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Tyr-phosphorylation signals translocate RIN3, the small GTPase Rab5-GEF, to early endocytic vesicles

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ABSTRACT

The small GTPase Rab5 plays a key role in early endocytic pathway, and its activation requires guanine-nucleotide exchange factors (GEFs). Rab5-GEFs share a conserved VPS9 domain for the GEF action, and RIN3 containing additional domains, such as *Src*-homology 2, RIN-family homology (RH), and Ras-association (RA), was identified as a new Rab5-GEF. However, precise functions of the additional domains and the activation mechanism of RIN3 remain unknown. Here, we found tyrosine-phosphorylation signals are involved in the Rab5-GEF activation. Treatment of HeLa cells with pervanadate translocates RIN3 from cytoplasm to the Rab5-positive vesicles. This RIN3 translocation was applied to various mutants lacking each domain of RIN3. Our present results suggest that a Ras GTPase(s) activated by tyrosine-phosphorylation signals interacts with the inhibitory RA domain, resulting in an active conformation of RIN3 as a Rab5-GEF and that RIN-unique RH domain constitutes a Rab5-binding region for the progress of GEF action.

The small GTPase Rab family plays central roles in intracellular membrane trafficking. At present, more than 60 members belonging to this family have been identified, and they appear to localize in distinct intracellular compartments and regulate the specific transport between donor and acceptor organelles [1–3]. Rab5, which is the most thoroughly characterized member of this family, functions not only in the homotypic fusion process of early endosomes but also in the budding of clathrin-coated vesicles from plasma membranes and its transport to early endosomes. Like other Rab GTPases, the functional state of Rab5 is determined by its binding to guanine nucleotides. The exchange from an inactive (GDP-bound) state to an active (GTP-bound) state is mediated by the guanine-nucleotide exchange factors (GEFs). Thus Rab5-GEFs are very important in the activation of Rab5 and have been extensively analyzed [4]. To date, all Rab5-GEFs share a conserved the vacuolar protein sorting 9 (VPS9) domain, which is required for their bindings to and the nucleotide-exchange reaction on Rab5 [5].

The RIN (Ras and Rab interactor, or Ras interaction/interference) family composed of RIN1-3 also has VPS9 domain and functions as a Rab5-GEF [6–8]. The unique feature of RIN family is the existence of many functional domains: Besides VPS9 domain, they have *Src*-homology 2 (SH2), proline-rich (PR) domains in their N-termini, RIN-family homology (RH) and Ras-association (RA) domains in their C-termini [6,7]. Previous studies have shown that RIN1 and RIN2 are involved in the signaling pathways of receptor tyrosine kinases (RTKs), such as EGF and HGF receptors, and they regulate the membrane trafficking and degradation of RTKs [9,10]. We have shown in the previous study that another member of RIN family, RIN3, mainly disperses throughout cell cytoplasm and partly colocalizes with Rab5-positive vesicles in non-stimulated culture cells [6]. However, the molecular mechanisms underlying RIN3-induced Rab5 activation and precise functions of each domain in RIN3 have not been fully elucidated.

In the present study, we investigated how the subcellular localization of RIN3 is affected by the elevation of intracellular tyrosine-phosphorylated proteins. For the analysis, we used the treatment of HeLa cells with pervanadate (PV), a non-selective tyrosine-phosphatase inhibitor, and found that tyrosine-phosphorylation signals translocate the cytoplasmic RIN3 to Rab5-positive early endocytic vesicles. This PV-induced RIN3 translocation was applied to various mutants lacking each domain of RIN3 to elucidate their functions. Our present findings suggest that a Ras GTPase(s) activated by tyrosine-phosphorylation signals interacts with RA domain and masks its inhibitory effect, resulting in an active conformation of RIN3 as Rab5-GEF. Furthermore, we identified that the RIN-unique RH domain directly interacts with Rab5 to progress the GEF action.

