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Characterization of RIN3 as a Guanine Nucleotide Exchange Factor for the Rab5 Subfamily GTPase Rab31*[S]

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The small GTPase Rab5, which cycles between GDP-bound inactive and GTP-bound active forms, plays essential roles in membrane budding and trafficking in the early endocytic pathway. Rab5 is activated by various vacuolar protein sorting 9 (VPS9) domain-containing guanine nucleotide exchange factors. Rab21, Rab22, and Rab31 (members of the Rab5 subfamily) are also involved in the trafficking of early endosomes. Mechanisms controlling the activation Rab5 subfamily members remain unclear. RIN (Ras and Rab interactor) represents a family of multifunctional proteins that have a VPS9 domain in addition to Src homology 2 (SH2) and Ras association domains. We investigated whether RIN family members act as guanine nucleotide exchange factors (GEFs) for the Rab5 subfamily on biochemical and cell morphological levels. RIN3 stimulated the formation of GTP-bound Rab31 in cell-free and *in cell* GEF activity assays. RIN3 also formed enlarged vesicles and tubular structures, where it colocalized with Rab31 in HeLa cells. In contrast, RIN3 did not exhibit any apparent effects on Rab21. We also found that serine to alanine substitutions in the sequences between SH2 and RIN family homology domain of RIN3 specifically abolished its GEF action on Rab31 but not Rab5. We examined whether RIN3 affects localization of the cation-dependent mannose 6-phosphate receptor (CD-MPR), which is transported between trans-Golgi network and endocytic compartments. We found that RIN3 partially translocates CD-MPR from the trans-Golgi network to peripheral vesicles and that this is dependent on its Rab31-GEF activity. These results indicate that RIN3 specifically acts as a GEF for Rab31.

The small GTPase Rab family plays pivotal roles in intracellular membrane trafficking. At present, >60 Rab GTPases have been identified, and they localize to distinct intracellular compartments that organize transport in specific organelles (1–3). The functional state of Rab GTPases depends on structural conformation, which is determined by binding to guanine nucleotides. In the GDP-bound state, Rab forms a cytoplasmic complex with Rab-GDI,[2] which regulates association with cellular components. After dissociation of Rab-GDI by a GDI dissociation factor on donor membranes, Rab replaces GDP with GTP through its interaction with a GEF at target membranes. This causes a conformational change in Rab that allows recruiting of a variety of downstream effectors onto membranes. During or after membrane fusion, a regulatory protein called Rab-GAP enhances the intrinsic GTPase activity of Rab and promotes GTP hydrolysis. Once this has occurred, GDP-bound Rab re-forms the complex with Rab-GDI and dissociates from membranes.

Rab5, the most thoroughly characterized member of the Rab family, is localized mainly to early endosomes (4–6). Rab5 is involved in the homotypic fusion process of early endosomes and in the budding of clathrin-coated vesicles from plasma membranes and transport to early endosomes (7–9). Therefore, Rab5-GEF plays a key role in the regulation of Rab5 function. To date, a large number of proteins have been identified as potential Rab5-GEFs (10–18), and they contain a highly conserved VPS9 domain, which is required for nucleotide exchange of Rab5 (19). Domains other than VPS9 on Rab5-GEFs primarily mediate their interaction with proteins, including Ras GTPases, that determine the specificity and location of Rab5-GEF (20, 21). Thus, the diversity of Rab5-GEFs may partly account for the temporal and spatial aspects of Rab5 function.

The RIN family is composed of RIN1, RIN2, and RIN3. These proteins have multifunctional domains including SH2 and proline-rich domains in the N-terminal region, and RH, VPS9, and RA domains in the C-terminal region. RIN proteins also function as Rab5-GEFs (13, 15, 17). Previous studies showed that RIN1 interacts with EGF receptors via its SH2 domain and regulates trafficking and degradation of EGF receptors via its interaction with signal-transducing adaptor molecule (20, 22), indicating a vital role for RIN1 in regulating endosomal trafficking of receptor tyrosine kinases. Previous work has also shown that RIN2 and RIN3 interact with amphiphysin II mediating receptor-induced endocytosis and that cytoplasmic amphiphysin II is translocated into the RIN3-positive vesicles (13).

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[2] The abbreviations used are: GDI, GDP dissociation inhibitor; CD-MPR, cation-dependent mannose 6-phosphate receptor; CFP, cyan fluorescence protein; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; iSR, internal sequence between SH2 and RH domains; RA, Ras association; RH, RIN family homology; RIN, Ras and Rab interactor or Ras interaction/interference; SH2, Src homology 2; TGN, trans-Golgi network; VPS9, vacuolar protein sorting 9.

Recently, Rab21, Rab22, Rab24, and Rab31 have been identified as novel Rab GTPases that show amino acid sequence homology to Rab5 (23–25). Physiological functions of these Rab GTPases are still being defined, but all appear to localize to the early endocytic pathway (26–28). Thus, they can be functionally categorized into the Rab5 subfamily (29). Recent reports show that Rab21 is activated by Varp and Rabex-5 (18, 30) and that Rab31 is activated by Gapex-5 (26), with each of these proteins containing a VPS9 domain. However, the activation mechanisms of Rab31 are poorly understood, and other VPS9 domain proteins might act as Rab31-GEFs in this paradigm. In the present study, we detail the interaction between RIN family members and the Rab5 subfamily by measuring the following GEF-related activities: (i) cell-free GTPγS binding to the Rab5 isoforms with recombinant RIN proteins, (ii) *in cell* GTP-bound Rab formation by the overexpression of RIN proteins under nonstimulated cell conditions, and (iii) morphological changes in Rab-containing vesicles in overexpressing cells. Our present findings show that RIN3 is capable of acting as a GEF for Rab31.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Monoclonal anti-FLAG (M2) and anti-c-myc (9E10) antibodies were purchased from Sigma. All other reagents were from commercial sources and analytical grade.

Construction of Expression Vectors—pECFP-C1, pEGFP-C1, and pDsRed-monomer vectors, as well as a human leukocyte MATCHMAKER cDNA library were obtained from BD Biosciences. pCMV-FLAG-RIN1, RIN2, RIN3, and pCMV-myc-Rab5 were constructed as described previously (13, 15). Rabex-5, Gapex-5, Varp, and Rab31 were amplified from the human leukocyte cDNA library. RIN/Rabex-5 mutants lacking GEF activity, pCMV-FLAG-RIN1/D537A,P541A, pCMV-FLAG-RIN1/Y577A,T580A, pCMV-FLAG-RIN2/D696A,P700A, pCMV-FLAG-RIN2/Y736A,T739A, pCMV-FLAG-RIN3/D785A,P789A, pCMV-FLAG-RIN3/Y825A,T828A, pCMV-FLAG-Rabex-5/D313A,P317A, and pCMV-FLAG-Rabex-5/Y354A,T357A were generated by PCR-mediated mutagenesis (31). ALS2 and ALS2CL plasmids were provided as gifts by J. Ikeda (14, 32).

Cell Culture and Transfection—HeLa and HEK293T cells were cultured in DMEM containing 10% FCS, 0.16% (w/v) $NaHCO_3$, 0.6 mg ml^{-1} L-glutamine, 100 μg ml^{-1} streptomycin, and 100 units ml^{-1} penicillin at 37 °C in 95% air and 5% CO_2. Cells were transfected with plasmid constructs using Lipofectamine 2000 (Invitrogen), FuGENE 6 (Roche Diagnostics), or HEKFectin (Bio-Rad).

