RIN3: a novel Rab5 GEF interacting with amphiphysin II involved in the early endocytic pathway

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Accepted 19 June 2003
Journal of Cell Science 116, 4159-4168 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00718

Summary

The small GTPase Rab5, which cycles between active (GTP-bound) and inactive (GDP-bound) states, plays essential roles in membrane budding and trafficking in the early endocytic pathway. However, the molecular mechanisms underlying the Rab5-regulated processes are not fully understood other than the targeting event to early endosomes. Here, we report a novel Rab5-binding protein, RIN3, that contains many functional domains shared with other RIN members and additional Pro-rich domains. RIN3 displays the same biochemical properties as RIN2, the stimulator and stabilizer of GTP-Rab5. In addition, RIN3 exhibits its unique intracellular localization. RIN3 expressed in HeLa cells localized to cytoplasmic vesicles and the RIN3-positive vesicles contained Rab5 but not the early endosomal marker EEA1. Transferrin appeared to be transported partly through the RIN3-positive vesicles to early endosomes. RIN3 was also capable of interacting via its Pro-rich domain with amphiphysin II, which contains SH3 domain and participates in receptor-mediated endocytosis. Interestingly, cytoplasmic amphiphysin II was translocated into the RIN3- and Rab5-positive vesicles when co-expressed with RIN3. These results indicate that RIN3 biochemically characterized as the stimulator and stabilizer for GTP-Rab5 plays an important role in the transport pathway from plasma membrane to early endosomes.

Supplemental data available online

Key words: Small GTPase Rab5, RIN, Amphiphysin II, Endocytosis, Guanine nucleotide exchange factor

Introduction

Rab proteins constitute a subfamily of small GTPases that play central roles in intracellular membrane trafficking. At present, more than 40 members of the Rab GTPases have been identified, and they localize to distinct intracellular compartments and regulate the transport between specific organelles (Olkkonen and Stenmark, 1997; Zerial and McBride, 2001). The functional state of Rab GTPases depends on the conformation, which is determined by their bindings to guanine nucleotides. In the GDP-bound state, Rab forms a cytoplasmic complex with the regulatory protein Rab GDP-dissociation inhibitor (RabGDI), which prevents the association with improper cellular compartments. However, Rab replaces GDP with GTP through its interaction with the guanine nucleotide exchange factor (GEF) at the target membranes. This causes a conformational change of Rab that allows the recruitment of a range of downstream effectors onto the membranes. During or after the membrane fusion, a regulatory protein called Rab-GAP enhances the intrinsic GTPase activity of Rab and promotes the hydrolysis of GTP. Once this has occurred, GDP-bound Rab reforms the complex with RabGDI and dissociates from the membranes.

Rab5, the most thoroughly characterized member of the Rab GTPase subfamily, is mainly localized to early endosomes (Gorvel et al., 1991). Rab5 is involved not only in the homotypic fusion process of early endosomes but also in the budding of clathrin-coated vesicle from plasma membranes and its transport to early endosomes (Barbieri et al., 1998; Bucci et al., 1992; Gorvel et al., 1991; Stenmark et al., 1994). In the process of homotypic early endosomal fusion, the complex of Rabex-5 and Rabaptin-5 functions as both the stimulator and the stabilizer for Rab5 (Horiuchi et al., 1997), and the resultant GTP-bound Rab5 recruits hVps34, a class-II phosphatidylinositol (PtdIns) 3(OH)-kinase that specifically generates PtdIns(3)phosphate [PtdIns(3)*P*], to early endosomes (Christoforidis et al., 1999). The localized lipid production also allows the recruitment of Rab5 effectors such as EEA1 (Stenmark et al., 1996) and Rabenosyn-5 (Nielsen et al., 2000), because these proteins contain the FYVE domain, which binds to PtdIns(3)*P* with high affinity (Burd and Emr, 1998). Thus, Rab5 organizes a specific membrane domain that defines the entry site to early endosomes, leading to the fusion event by the SNARE machinery (McBride et al., 1999).

