cells depleted of Rab3GEP by immunoblotting (Fig. 4B) or by immunofluorescence (Fig. 4, E and H), similar to what was observed in cells treated with Mlph siRNA (Fig. 4, B, D, and G). These results support the idea that in Rab3GEP-depleted cells, Rab27a is predominantly in the GDP-bound form, precluding interaction with Mlph and resulting in its destabilization.
Rab3GEP Is the Nucleotide Exchange Factor for Rab27a

A

siRNA

pull-down

input

GST

GST-Slp1

recomb.

protein

pull-down

Rab27a

Rab27a

B

siRNA

NT

Mlph

Rab3GEP

NT

Mlph

Rab3GEP

S

P

S

P

S

P

Mlph

calnexin

FIGURE 4. Down-regulation of Rab3GEP results in reduced levels of activated Rab27a. A, Rab3GEP-depleted melanocytes contain lower levels of Rab27a in the GTP-bound form. Cell lysates of melan-ink4a melanocytes transfected with either non-targeting (NT), Mlph-specific, or Rab3GEP-specific siRNA oligonucleotides were incubated with GST or GST-Slp1 proteins in the presence of glutathione-Sepharose. Rab27a pulled down by GST-Slp1 was detected by immunoblot analysis with antibody to Rab27a. Recombinant GST or GST-Slp1 proteins were visualized by Amido Black staining. Results are representative of three independent experiments. B, reduced levels of melanophilin in Rab3GEP-depleted melanocytes are shown. Melan-ink4a cells were transfected with non-targeting, Mlph-specific, or Rab3GEP-specific siRNA oligonucleotides. After 72 h, cells were homogenized and separated into soluble (S) and pellet (P) fractions by centrifugation at 100,000 × g. The presence of melanophilin and calnexin (as a loading control) was analyzed by immunoblotting. C–H, melan-ink4a melanocytes treated with non-targeting (C and F), Mlph-specific (D and G), or Rab3GEP-specific (E and H) siRNA oligonucleotides were fixed 72 h after transfection and labeled with antibodies to Mlph (F–H). Panels C–E show the corresponding phase contrast images. Arrows indicate siRNA-transfected cells. Scale bar = 10 μm.

FIGUR ements of Rab3GEP interaction with its Rab substrates.

The regulation of two related Rab subfamilies, Rab3 and Rab27, by a common exchange factor is similar to what is observed for the Vps9 domain-containing family of Rab GEFs. At least some Vps9 proteins, such as Rabex-5, recognize multiple members of the same Rab functional group, consisting of the Rab5 isoforms, Rab21, and Rab22 (36). Thus, a pattern may be emerging for the Rab family whereby a large number of Rab proteins are activated by a much smaller number of Rab GEFs with some substrate promiscuity. This is in marked contrast to the Rho family, in which a relatively small set of ~20 Rho GTPases are activated by a large number of at least 70 Rho GEFs (39). An intriguing question is how a small number of Rab GEFs could regulate the many Rab family members involved in controlling diverse and dynamic intracellular trafficking pathways under a wide variety of conditions. To gain a better understanding of this, much more will need to be learned about how the activity of Rab exchange factors themselves is regulated. Many are multi-domain proteins that, in addition to the Rab domain, might contain a proline-rich domain (PRD) and a C-terminal Pleckstrin homology (PH) domain, which might be involved in Rab exchange factor localization and regulation of Rab activity. In this issue of the Journal of Biological Chemistry, a study by Watanabe et al. (40) describes a new Rab GEF that imparts distinct membrane targeting and regulation over other members of the same family. The study by Watanabe et al. (40) is an example of how studies of Rab GEFs are shedding light on the regulation of Rab proteins in endocytic pathway and beyond.
Rab3GEP Is the Nucleotide Exchange Factor for Rab27a

A

B

FIGURE 5. Recombinant Rab3GEP is a nucleotide exchange factor for Rab27a. A, His6-Rab1a ( ), His6-Rab3a ( ), His6-Rab5a ( ), His6-Rab3a ( ), His6-Rab27b ( ), and His6-Rab8a ( ) were incubated with [35S]GTP-γ-S at 30 °C for the indicated periods of time in the presence of recombinant Rab3GEP. [35S]GTP-γ-S binding was quantified by filter binding assay followed by scintillation counting. Each reaction contained 80 pmol of Rab. Values shown are pmol of Rab-GTP after subtraction of values obtained in the absence of Rab3GEP. B, GRAB is an exchange factor for Rab3a but not Rab27a. His6-Rab3a ( ), His6-Rab5a ( ), and His6-Rab27a ( ) were incubated with [35S]GTP-γ-S at 30 °C for the indicated periods of time in the presence or absence of recombinant GRAB. [35S]GTP-γ-S binding was quantified by filter binding assay followed by scintillation counting. The data shown represent the mean of duplicate determinations from a single experiment, which is representative of three such experiments. Each reaction contained 80 pmol of Rab. Values shown are pmol of Rab-GTP after subtraction of values obtained in the absence of GRAB.

interact with a variety of intracellular regulatory molecules. A further interesting example is the yeast TRAPP complex, which shows GEF activity for two different Rab GTPases, Ypt1 and Ypt31/32, with a switch in substrate specificity dependent on the presence or absence of recombinant GRAB. A further interesting example is the yeast TRAPP complex, which shows GEF activity for two different Rab GTPases, Ypt1 and Ypt31/32, with a switch in substrate specificity dependent on the presence or absence of recombinant GRAB. A further interesting example is the yeast TRAPP complex, which shows GEF activity for two different Rab GTPases, Ypt1 and Ypt31/32, with a switch in substrate specificity dependent on the presence or absence of recombinant GRAB.

Acknowledgments—We thank Yoshimi Takai and Jun Miyoshi for the gift of the Rab3GEP cDNA and antibody and Vanda S. Lopes for the GRAB and Rab8 plasmid constructs.

REFERENCES

35. Zhao, S., Torii, S., Yokota-Hashimoto, H., Takeuchi, T., and Izumi, T.
Rab3GEP Is the Nucleotide Exchange Factor for Rab27a