Production of Recombinant Proteins—FLAG-RIN1, RIN2, RIN3, and Rabex-5 were purified from baculovirus-infected Sf9 cells with anti-FLAG M2 agarose beads as described previously (33). GST-fused Rab5, Rab21, and Rab31 were expressed in and purified from the cytoplasmic fraction of pGEX6P-1-transformed *Escherichia coli* BL21-CodonPlus (DE3)-RIL (Stratagene) using glutathione-Sepharose 4B resin (GE Healthcare). Protein concentrations were determined using the Amido Black 10B staining method.

Cell-free GTPγS Binding Assay—The GTPγS binding assay was performed by the filter method as described previously (13). Briefly, GST-Rab5, Rab21, and Rab31 (4–6 pmol of alive GTPγS binding activity) purified from *E. coli* were incubated with 1 μM [^{35}S]GTPγS (20,000 cpm $pmol^{-1}$) at 30 °C for the indicated times in the presence or absence of FLAG-RIN1, RIN2, RIN3, or Rabex-5 (8 pmol) purified from baculovirus-infected Sf9 cells in a reaction mixture (50 μl) consisting of 40 mM Tris-HCl (pH 8.0), 62.5 mM NaCl, 0.5 mM DTT, 0.36% (w/v) CHAPS, 50 μg ml^{-1} BSA, 5 mM EDTA, and 15 mM $MgCl_2$. The reaction was terminated by the addition of ice-cold 20 mM $MgCl_2$ and 20 mM NaCl at the final concentrations.

Radiolabeling of Nucleotides Associated with the Rab5 Subfamily in Intact Cells and Identification of the Nucleotide-bound Forms—cDNAs of the Rab5 subfamily and Rab5-GEFs were inserted into pCMV5-myc and FLAG vectors, respectively. These vectors were cotransfected into HEK293T cells using HEKFectin Reagent. Expression levels were confirmed by immunoblot analysis with anti-c-myc and anti-FLAG monoclonal antibodies. Guanine nucleotides associated with the GTP-binding proteins were analyzed as described previously (34).

Immunostaining and Fluorescence Microscopy—Immunostaining was performed as described previously (34). Briefly, HeLa cells grown on a polylysine-coated glass coverslip (15-mm diameter) were fixed with 4% paraformaldehyde in PBS for 15 min and treated with 0.1% Triton X-100 in PBS. Cells were incubated with a blocking solution consisting of PBS supplemented with 2% BSA and 2% FBS and then probed with primary antibodies (1 μg/ml in the blocking solution) for 2 h at 37 °C followed by subsequent incubation for 30 min with Alexa Fluor 488- or 568-conjugated secondary antibodies diluted with blocking solution. After washing three times with PBS, the coverslip was mounted onto a glass slide in Permafluor-mounting medium (IMMUNON) and viewed on Carl Zeiss LSM700 or Leica TCS-SP5 confocal microscopy systems.

Yeast Two-hybrid Analysis—A yeast two-hybrid assay was performed according to the method described previously (34). Briefly, yeast strain Y190 was cotransformed with plasmids containing pGBT9-RIN3 mutants and pGAD10-Rab5, or Rab31. Cotransformants grown on synthetic medium lacking leucine and tryptophan were subjected to quantitative β-galactosidase assays using 4-methylumbelliferyl-β-D-galactoside as a substrate.

RESULTS

RIN3 Exerts Guanine Exchange Activity for Rab31 in a Cell-free GTPγS Binding Assay—Analysis of Rab GTPase sequences suggested that Rab31 has motifs that are highly homologous to Rab5, Rab21, Rab22, and Rab24. Previous studies have shown that RIN1, RIN2, and RIN3 function as GEFs for Rab5 (13, 15, 17). We first investigated whether RIN proteins might exhibit GEF activity for Rab5 proteins. For this analysis, we also included Rabex-5, which is another VPS9 domain-containing GEF for Rab5 and Rab21 (35), as a control. We evaluated their biochemical abilities to stimulate [^{35}S]GTPγS binding to Rab5 subfamily members in a cell-free assay. Recombinant Rab5 subfamily proteins were expressed in, and purified from, *E. coli*

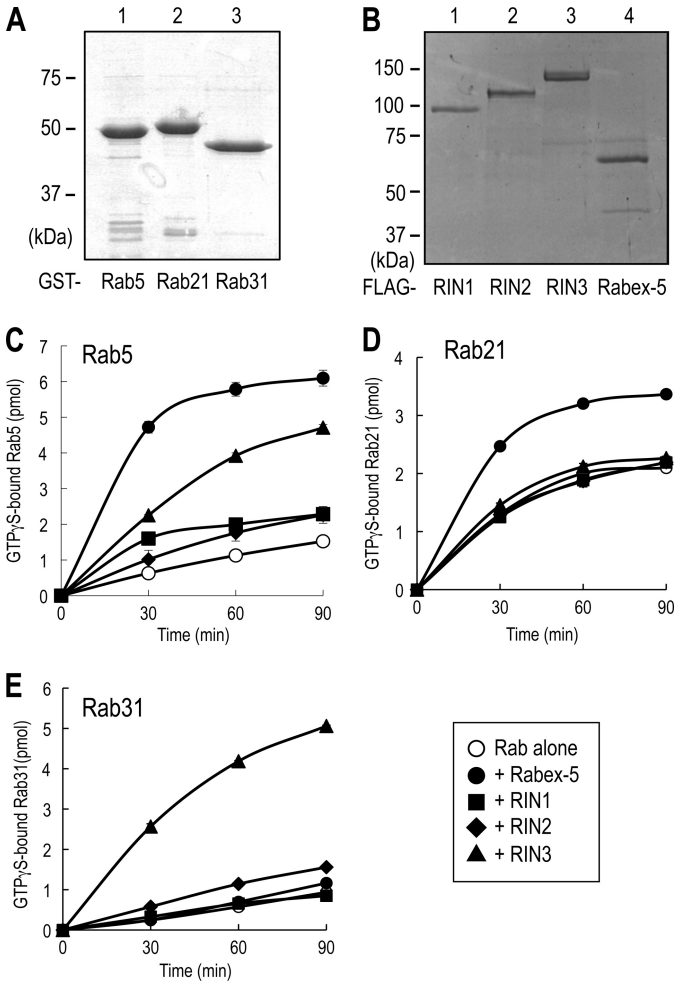

FIGURE 1. **Cell-free GEF activity of the RIN proteins and Rabex-5 for Rab5 proteins.** The Rab5 proteins (*A*, GST-Rab5, Rab21, and Rab31) and GEF proteins (*B*, FLAG-tagged RIN1–3 and Rabex-5) purified from *E. coli* and baculovirus-infected Sf9 cells, respectively, were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. GST-Rab5 (*C*, 6 pmol of alive GTPγS binding activity), Rab21 (*D*, 4 pmol), or Rab31 (*E*, 6 pmol) were incubated at 30 °C with 1 μM [^{35}S]GTPγS for the indicated times in the absence (FLAG peptide alone) and presence of 8 pmol of FLAG-Rabex-5 (*filled circles*), RIN1 (*filled squares*), RIN2 (*filled diamonds*), or RIN3 (*filled triangles*). The amounts of [^{35}S]GTPγS bound to Rab5 proteins at each time point are presented. No [^{35}S]GTPγS binding activity was detected in the fractions containing RIN proteins or Rabex-5 (data not shown). The data obtained from three independent experiments are shown with mean ± S.E. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ *versus* Rab alone samples.