In contrast to the homotypic fusion process of early endosomes, the molecular mechanism underlying the Rab5-dependent regulation of fission and initial vesicular transport is poorly understood. Possible candidates for Rab5 regulators responsible for these early endocytic processes have not been reported yet. In the present study, we have identified and characterized a novel Rab5-binding protein, RIN3, whose sequence is similar to those of RIN1 and RIN2 (Colicelli et al., 1991; Saito et al., 2002). We first confirmed that the

biochemical properties of RIN3 are similar to those of other RIN members and further found that it displays unique intracellular localization. RIN3 localizes to Rab5-positive, but not EEA1-positive, vesicles in HeLa cells, and transferrin appears to be transported partly through the RIN3-positive vesicles to early endosomes. Furthermore, we found that amphiphysin II, which mediates receptor-induced endocytosis, interacts with RIN3 and that cytoplasmic amphiphysin II is translocated into the RIN3- and Rab5-positive vesicles. Collectively, these results suggest that RIN3 is a novel player in the transport pathway from plasma membranes to early endosomes.

Materials and Methods

Materials

Alexa-488 secondary antibody and human Alexa-488 transferrin were purchased from Molecular Probes (Eugene, OR). pFastBacHTa vector was from Invitrogen. pEGFP-C1 and pDsRed-1 vectors and human leukocyte MATCHMAKER cDNA library were obtained from BD Biosciences. Antibody sources were as follows: monoclonal antibody against the Flag epitope (M2) (Sigma); monoclonal antibodies against DsRed and Rab5 (BD biosciences); polyclonal antibody against glutathione-*S*-transferase (GST) (Santa Cruz Biotechnology).

cDNA constructs

A DNA fragment encoding the Flag epitope (MDYKDDDDK) was substituted for 6× histidine affinity tag of pFastBacHTa and constructed into the pFastbac-Flag vector. To express Flag-RIN3 and Flag-RIN2, fragments coding RIN3 and RIN2 were inserted between the *Eco*RI site and *Sal*I site of the pFastBac-Flag vector. pCMV5-Flag vector was obtained by inserting a DNA fragment encoding a start methionine followed by the Flag epitope between the *Eco*RI and *Bam*HI sites of pCMV5. Human amphiphysin II was obtained using the human leukocyte MATCHMAKER cDNA library as a template. Deletion mutants of RIN3 and amphiphysin II were constructed using a PCR-based strategy.

Yeast two-hybrid screening

A yeast two-hybrid assay was performed according to the method described previously (Hoshino et al., 1999; Kontani et al., 2002). The yeast reporter-strain Hf7c was transformed with pGBT9-Rab5b/Q79L using a lithium-acetate-based method and grown in synthetic medium lacking tryptophan at 30°C for 5 days. The cells were transformed with the human leukocyte MATCHMAKER cDNA library and plated on synthetic medium lacking leucine, tryptophan and histidine at 30°C for 7 days. For interaction analysis, the transformed yeasts were lifted onto filter papers and lysed by brief liquid-nitrogen treatment and incubated with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. Library plasmids from positive clones were rescued into *Escherichia coli* HB101 cells plated on leucine-free medium. True Rab5b/Q79L-interacting clones were sequenced by the dideoxynucleotide chain termination method on both strands. When RIN3 was screened as bait, the same method described above was adopted.

Cloning of the full-length RIN3 cDNA

Upstream and downstream sequences of HK281 clone were obtained by 5′ and 3′ rapid amplification of cDNA end (RACE) PCR using the same library as described above, according to the manufacturer's protocols. The RACE PCR fragment was purified and subcloned into pGEM-T easy (Promega). The complete nucleotide sequence was confirmed by isolating and sequencing multiple clones, and designated as RIN3.

Northern blot analysis

Human 12-lane multiple tissue northern blot containing poly(A) mRNA was obtained from Clontech. An [α-^{32}P] dCTP-labelled fragment encoding amino acids 434-678 of RIN3 was hybridized to the membrane overnight at 65°C in ExpressHyb Solution (BD Biosciences). The membrane was washed twice with 2× SSC (sodium chloride/sodium citrate) containing 0.1% SDS for 10 minutes each and twice with 1× SSC containing 0.1% SDS for 20 minutes each. The filter was autoradiographed for 12 hours at –80°C and analysed by BAS1800 (FujiFilm).