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Materials and methods

Antibodies and reagents. Anti-FLAG and anti-c-myc (9E10) antibodies were purchased from Sigma. Anti-Rab5 and anti-TGN38 antibodies were from BD Biosciences. Anti-transferrin receptor and anti-Rab11 antibodies were from Zymed Laboratories. Anti-EEA1, anti-Rab9, and anti-GAPDH antibodies were from Transduction Laboratories, Calbiochem, and Millipore, respectively. Alexa Flour 488 and 568 anti-mouse and anti-rabbit IgG secondary antibodies were purchased from Molecular Probes. All other reagents were from commercial sources and of analytical grade.

Construction of expression vectors. pCMV5-FLAG-RIN3, pCMV5-myc-Rab5, and pGAD10-Rab5 plasmids were constructed as described previously [6]. Full length of RIN3 was subcloned into pEGFP-C1 vector (BD Biosciences). cDNAs of RIN3 fragments were amplified by PCR from full-length RIN3, and the PCR-fragments were subcloned into pCMV5-FLAG, pEGFP-C1, and pGBT9 vectors.

Cell culture and transfection. HeLa and HEK293T cells were maintained as described previously [6,11] and transfected with the plasmid constructs using LipofectAMINE 2000 (Invitrogen) and HEKFectin (Bio-Rad), respectively.

PV treatment. PV solution (50 μM) was freshly prepared by mixing 2.5 μl of 200 mM sodium orthovanadate with 17 μl of 30% (w/v) hydrogen peroxide and diluting with 10 ml of PBS. HeLa cells cultured in 800 μl of DMEM were stimulated by adding 200 μl of the PV solution and further incubated at 37 °C for indicated periods.

Immunofluorescence staining. 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) overnight at 4 °C followed by subsequent incubation for 1 h with Alexa Fluor 488- or 568-conjugated secondary antibodies diluted with the blocking solution. After washing three times with PBS, the coverslip was mounted onto a glass slide in Permafluor-mounting medium (IMMUNON) and viewed on a Carl Zeiss confocal microscope with LSM510 software using excitation wavelength of 488 or 546 nm. The images were merged by using the Adobe Photoshop (Adobe Systems, Mountain View, CA).

Yeast two-hybrid analysis. A yeast two-hybrid assay was performed according to the method described previously [12]. Briefly, yeast strain Y190 was cotransformed with plasmids containing pGBT9-RIN3 deletion mutants and pGAD10-Rab5. Cotransformants grown on a synthetic medium lacking leucine and tryptophan were subjected to quantitative β-galactosidase assays using 4-methylumbelliferyl-β-D-galactoside as a substrate.

Radiolabeling of intracellular nucleotides and identification of the nucleotide-bound forms of Rab5. Guanine nucleotides bound to Rab5 proteins were analyzed essentially as described previously [13,14]. Briefly, HEK293T cells that had been cultured in 60-mm dishes for 48 h after transfection were radiolabeled with $^{32}P_i$ (1.85 MBq per dish) in phosphate-free DMEM for 4 h. The expression levels of Rab5 and RIN3 proteins were confirmed by immunoblot analysis with the anti-c-myc monoclonal and the anti-FLAG polyclonal antibodies, respectively. The labeled cells (3×10^6 cells) were lysed with 1 ml of an ice-cold solubilizing buffer consisting of 40 mM Tris–HCl (pH 7.5), 100 mM NaCl, 20 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1% (w/v) Triton X-100, and 2 μg