(Fig. 1*A*). FLAG-tagged RIN family and Rabex-5 proteins were purified from baculovirus-infected Sf9 cells (Fig. 1*B*). Rates of GTPγS binding to Rab5 proteins were relatively slow at physiological concentrations (millimolar order) of Mg^{2+} (Fig. 1, *C*–E) due to slow GDP-GTP exchange, and no detectable GTPγS binding activity was observed for RIN isoforms or for Rabex-5 alone (data not shown). Under these conditions, Rabex-5 markedly accelerated GTPγS binding to Rab5 and Rab21, but not to Rab31, consistent with a previous report (35). RIN3 stimulated GTPγS binding to Rab5 slowly, and RIN1 and RIN2 exhibited weak activity compared with Rabex-5 (Fig. 1*C*). No RIN family members exhibited detectable GEF activity for Rab21 (Fig. 1*D*). RIN3 potently and RIN2 weakly accelerated GTPγS binding to Rab31 (Fig. 1*E*). We further analyzed the kinetics of RIN3 guanine exchange activity. Rab5 and Rab31 (2 pmol) were incubated with various concentrations of RIN3 in the presence of [^{35}S]GTPγS. From the quantities of [^{35}S]GTPγS-bound Rab5 and Rab31, the catalytic activities of RIN3 were calculated. We found that the preference for Rab31 binding and the catalytic activity for Rab31 were as much as that for Rab5 (supplemental Fig. S1*A*). However, the basal nucleotide exchange activity of Rab31 (0.005 pmol/min) is apparently slower than that of Rab5 (0.015 pmol/min). Thus, the increasing rate of GTPγS-Rab31 formation by RIN3 is higher than that of GTPγS-Rab5 formation (supplemental Fig. S1*B*), suggesting that RIN3 enhances the nucleotide exchange reaction of Rab31 more potently than for Rab5. In contrast, RIN1 or Rabex-5 exhibited no apparent GEF activity for Rab31. Collectively, these results indicated that RIN3 exerts guanine exchange activity for Rab31 in the cell-free assay.

RIN2 and RIN3 Have the Ability to Stimulate GTP-bound Rab31 Formation in in Cell GEF Activity Assays—We next investigated the GEF activities of RIN and Rabex-5 for Rab5 subfamily proteins in intact cells. For the *in cell* GEF activity assay, HEK293T cells expressing myc-tagged Rab5 proteins and FLAG-tagged RIN proteins, or Rabex-5, were metabolically radiolabeled with $^{32}P_i$, and the cell lysate containing the myc-Rab5 subfamily was immunoprecipitated with an anti-myc antibody. Guanine nucleotides bound to the immunoprecipitated Rab5 subfamily were separated by thin layer chromatography and quantitated with an image analyzer. An example experiment performed with Rab31 is shown in Fig. 2*A*. Approximately 5% of Rab31 was present as the GTP-bound form in mock-transfected cells (*lane 1*), whereas the expression of RIN proteins (*lanes 2–4*) or Rabex-5 (*lane 5*) increased the formation of GTP-bound Rab31, in which RIN2 and RIN3 were the most effective isoforms (>60%). The same experiments were applied to Rab5 and Rab21, together with Rab31, and the results are summarized in Fig. 2*B*. RIN proteins and Rabex-5 increased GTP-bound Rab5 levels, although RIN proteins appeared to have less effect than Rabex-5 and did not increase GTP-bound Rab21 effectively. These results are consistent with the cell-free GEF activity assay data in Fig. 1.

We investigated further whether other Rab5-GEFs possess GEF activity for Rab5 proteins in mammalian cells. We applied the same assay to all known VPS9 domain-containing proteins. To our surprise, Gapex-5, which has been reported to have GEF activity for Rab31 (26), did not increase GTP-bound forms of any Rab5 proteins under the present conditions. To investigate whether domains other than VPS9 might inhibit Gapex-5 GEF activity, we expressed only the VPS9 domain of Gapex-5, together with Rab5 subfamily proteins, in HEK293T cells. In this case, GEF activity was as little as that observed with the full-length protein (data not shown). Recombinant Gapex-5 was purified from baculovirus-infected Sf9 cells to monitor its GEF activity for Rab5 and Rab31, but no activity was detected (supplemental Fig. S2). ALS2 and ALS2CL, which are highly homologous in their C-terminal domains and form a heteromeric complex (32), tended to exert GEF activities for Rab5 proteins at levels comparable with those observed for RIN2 and RIN3. Varp, which was identified as a Rab5- and Rab21-GEF (18), enhanced GTP-bound Rab21 as well as Rabex-5. Because Varp was not expressed well in HEK293T cells relative to other

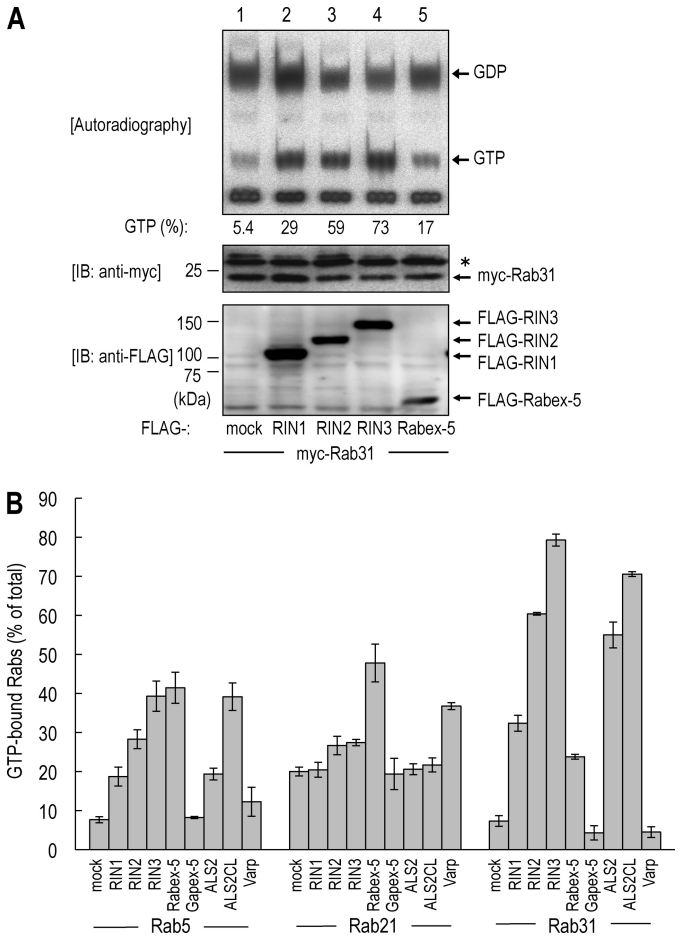

FIGURE 2. ***In cell* GEF activity of the VPS9 domain proteins for the Rab5 proteins.** *A*, HEK293T cells expressing myc-Rab31 and FLAG-mock, RIN1–3, or Rabex-5 were metabolically radiolabeled with $^{32}P_i$ for 4 h. Myc-tagged Rab31 was immunoprecipitated with an anti-myc monoclonal antibody, and nucleotides associating with Rab31 were separated by thin layer chromatography (*top*). The radioactivity of GTP and GDP was quantified, and the percentages (%) of GTP-bound Rab31 are shown in the *bottom lanes*. Immunoprecipitated samples (*middle*) and total lysates (*bottom*) from the radiolabeled cells were separated by SDS-PAGE and immunoblotted with anti-myc and anti-FLAG antibodies, respectively. *Asterisk* (*) shows a nonspecific band. *B*, HEK293T cells expressing myc-Rab5 subfamily (Rab5, 21, and 31) and the FLAG-VPS9 domain proteins (mock, RIN1, RIN2, RIN3, Rabex-5, Gapex-5, ALS2, ALS2CL, and Varp) were metabolically radiolabeled with $^{32}P_i$, and the percentages (%) of GTP-bound Rab GTPases were determined as described in *A*. The data obtained from more than three independent experiments are shown with mean ± S.E. (*error bars*).