Cell culture and transient transfection

COS7 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 0.16% (w/v) $NaHCO_3$, 0.6 mg ml^{-1} L-glutamine, 100 μg ml^{-1} streptomycin and 100 IU ml^{-1} of penicillin at 37°C in 95% air and 5% CO_2. For electroporation, the cells (1×10^7 cells) were washed twice and resuspended in 0.2 ml Opti-MEM. The cell suspension was mixed with 10 μg of plasmids and transferred to a 0.4-cm gap cuvette (BioRad). After being electroporated (220 V, 960 μF), the cells were diluted into 20 ml of DMEM and cultured at 37°C for 2-3 days.

Production of recombinant proteins

Prenylated Rab5b was purified from baculovirus-infected Sf9 cells according to the method described previously (Horiuchi et al., 1995). To prepare guanosine 5′-[γ-thio]triphosphate (GTPγS)- and GDP-bound forms of Rab5b, the purified protein was incubated with the nucleotides (250 μM) at 30°C for 45 minutes in 11.2 mM Tris-HCl (pH 8.0), 50 mM Hepes-NaOH (pH 7.5), 110 mM NaCl, 0.5 mM DTT, 0.27% (w/v) CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate), 5 mM EDTA and 2.2 mM $MgCl_2$. The reaction was terminated by the addition of $MgCl_2$ at the final concentration of 10 mM. Flag-RIN3 and RIN2 were purified from baculovirus-infected Sf9 cells with anti-Flag M2 agarose beads. GST-fused amphiphysin II was purified from the cytoplasmic fraction of pGEX4T-1-transformed *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene) using glutathione Sepharose 4B (Amersham Biosciences).

Assays for guanine nucleotide exchange and GDP-dissociation reactions

The GDP-dissociation assay was performed by monitoring the time-dependent release of [^{3}H]GDP from Rab5 as described previously (Hama et al., 1999). Prenylated Rab5 (18 nM) that had been treated with 5 μM [^{3}H]GDP (10,000 cpm pmol^{-1}) at 30°C for 30 minutes was incubated in the presence or absence of Flag-RIN3 or RIN2 in a reaction mixture (65 μl) consisting of 40 mM Tris-HCl (pH 8.0), 62.5 mM NaCl, 0.5 mM DTT, 0.36% (w/v) CHAPS, 120 μM unlabelled GDP, 40 μM GTP, 5 mM EDTA and 15 mM $MgCl_2$. The GTPγS-binding assay was performed by the filter method as described previously (Araki et al., 1990). Prenylated Rab5 (18 nM) was 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-RIN3 or RIN2 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 μM ATP, 5 mM EDTA and 15 mM $MgCl_2$.

Assay for the in vitro association between Rab5 and RIN3

The association between Rab5 and Flag-tagged RIN3 was assayed as described previously (Saito et al., 2002). The GDP- or GTPγS-bound Rab5 was incubated with agarose resin (10 μl) conjugated with Flag-RIN3 at 30°C for 60 minutes. The resin was washed, and proteins

were eluted from the resin with 30 μl of the washing buffer containing 100 ng ml^{-1} of Flag peptide. After centrifugation, the supernatant (24 μl) was mixed with 8 μl of 4× SDS sample buffer, boiled for 5 minutes and subjected to SDS-PAGE. Immunoblotting was performed with anti-Flag monoclonal and anti-Rab5 polyclonal antibodies.