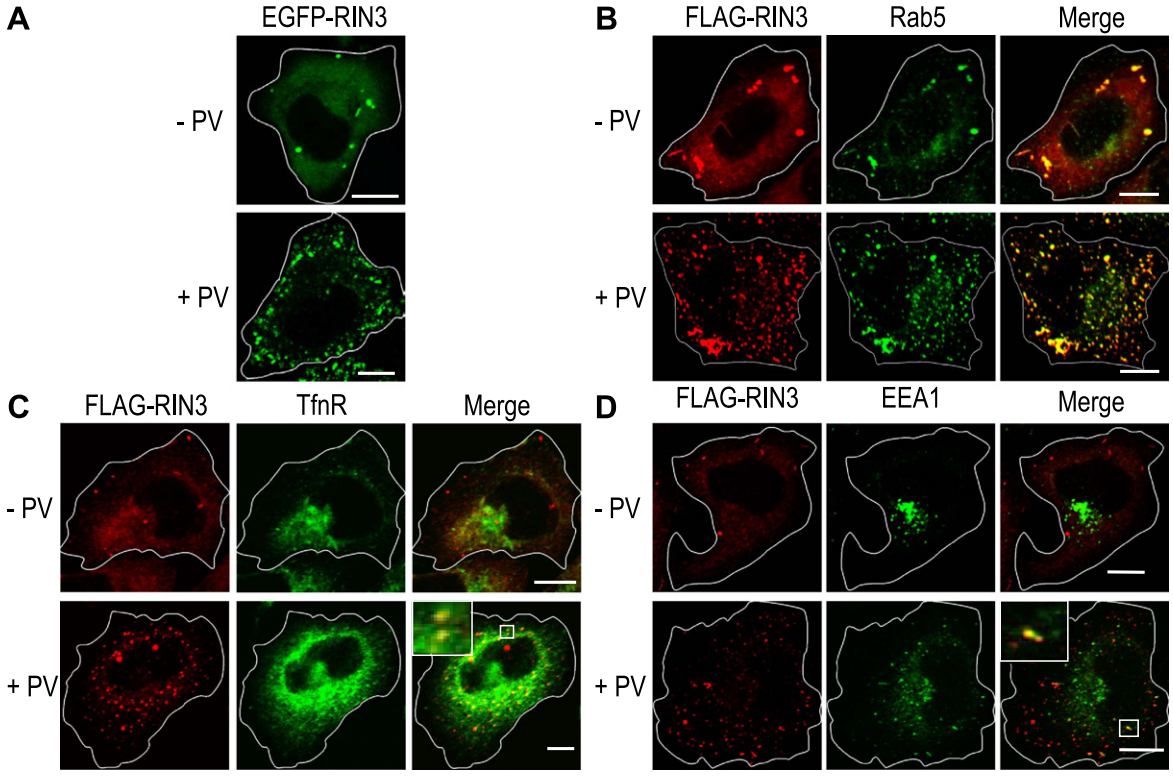

Fig. 1. Treatment of HeLa cells with PV induces the translocation of cytoplasmic RIN3 to Rab5-positive early endocytic vesicles. (A) HeLa cells transiently expressing EGFP-RIN3 were treated with (+) or without (−) PV for 10 min, and the fluorescence signals of GFP were recorded in a Carl Zeiss LSM-510 confocal microscope. (B–D) HeLa cells transiently expressing FLAG-RIN3 were treated with (lower panels) or without (upper panels) PV for 10 min and immunostained with anti-FLAG (B–C) and anti-Rab5 (B), anti-transferrin receptor (TfnR, C) or anti-EEA1 (D) antibodies. The fluorescence signals of Alexa Flour 488 (left) and Alexa Flour 568 (middle) secondary antibodies were visualized by the confocal microscope, and merged images of the two signals are displayed in yellow (right). The insets show the expanded images of the indicated squares. Bars indicate 10 μm.

ml^{-1} aprotinin, and clarified. The precleared lysates were incubated with anti-myc antibody-immobilized Protein G–Sepharose beads (GE Healthcare) at 4 °C for 10 min. After extensive washing of the immunocomplexes, associated nucleotides were separated by thin layer chromatography and quantified with a BAS-1800 image analyzer (Fujifilm).

Time-lapse microscopic analysis. The subcellular movement of EGFP-RIN3 in HeLa cells was obtained by using a confocal system (Yokogawa CSU-10 spinning disk scanhead, Tokyo, Japan) attached to an Eclipse TE2000-E microscope (Nikon, Tokyo, Japan). The fluorescence was registered by iXon Electron Multiplying CCD (Andor Technology, Belfast, Northern Ireland) under Andor IQ software control. Images were acquired by 250 ms exposure every 10 sec.