Rab5-GEFs (data not shown), we still do not know whether Varp has GEF activity for Rab5 or Rab31. Taken together, these results indicate that RIN2/3 and ALS2/ALS2CL are capable of acting as GEFs for Rab31, but not for Rab21, in intact-cell assays.

VPS9 Is Responsible for the GEF Activity of RIN3—We next investigated whether VPS9 domains of RIN proteins are responsible for the GEF activity for Rab31. The VPS9 domain structure of Rabex-5 has been determined by x-ray analysis, and four amino acids (D, P, Y, and T) have been identified to be critical for GEF activity (35). Because all four amino acids are conserved among RIN family VPS9 domains (supplemental Fig. S3*A*), we generated two mutants of each RIN that would reduce GEF activity: RIN1/D537A,P541A (DP_AA), RIN1/Y577A,T580A (YT_AA), RIN2/D696A,P700A (DP_AA), RIN2/Y736A,T739A (YT_AA), RIN3/D785A,P789A (DP_AA), and RIN3/Y825A,T828A (YT_AA). FLAG-tagged wild-type or each RIN family mutant was cotransfected with myc-tagged Rab31 into HEK293T cells, and the radiolabeled nucleotides bound to Rab31 were analyzed as described above. The increase in GTP-bound Rab31 from coexpression of RIN proteins was completely abolished by the mutations of their VPS9 domains (Fig. 3, *A* and *B*), except for a moderate abolishment in RIN3/DP_AA. The modest increase of GTP-bound Rab31 by Rabex-5 was also attenuated by mutations in its VPS9 domain (supplemental Fig. S3, *B* and *C*). Mutations in RIN proteins also attenuated, although not completely, their GEF activities for Rab5 (data not shown). Next, we investigated whether the GEF activity of these mutants for Rab31 decreased in the cell-free GTPγS binding assay by using purified wild-type, RIN3/YT_AA and DP_AA (Fig. 3*C* and supplemental Fig. S4*A*). Wild-type RIN3 increased GTP-bound Rab31, but neither the YT_AA nor DP_AA mutant exhibited any significant activity (Fig. 3*D* and supplemental Fig. S4*B*).

RIN3 Is Capable of Enlarging Rab31-positive Tubulovesicular Organelles in HeLa Cells—It was previously observed that coexpression of RIN3 and Rab5 enlarged Rab5-positive vesicles (13). We investigated whether Rab31 localization was also affected by coexpression of RIN3 in mammalian cells. Rab31 fused with EGFP localized mainly to perinuclear vesicles and reticulate structures located throughout the cytoplasm (Fig. 4*A*, *left*), as reported previously (36). Partial colocalization of EGFP-Rab31-positive vesicles with EEA1 and TGN46 signals (supplemental Fig. S5) confirmed that Rab31 shuttles between TGN and early endosomes (37, 38). On the other hand, RIN3 was distributed throughout the cytoplasm and only somewhat localized to a few vesicle structures when expressed alone (Fig. 4*A*, *center*). Coexpression of RIN3 with Rab31 induced extended and bundled tubulovesicular structures, as well as many enlarged vesicles, where colocalized (Fig. 4*C*). This colocalization appeared to be dependent on GEF activity of RIN3, because the RIN3/YT_AA mutant did not have an effect on the localization of Rab31 (Fig. 4*D*). In contrast, neither RIN1 nor Rabex-5 altered the morphology of Rab31 (data not shown). Next, we examined whether other VPS9 domain proteins that possess GEF activities for Rab31 generate similar structures. RIN2 induced enlarged Rab31-positive vesicles near the nucleus but did not colocalize with Rab31. ALS2CL induced many Rab31-positive vesicles throughout the cytoplasm where they colocalized (supplemental Fig. S6). These results indicate that RIN3 specifically induced tubulovesicular structures and suggest that each Rab31-GEF activates Rab31 under different spatio-temporal conditions.

To characterize enlarged vesicles and tubulovesicular structures, HeLa cells expressing ECFP-Rab31 and EYFP-RIN3 were immunostained with organelle-specific markers. As shown in Fig. 5, EEA1 and the transferrin receptor were partially colocalized with RIN3/Rab31-positive organelles, but TGN46 or Lamp1 was not, indicating that these structures are similar to early endosomes. However, these structures did not colocalize with fluorescent-labeled EGF, transferrin, or Shiga toxin (data not shown). Taking into consideration that