Results

Tyrosine-phosphorylation signals induce the translocation of RIN3 from cytoplasm to Rab5-positive early endocytic vesicles

RIN3 identified as a Rab5-GEF mainly localized throughout cytoplasm and partly colocalized with Rab5-positive vesicles in non-stimulated HeLa cells [6]. To investigate how tyrosine-phosphorylation signals exert its influence on the localization of RIN3, treatment with PV, a non-selective tyrosine-phosphatase inhibitor, was applied to HeLa cells transiently expressing EGFP-fused RIN3. Although EGFP-RIN3 proteins were present throughout the cytoplasm and at a few of vesicles in the non-treated cells, they were mostly located in a large number of small vesicles after PV treatment (Fig. 1A). We investigated by means of time-lapse microscopic analysis how EGFP-RIN3 proteins behave in response to PV treatment (see Supplementary Movie S1). After PV treatment, the RIN3-positive vesicles appeared synchronously with attenuation of the cytoplasmic signals and moved dynamically. These results indicate that cytoplasmic RIN3 assembles functional vesicles by the tyrosine phosphorylation of intracellular proteins.

We next characterized the RIN3-positive vesicles induced by PV treatment in view of their assignment to organelle(s). For the analysis, HeLa cells transiently expressing FLAG-tagged RIN3 were immunostained with various organelle markers (Fig. 1B–D). A few of RIN3-positive vesicles observed without PV colocalized with endogenous Rab5 as described previously [6], and the large number of RIN3-positive vesicles formed with PV extensively colocalized with Rab5 (Fig. 1B). The PV-induced RIN3-positive vesicles also partly colocalized with transferrin receptor, which is constitutively endocytosed from clathrin-coated pits and subsequently recycled from early endosomes to plasma membrane via recycling endosomes (Fig. 1C). Furthermore, the vesicles overlapped partly with EEA1, which is a marker for early endosomes (Fig. 1D). However, the RIN3-positive vesicles appeared to be excluded from late endosomes (Rab9), recycling endosomes (Rab11) or trans-Golgi networks (TGN38) (see Supplementary Fig. S1). Collectively, these results indicate that tyrosine-phosphorylation signals induce the translocation of RIN3 from cytoplasm mainly to the Rab5-positive vesicles that move from plasma membrane to early endosomes. We can safely state that PV treatment did not have any apparent effects on the numbers and shapes of Rab9-, Rab11-, or TGN38-po-

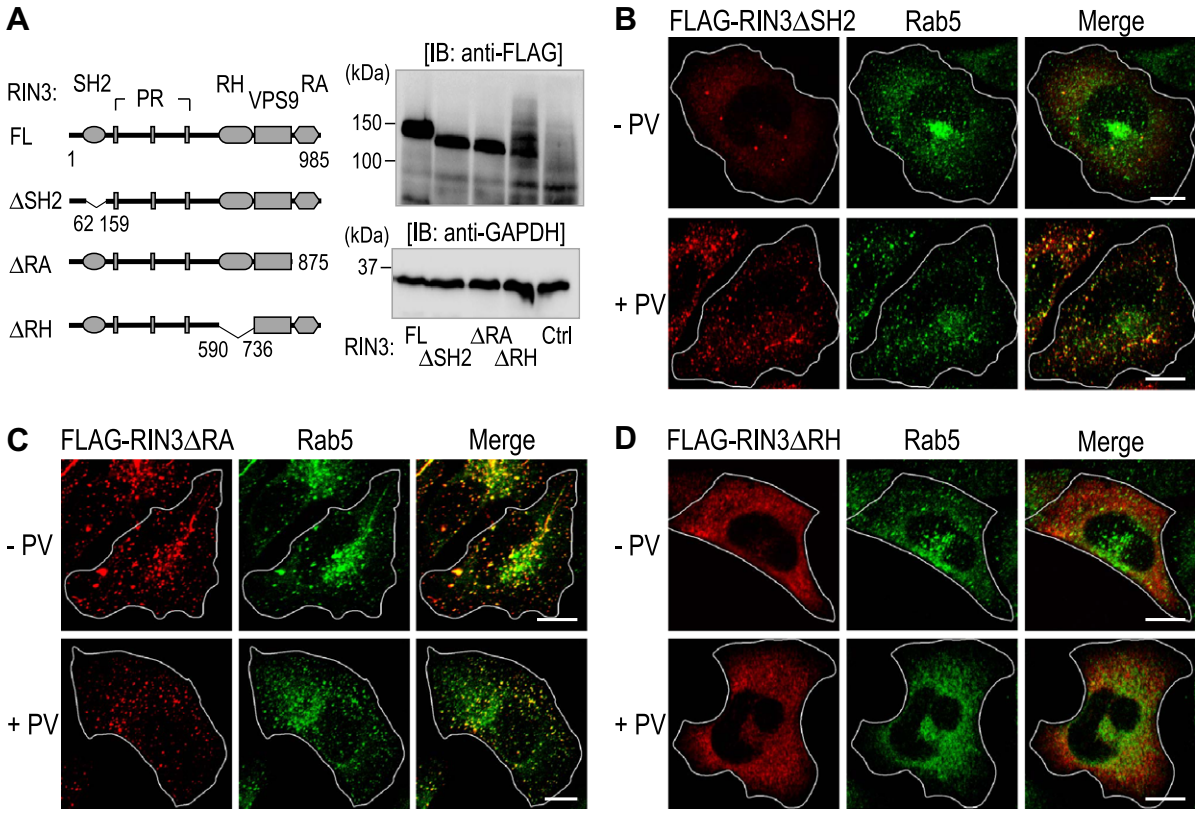

Fig. 2. PV-induced translocation of RIN3 to Rab5-positive vesicles is affected by the deletions of various domains from RIN3. (A) Expression levels of the RIN3-deletion mutants. Full-length (FL), ΔSH2, ΔRA, and ΔRH mutants of FLAG-RIN3 were constructed (left panel) and transiently transfected into HeLa cells. The lysates prepared from these cells were separated by SDS–PAGE and immunoblotted (IB) with anti-FLAG and anti-GAPDH antibodies (right panel). Non-transfected cells were loaded as a negative control (Ctrl). (B–D) Localizations of the RIN3-deletion mutants in HeLa cells. HeLa cells expressing ΔSH2 (B), ΔRA (C), and ΔRH (D) mutants of FLAG-RIN3 were treated with (+) or without (−) PV for 10 min and immunostained with anti-FLAG and anti-Rab5 antibodies. Merged images of the two signals are displayed in yellow (right). Bars indicate 10 μm.

sitive vesicles (see Supplementary Fig. S1), suggesting that the PV-induced RIN3 translocation is a specific phenomenon rather than due to the cell perturbation by the reagent or an artificial effect.

The RIN3 translocation is affected by the deletions of its RA or RH domain

As mentioned before, RIN family has many functional regions including SH2, PR, RH, VPS9, and RA domains. To elucidate which domain in RIN3 is responsible for its translocation by PV treatment, we generated various mutants lacking each domain of RIN3 and investigated their behaviors. The expression levels of the deletion mutants were confirmed by immunoblot analysis (Fig. 2A, right panel). We first examined the effect of SH2 deletion, since this domain is generally responsible for the interaction with tyrosine-phosphorylated proteins. However, RIN3ΔSH2 exhibited the same distribution pattern as wild-type RIN3, regardless of whether the cells were treated with or without PV (Fig. 2B). We next examined the localization of RIN3ΔRA that would lose the ability to bind to GTP-bound Ras GTPases. Quite interestingly, RIN3ΔRA localized at many small vesicles even without PV treatment (Fig. 2C, upper panels) and also colocalized with endogenous Rab5, indicating that RIN3ΔRA mimics the PV-treated localization of wild-type RIN3. Treatment with PV did not alter the Rab5-positive vesicular localization of RIN3-ΔRA. These results support an idea that RA domain plays an inhibitory role in the RIN3 translocation and that the binding of a GTP-bound Ras(s) may unlock the inhibitory action of RA leading to the translocation of RIN3 to the Rab5-positive vesicles. We also investigated the significance of RIN-unique RH domain, whose function has not been elucidated yet. RIN3ΔRH localized throughout the cytoplasm under no PV-treated conditions. Furthermore, PV treatment failed to induce the translocation of the mutant RIN3 or its colocalization with Rab5 (Fig. 2D), suggesting that RH domain is necessary for the proper localization of RIN3 to the Rab5-positive vesicles. Thus RA and RH domains rather than SH2 domain appeared to be important for the regulation of RIN3 translocation in response to tyrosine-phosphorylation signals.