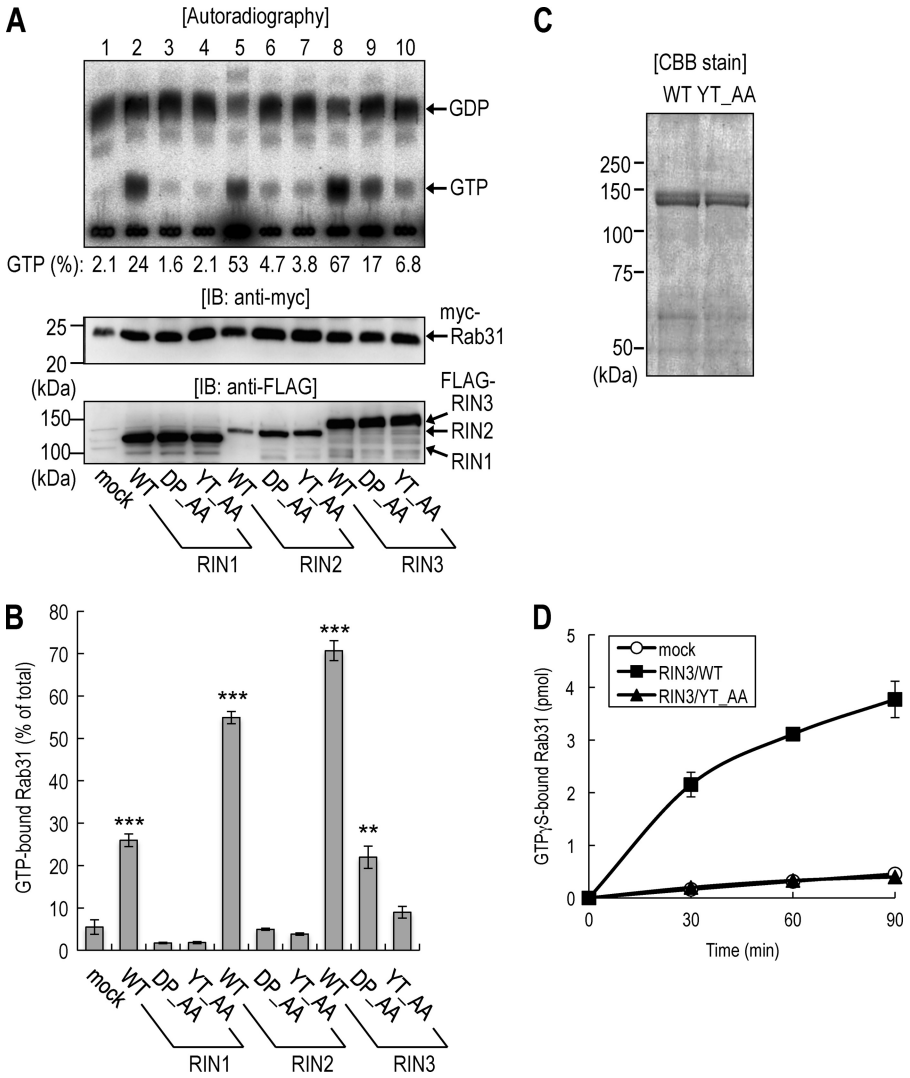

FIGURE 3. **Mutations in the VPS9 domains of RIN proteins impair their GEF activities for Rab31.** *A* and *B*, myc-Rab31 was cotransfected with wild-type (*WT*), DP_AA, or YT_AA mutant of FLAG-RIN1–3 into HEK293T cells. Cells were metabolically radiolabeled with $^{32}P_i$, and the percentages (%) of GTP-bound Rab31 were determined (*top*) as shown in Fig. 2. Immunoprecipitated samples (*middle*) and total lysates (*bottom*) from the radiolabeled cells were also separated by SDS-PAGE and immunoblotted with anti-myc and anti-FLAG antibodies, respectively. The data obtained from three independent experiments are shown (*B*) with mean ± S.E. (*error bars*). ***, $p < 0.001$; **, $p < 0.01$ *versus* mock-transfected cells. *C*, wild-type and YT_AA mutant versions of FLAG-RIN3 were purified from baculovirus-infected Sf9 cells. Recombinant proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. *D*, GST-Rab31 (4 pmol of alive GTPγS binding activity) was incubated at 30 °C with 1 μM [^{35}S]GTPγS for the indicated times in the presence of wild-type (*filled squares*) and YT_AA mutant (*filled triangles*) of FLAG-RIN3 (8 pmol) for 90 min. The data obtained from three independent experiments are shown with mean ± S.E.

Rab31 localizes to both TGN and early endosomes, RIN3 might enhance the movement of Rab31 from TGN to early endosomal compartments.

Vesicle enlargement by the overexpression of RIN3 was rather specific for Rab31 and Rab5 (13), and RIN3 certainly exhibited GEF activity for these Rab members (Fig. 2*B*). As expected from the finding that RIN3 exhibited no GEF activity for Rab21, coexpression of RIN3 did not show a potent enlarging effect on Rab21-positive vesicles (supplemental Fig. S7*E*). Furthermore, no significant overlap was observed between RIN3 and other Rab GTPases, including Rab4, Rab7, and Rab11, which are involved in retrograde transport (supplemental Fig. S7, *F–H*). Collectively, the characteristics of RIN3-induced formation of enlarged Rab31-positive vesicles and tubulovesicular organelles were well correlated with the biochemical properties observed in the cell-free and *in cell* GEF-activity assays.

Substitution of Serine Residues with Alanine in RIN3-iSR Specifically Attenuates Interaction with, and GEF Activity for, Rab31—We investigated the conformational regulation of RIN3 GEF activity for Rab31. We focused on the internal sequence between SH2 and RH domains, iSR, that contains proline-rich domains because this region is variable across RIN proteins and is adjacent to RH and VPS9 domains, which are responsible for GEF activity (13). We generated a number of point mutants in the iSR to investigate whether mutations affect the ability to interact with Rab31 as evaluated using the yeast two-hybrid system. We found that mutations of eight serine sites (523, 524, 530, 533, 535, 537, 583, 586) to alanine (RIN3/S_A) decreased interaction with Rab31, but not Rab5 (Fig. 6*A*). To verify GEF activity for this mutant, recombinant RIN3/S_A was purified from baculovirus-infected Sf9 cells and incubated with Rab5 or Rab31 in the presence of [^{35}S]GTPγS. The GEF activity of RIN3/S_A for Rab31 was strongly inhibited,

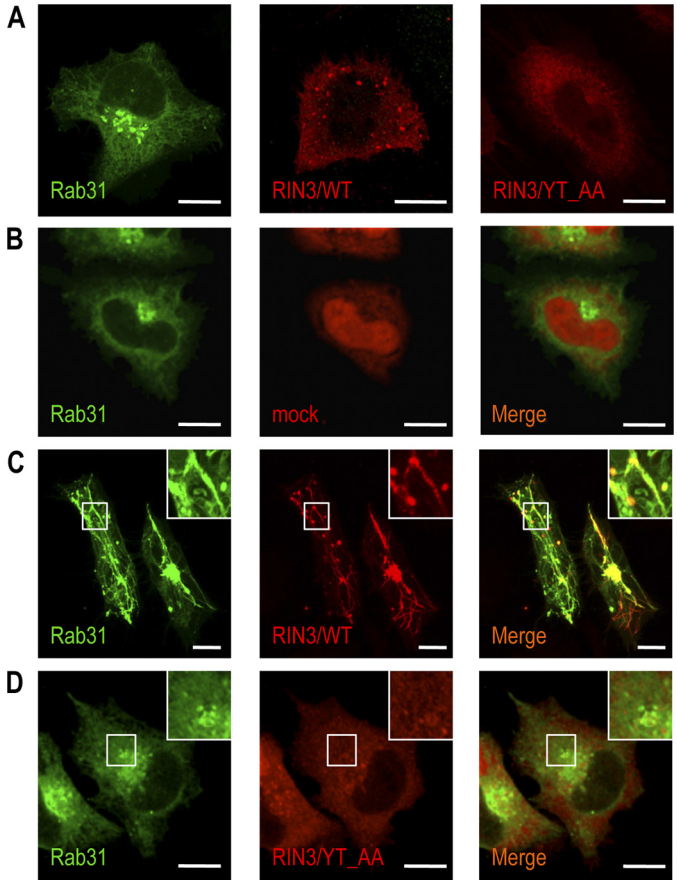

FIGURE 4. **Expression of RIN3 induces the enlargement of Rab31-positive endosomes and tubulovesicular structures.** *A*, EGFP-Rab31 (*right*), wild-type (*center*), and YT_AA mutant (*right*) versions of DsRedm-RIN3 were transiently transfected into HeLa cells. *B–D*, EGFP-Rab31 was cotransfected with DsRedm-mock (*B*), wild-type (*C*), and YT_AA mutant (*D*) versions of RIN3 in HeLa cells. Merged images of the two signals are displayed in the *right panels*, and *insets* in *C* and *D* are magnifications of the areas highlighted by *white squares*. *Scale bars*: 10 μm.

but GEF activity for Rab5 was only slightly reduced compared with wild type (Fig. 6, *B* and *C*). This trend was confirmed using *in cell* GEF activity assays (Fig. 6*D*). Wild-type RIN3 colocalized with both Rab5 and Rab31, but RIN3/S_A significantly abolished colocalization with Rab31 (Fig. 6, *E–I*). Although we found that both endogenous and overexpressing RIN3 are constitutively phosphorylated, RIN3/S_A was phosphorylated to the same degree as wild-type in HEK293T cells (data not shown), suggesting that the decrease of its interaction with Rab31 was not due to the phosphorylation state. Taken together, these results indicate that RIN3/S_A specifically attenuates GEF activity for Rab31, and the conformation change in iSR region by some modifications other than phosphorylation might regulate GEF activity for Rab5 proteins.