Identification of RIN-unique RH domain as a Rab5-binding region

The inability of RIN3ΔRH to move into Rab5-positive vesicles upon PV treatment (Fig. 2D) allowed us to speculate that RIN family may interact with Rab5 via RH domain. To verify this idea, various deletion mutants of RIN3 were generated (Fig. 3A, left) and their interaction with Rab5 was investigated by the yeast two-hybrid analysis. In addition to the full-length RIN3, all the deletion mutants containing RH domain were capable of interacting with Rab5 (Fig. 3A, right). However, VPS9 domain responsible for GEF activity had no binding ability by itself. Consistent with the finding that RIN3ΔRH lost its ability to bind to Rab5 (Fig. 3A, lane 5), this mutant did not exhibit GEF activity for Rab5 in HEK293T cells (Fig. 3B). These results clearly indicate that RH domain is necessary and sufficient for the interaction of RIN3 with Rab5.

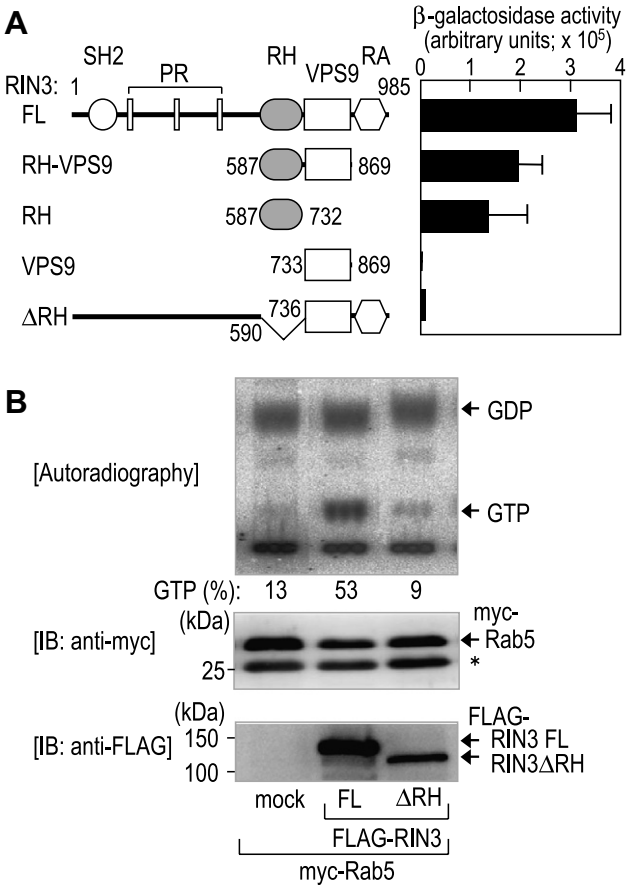

Fig. 3. (A) Schematic diagram of the truncated RIN3 proteins and analysis of the RIN3–Rab5 interactions in yeast two-hybrid system. Bars in the left panels represent the protein product of deleted RIN3 gene with their amino-acid numbers. The yeast two-hybrid assay was performed as described in Materials and methods. The data obtained from three independent experiments are shown with means ± SEM (B) RH domain of RIN3 is required for its Rab5-GEF activity. myc-Rab5 was cotransfected with full-length (FL) or RH domain-deleted mutant (ΔRH) of FLAG-RIN3 in HEK293T cells, and the cells were metabolically radiolabeled with $^{32}P_i$ for 4 h. The myc-tagged Rab5 protein was immunoprecipitated with an anti-myc antibody, and nucleotides associating with Rab5 were separated by thin layer chromatography (top). The radioactivity of GTP and GDP was quantified, and the percentages (%) of GTP-bound Rab5 are shown in the bottom lanes. The immunoprecipitate (middle) and total lysate (bottom) of the radiolabeled cells were separated by SDS–PAGE and immunoblotted (IB) with anti-myc and anti-FLAG antibodies, respectively. Asterisk (*) shows a non-specific band.

Discussion

To date, VPS9 domain, which is a hallmark of GEFs for the small GTPase Rab5 subfamily, has been found in many proteins, including RIN family (RIN1-3), Vps9p, Rabex-5, ALS2/Alsin, Varp, and Gapex-5/RAP6/RME-6 [6–8,15–19]. In contrast that many VPS9-containing proteins localized in early endosomes, RIN family including RIN3 mainly dispersed throughout the cytoplasm (Fig. 1A). In the present study, we uncovered for the first time that the cytoplasmic RIN3 is translocated into Rab5-positive early endocytic vesicles by the activation of a tyrosine-phosphorylation signaling pathway(s) and that both RA and RH domains play critical roles in the proper translocation of the Rab5-GEF.

The deletion of RA domain from RIN3 caused constitutive localization of the mutant in many early endocytic vesicles similar to the localization of its wild type after PV treatment (Fig. 2C). This suggests that RA domain has an inhibitory effect on the RIN3 translocation to early endocytic vesicles. Considering that RA domain interacts with GTP-bound Ras GTPases and PV treatment increases tyrosine-phosphorylation level of many proteins including RTKs, it is very likely that the binding to RA domain of an activated Ras GTPase(s) resulting from RTK-signaling pathways triggers the translocation of RIN3. This speculation could be reasonable with the previous finding that RA domain of RalGDS, which is a GEF for Ral small GTPase, has an inhibitory effect on the GEF activity [20,21]. This idea is also supported by the reports that the GEF activity of RIN1 and RIN2 for Rab5 is stimulated by GTP-bound Ha-Ras [8,10]. We are currently under investigation to identify

Ras or Ras-like GTPase(s) and RTK(s) or tyrosine-phosphorylated adaptor protein(s) that are involved in RIN3 activation under physiological conditions.

The present study also reveals the significance of RH domain that is highly conserved among RIN family. RIN3ΔRH dispersed throughout the cytoplasm and did not colocalize with Rab5 (Fig. 2D) and that RH domain contained a Rab5-interacting region (Fig. 3A). The X-ray analysis of core catalytic region of Rabex-5 and the amino-acid sequence scrutiny of RIN family suggested that RH domain in RIN family corresponds to four helical bundle (HB) domains and one unit of VPS9 domain in Rabex-5 [5]. We found that the four HB domains within RIN3-RH are sufficient for the binding to Rab5 in yeast two-hybrid system (data not shown). Thus, this is the first report showing that RIN has a Rab5-binding region (i.e., RH domain) other than VPS9 domain among many Rab5-GEFs. Since RIN3ΔRH lost GEF activity for Rab5 (Fig. 3), the interaction between RH domain and Rab5 could constitute a critical step for the VPS9 domain-induced nucleotide-exchange reaction on Rab5. It is still unclear whether RH domain stabilizes the activated GTP-bound form of Rab5 after the GEF action by VPS9 domain or RH domain traps the inactive GDP-bound form of Rab5 before the VPS9-induced GEF action. Further analyses focused on the crystal structure of RIN3-Rab5 complex would reveal the precise molecular mechanism by which RIN3 activates Rab5.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.05.027.

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