RIN3 Promotes Transport of CD-MPR from TGN to Perinuclear Endosomes in a Way That Is Dependent on GEF Activity for Rab31—Rodriguez-Gabin *et al.* (39) have shown that Rab31 regulates transport of CD-MPR from the TGN to endosomes. We monitored the localization of CD-MPR to define the role of RIN3 for Rab31-GEF in the transport of CD-MPR in HeLa cells. CD-MPR-EGFP localized mainly to TGN (TGN46-positive tubular organelles) and to extending tubules spread throughout the cytoplasm (Fig. 7*A*). When RIN3 was coexpressed with CD-MPR-EGFP, it reduced the amount of CD-MPR in the TGN, but increased the amount of CD-MPR in peripheral compartments (Fig. 7*C*), which is consistent with the phenotype resulting from Rab31-Q64L expression (39). Translocation by RIN3 was dependent on its GEF activity for Rab31 because neither the RIN3/YT_AA nor S_A mutant had an effect (Fig. 7, *C* and *D*). These observations indicate that RIN3 regulates the transport of CD-MPR from the TGN to early endosomes by activating Rab31.

DISCUSSION

The VPS9 domain, which was initially reported to act as a GEF for Rab5, has been found in many proteins, including RIN family members (RIN1, RIN2, and RIN3), Vps9p, Rabex-5, ALS2/Alsin, ALS2CL, Varp, and Gapex-5/RAP6/RME-6 (10, 11, 13–18). Recent studies have shown that several VPS9 proteins also activate Rab21 and Rab31, in addition to Rab5. For example, Gapex-5 showed a GEF activity for Rab31, and Varp showed much more potent GEF activities for Rab21 than Rab5 (18, 26). On the contrary, Rabex-5 and RIN1 exhibit little GEF activity, and Gapex-5 exhibits none, for Rab22, which is also similar to Rab5 (35). These findings suggest that complex mechanisms are involved in the interaction between Rab5-like and VPS9-containing proteins. In the present study, we discovered that RIN3 is capable of acting as a GEF for Rab31 and Rab5, but not for Rab21. Our conclusion is based on the following results related to GEF activity: (i) RIN3 stimulated GTPγS bound to Rab31 in the cell-free assay (Fig. 1), (ii) RIN3 stimulated *in cell* GTP-bound Rab31 formation under nonstimulated cell conditions (Fig. 2), and (iii) the expression of RIN3 formed Rab31-containing tubulovesicular structures in intact cells, where they colocalized (Figs. 4 and 5).

It is interesting to note here that there are slight differences between the cell-free and *in cell* GEF activities of RIN proteins (and Rabex-5) for Rab5 proteins. For instance, Rabex-5 is much more efficient than RINs at loading GTPγS-bound Rab5 in the cell-free assay (Fig. 1*A*), whereas there is no significant difference between RIN3 and Rabex-5 in *in cell* GEF activities for Rab5 (Fig. 2*B*). The *in cell* assay might be superior to the cell-free GTPγS binding assay due to the experimental conditions close to native cell environments, but its quantitative analysis is rather difficult due to limitations in the expression control. The cell-free GTPγS activity was independent of intrinsic GTPase activities of Rab5 proteins, whereas the *in cell* GEF activities were affected by intrinsic GTPase activities that were dependent on the cellular conditions. For example, the attenuation of GTPase activities by the tight interaction of Rab5 proteins with GEFs may form the basis for differences between cell-free and *in cell* GEF activities. Consistent with this idea, it has been reported that RIN3 interacts with GTP-bound Rab5 more strongly than GDP-bound Rab5 (13). Thus, assays performed in the present study would be helpful for estimating GEF properties to elucidate molecular mechanisms and physiological significances.

We also found that quantification of GEF activity in intact cells would be informative for investigating the activities of these proteins. For example, Rab5-GEF activity of ALS2CL was significantly lower than that of ALS2, down to negligible levels

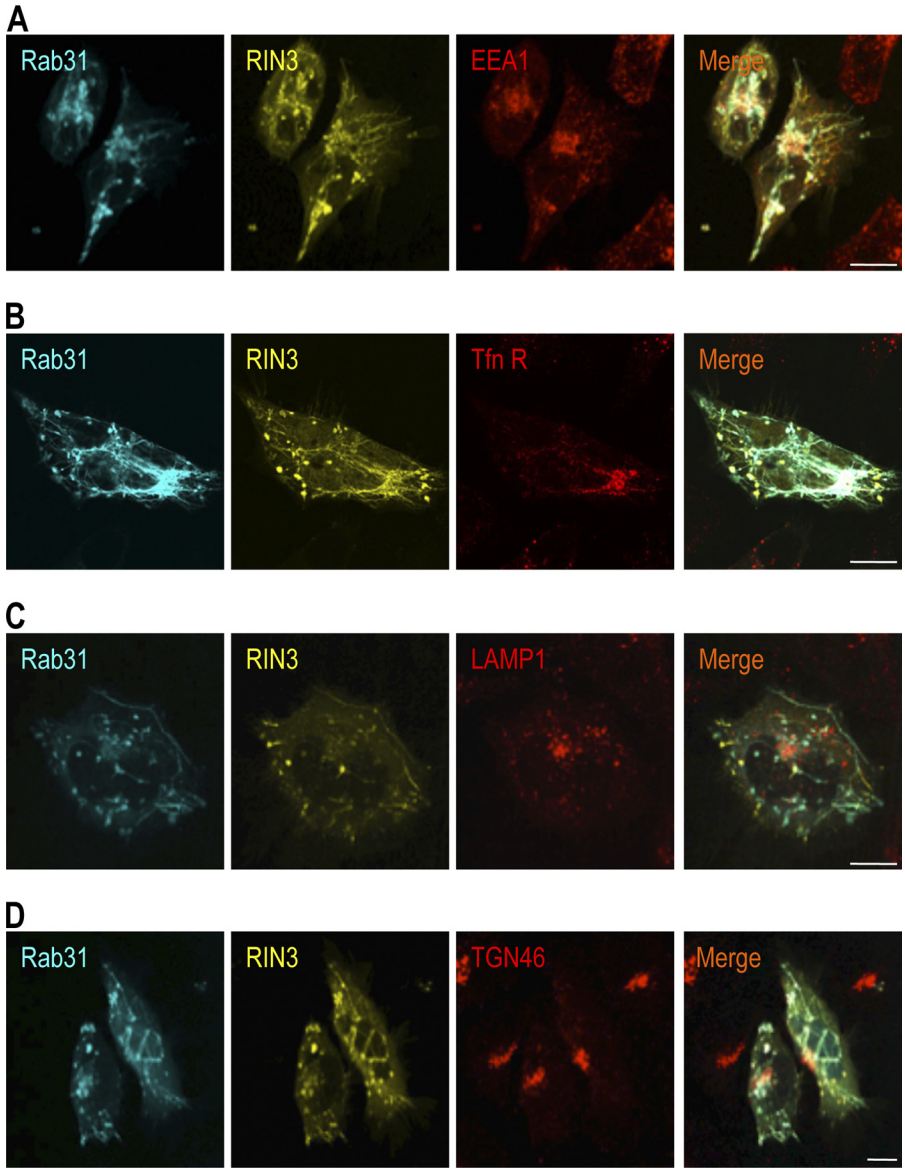

FIGURE 5. **Characterization of Rab31-positive enlarged endosomes and tubulovesicular structures induced by RIN3.** EYFP-RIN3 was transiently transfected with ECFP-Rab31 into HeLa cells. After incubation for 48 h, transfected cells were immunostained with anti-EEA1 (*A*), transferrin receptor (Tfn R, *B*), Lamp1 (*C*), and TGN46 (*D*) antibodies, and merged images of the three signals are displayed in the *right panels*. *Scale bars*: 10 μm.

in cell-free assays (40). However, expression of ALS2CL, as well as ALS2, increased GTP-bound forms of Rab5 and Rab31 in intact cells (Fig. 2*B*), indicating that some cofactors might be required for GEF activities.

Although it has been reported that Gapex-5 acts as a GEF for Rab31 (26) and that the VPS9 domain of Gapex-5 exhibited GEF activity for Rab5 in cell-free assays (12), we could not detect the activity of Gapex-5 for Rab5 or Rab31 in our assays (Fig. 2*B* and supplemental Fig. S2, *A–C*). The discrepancy might be due to differences in assay conditions. To date, GEF activities of VPS9 domain-containing proteins in cell-free assays have been monitored using recombinant VPS9 domains alone (12, 41). As the GEF activities of these proteins might be regulated by conformation changes due to upstream signals, structures other than VPS9 domains might alter their GEF activities. Actually, the exchange activities of Rabex-5 and Varp are suppressed by autoinhibitory elements present in the C terminus of VPS9 domains, and the elimination of these regions resulted in GEF activation (18, 30). Taking into consideration that down-regulation of Gapex-5 impaired insulin-stimulated PKB/Akt activation (42), the GEF activity of Gapex-5 might be elevated by stimulators, including growth factors.

Recent reports have suggested that Rab31 plays a role in trafficking vesicles between TGN and early endosomes. Rab31 colocalizes with TGN markers, early endosomes, and microtubules (36, 37) and interacts with EEA1 (26). Overexpression of active Rab31 enhanced the transport of CD-MPR, leading to a reduction in the amount of CD-MPR in TGN (39). Thus, Rab31 appears to regulate the heterotypic fusion of transported vesicles along with TGN and early endosomes. We observed that the expression of RIN3 reduced the amount of CD-MPR in TGN but increased the amount of CD-MPR in peripheral compartments (Fig. 7). We also observed, by means of time lapse imaging analysis, that mDsRed-RIN3 moved dynamically with

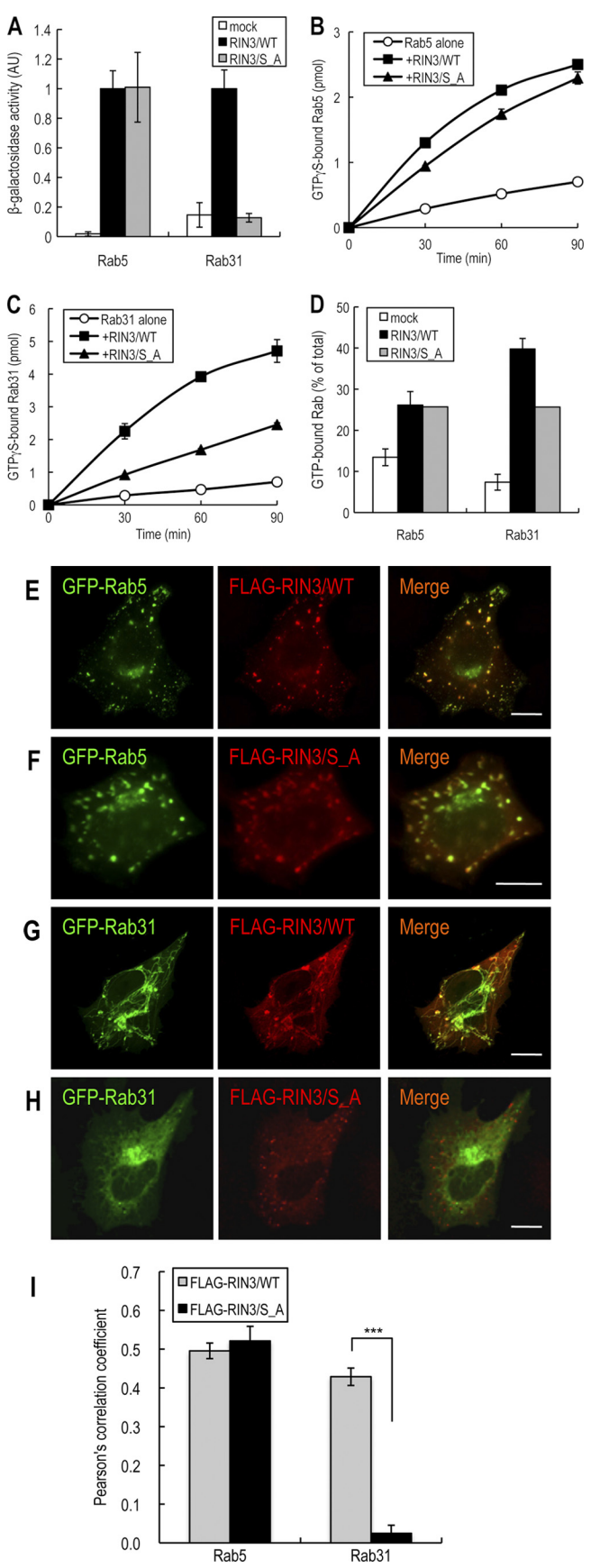

FIGURE 6. **RIN3/S_A mutant specifically attenuates interaction with, and GEF activity for, Rab31.** *A*, analysis of RIN3-Rab31 interactions using the yeast two-hybrid system. Two-hybrid bait plasmids encoding mock (*white*), RIN3/WT (*black*), and RIN3/S_A (*gray*) were cotransformed into the Y190 yeast strain with the Rab5 or Rab31 prey plasmid. Cotransformants were subjected to the β-galactosidase assay. The data obtained from three independent experiments are shown with the mean ± S.E. (*error bars*). *B* and *C*, The purified GST-Rab5 (*B*) or Rab31 (*C*) (4 or 5 pmol of alive GTPγS binding activity, respectively) were incubated at 30 °C with 1 μM [^{35}S]GTPγS for the indicated times in the presence of FLAG-RIN3/WT (*filled squares*), RIN3/S_A (*filled triangles*), or FLAG peptide alone (*open circles*) (each 8 pmol). *D*, *in cell* GEF activities of FLAG-mock (*white*), RIN3/WT (*black*), and RIN3/S_A (*gray*) for Rab5 or Rab31 in HeLa cells were determined as shown in Fig. 2. *E–I*, GFP-Rab5 (*E* and *F*) or Rab31 (*G* and *H*) were cotransfected with FLAG-RIN3/WT (*E* and *G*) or RIN3/S_A (*F* and *H*) in HeLa cells and immunostained with anti-FLAG antibody. The degrees of colocalization of 25 cells were evaluated by cross-correlation analyses and are shown as Pearson's coefficient (*I*). ***, $p < 0.001$. *Error bars*, S.E.

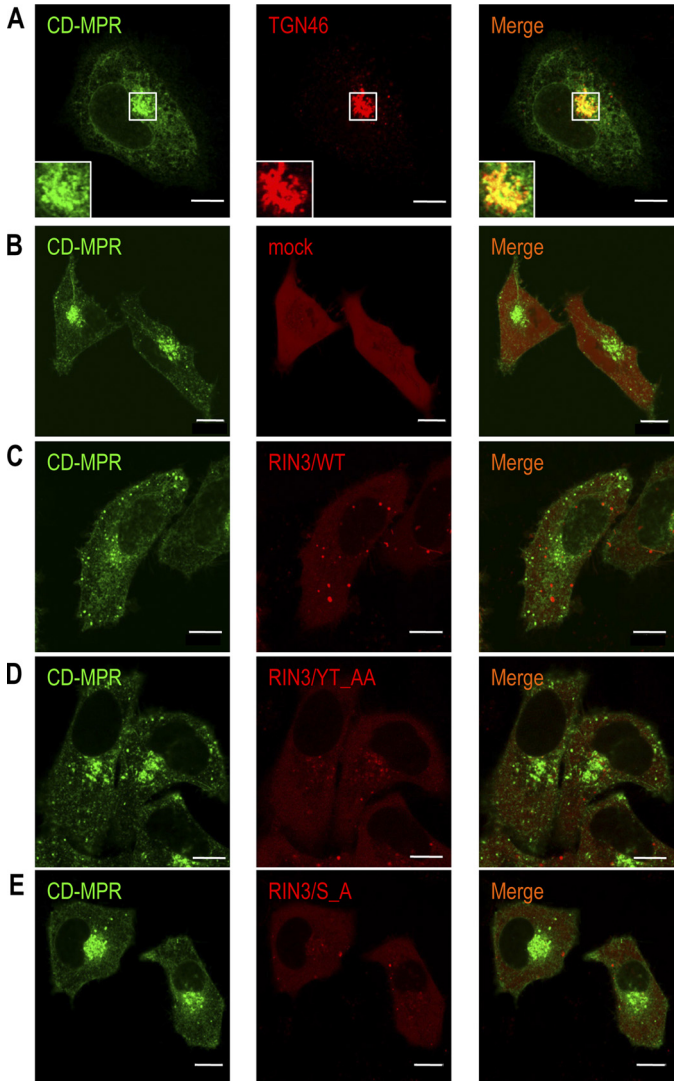

FIGURE 7. **Intracellular transport of the mannose 6-phosphate receptor from TGN to early endosome is enhanced by RIN3.** *A*, HeLa cells expressing CD-MPR-EGFP were immunostained with anti-TGN46 antibody. *B–E*, CD-MPR-EGFP was cotransfected with DsRedm-mock (*B*), RIN3/WT (*C*), RIN3/YT_AA (*D*), and RIN3/S_A (*E*) in HeLa cells. Merged images of the two signals are displayed in the *right*.

EGFP-Rab31 along the punctate vesicles and tubulovesicular organelles in HeLa cells (data not shown). These findings support the idea that RIN3 acts physiologically as a GEF to regulate Rab31-dependent transport between the TGN and early endosomes. Further studies will be necessary for elucidating the physiological roles of RIN3 as a GEF for both Rab5 and Rab31.

The structure of the Rabex-5 VPS9 domain has been elucidated (35), and four amino acids (Asp, Pro, Tyr, and Thr) that cluster near the N termini of αV4 and αV6 on the VPS9 domain appear to be binding pockets for conserved aromatic residues in the interswitch and switch II of Rab5 (30). Indeed, GEF activity of Rabex-5 was abolished by the mutations in these four amino acids of the VPS9 domain for Rab5 and Rab31 (supplemental Fig. S3, *B* and *C*). Moreover, mutations in corresponding amino acids of RIN proteins also attenuated GEF activity for Rab31 in both cell-free and *in cell* GEF activity assays (Fig. 3*D* and supplemental Fig. S4). These findings suggest that the characteristics of RIN-VPS9 domains as GEFs for Rab5 proteins are basically conserved among all VPS9 proteins including Rabex-5. Taking into consideration that the RH domain in RIN3 is sufficient and required for its interaction with Rab5 (34), RIN3 might recognize Rab5 proteins in a way that is slightly different from Rabex-5. The elucidation of the structure of the RH-VPS9 domain in RIN3 will provide insight into the specifics of this recognition.

Alternatively, differences within the Rab5 subfamily of proteins might be crucial determinants. Rab5 proteins have common functional features, including their interaction with EEA1. However, they differ from one another in the following ways: (i) Rab5 and Rab21, but not Rab22, interact with APPL1 (43); (ii) Rabex-5 has much higher GEF activity for Rab5 and Rab21 than for Rab22 (35); (iii) Rab5 and Rab22, but not Rab21, interact with Rabenosyn-5; and (iv) Rab5 and Rab21 localize to early endosomes, but Rab31 localizes to both vesicles and tubulovesicular structures. In context of the structure of Rab5 proteins, a detailed study using point mutations has shown that the switch I, interswitch, and switch II regions of Rab5, Rab21, and Rab22 are similar but differ in subtle ways. Such subtle differences could contribute to the reasons that support an interaction with the Rab5 effector Rabenosyn-5 (44).

Another unsolved key question is the molecular mechanisms by which these RIN-GEFs recognize and activate each Rab5 protein in response to the upstream signals. Both RIN2 and RIN3 have an RA domain in their C terminus that can interact with the GTP-bound form of Ras, so the Ras family is a possible modulator of RIN-GEF activity upon cell stimulation. This idea is supported by reports that RIN1 and RIN2 interact with Ha-Ras via their RA domain, and this interaction potentiates their GEF activities for Rab5 (17, 45). However, the GEF activity of RIN3 for Rab31 was not altered in the absence or presence of activated Ha-Ras/G12V in mammalian cells (data not shown). Moreover, the deletion of the RA domain from RIN3 did not attenuate its GEF activity for Rab5 or Rab31 (supplemental Fig. S8), indicating that Ras proteins do not appear to enhance the GEF activity of RIN3. However, Rabex-5 ubiquitinates Ha-Ras and attenuates Ras signaling, and RIN1 is required for Rabex-5-mediated Ha-Ras ubiquitination (46, 47). Gapex-5 has a Ras GAP domain and shows GAP activity for Ha-Ras (12), indicating that some Rab5-GEF proteins contribute to Ras signaling. RIN3 may interact with some Ras proteins that have not been identified yet and affect related signaling pathways.

The SH2 domain could be responsible for the rigid regulation of RIN family. RIN1 forms a complex via its N-terminal SH2 domain with a number of receptor tyrosine kinases but not with cargo endocytic receptors (20, 48), and knockdown of RIN1 significantly reduced the internalization of insulin receptors and ERK1/2 activation (49). We examined whether the SH2 domain of RIN3 affects its GEF activity and found that the deletion of the SH2 domain significantly attenuated GEF activity for both Rab5 and Rab31 in mammalian cells (supplemental Fig. S8). These results suggest that RIN3 functions in the receptor tyrosine kinase-linked early endocytic pathway in response to stimulation via its SH2 domain. Further study to identify the stimulus that regulates GEF activity will be necessary.

Two potential models are supported by our study. The first is that a single member of RIN acts as a GEF for multiple Rab members through the full range of vesicular transport pathways. The second is that multiple RIN proteins switch target Rab members to regulate specific pathways. Additional studies will be necessary to define the interactions between these types of signaling molecules and to elucidate the molecular mechanism through which each cognate GEF is activated in intracellular microenvironments.

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