GST pull-down assay

HeLa cells were transfected with pCMV5 that contains the cDNA encoding Flag-RIN3 or its deletion mutants. The cells were harvested, washed twice with PBS and solubilized with 2 ml of buffer A, consisting of 40 mM Hepes-NaOH (pH 7.4), 75 mM NaCl, 15 mM NaF, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 2 mM EDTA, 1 μg ml^{-1} leupeptin, 2 μg ml^{-1} aprotinin and 1% (w/v) NP-40. Supernatants pre-cleared with Sepharose 4B were incubated with 5 μg of GST-fused amphiphysin II, its SH3 domain or GST alone, together with glutathione Sepharose 4B resin (10 μl) for 30 minutes at 4°C. The resin was washed three times with 100 μl TBS containing 0.1% (w/v) NP-40 and three more times with 100 μl of buffer B, consisting of 100 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA and 0.1% (w/v) NP-40. Proteins were eluted from the resin with 30 μl buffer B containing 7.5 mM glutathione. After centrifugation, the supernatant (24 μl) was mixed with 8 μl of 4× SDS sample buffer, boiled for 5 minutes and subjected to SDS-PAGE. Immunoblotting was performed with anti-GST polyclonal and anti-Flag M2 monoclonal antibodies.

Confocal microscopy

HeLa cells transiently expressing red fluorescent protein (RFP)-RIN3 were cultured on a polylysine-coated glass coverslip (15-mm diameter) and washed three times with PBS before fixation with 4% paraformaldehyde in PBS for 15 minutes at 4°C. After treatment with 0.1 mM glycine in PBS for 15 minutes, the cells were permeabilized with 0.1% Triton X-100 in blocking solution [3% bovine serum albumin (BSA) in PBS] before incubation with a primary antibody (1 μg ml^{-1} diluted with blocking solution) for 1 hour at room temperature. The cells were washed three times with PBS and incubated for 1 hour with Alexa-488-conjugated secondary antibodies diluted with the blocking solution. After washed 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 wavelengths of 488 nm or 546 nm. The images were merged using Photoshop (Adobe Systems, Mountain View, CA). In co-expressing experiments of green fluorescent protein (GFP), yellow fluorescent protein (YFP) and RFP fusion proteins, the transfected HeLa cells were cultured for 48 hours in a glass-based dish (35-mm diameter, Iwaki) and examined by confocal microscopy.

Analysis of transferrin uptake and internalization

For the steady-state internalization of Alexa-488 transferrin, HeLa cells transiently expressing RFP-RIN3 (or RFP-mock) were cultured in the 35-mm glass dish. The cells were incubated at 37°C for 60 minutes in internalization medium (IM) consisting of DMEM plus 20 mM Hepes-NaOH (pH 7.4) and 2 mg ml^{-1} BSA to deplete endogenous transferrin, and further incubated at 4°C for 45 minutes in IM containing 10 μg ml^{-1} Alexa-488/transferrin. The cells were washed and incubated in IM at 25°C for the indicated times to allow internalization.

Interaction between RIN family and amphiphysin II in transfected HeLa cells

HeLa cells that had been co-transfected with pCMV5 containing RIN3 and Flag-tagged amphiphysin II (the full-length or with the SH3 domain deleted (ΔSH3)) were solubilized with 2 ml buffer A. The supernatants were pre-cleared and immunoprecipitated with agarose resin (10 μl) conjugated with 0.5 μg of the anti-Flag monoclonal antibody. After incubation at 4°C for 90 minutes, the resin was washed three times with 100 μl TBS containing 0.1% (w/v) NP-40 and three more times with 100 μl buffer C, consisting of 75 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA and 0.1% (w/v) NP-40. Proteins were eluted from the resin with the buffer C containing 50 ng ml^{-1} Flag peptide. After centrifugation, the supernatant (24 μl) was mixed with 8 μl 4× SDS sample buffer, boiled for 5 minutes and subjected to SDS-PAGE. Immunoblotting was performed with anti-RIN3 polyclonal and anti-Flag monoclonal antibodies.

Western blot analysis

Proteins were transferred to a polyvinylidene difluoride membrane (BioRad), blocked in 5% BSA in TBS for 1 hour and incubated with primary antibodies for 1 hour at room temperature. After washing three times with 0.2% Triton X-100/TBS, the membrane was further incubated with a horseradish-peroxidase-conjugated secondary antibody for 1 hour. The immuno-positive signal was visualized in the presence of luminol (Pierce).

DDBL/EMBL/GenBank accession number

The accession number for the full-length RIN3 is AB081753.

Results

Complementary DNA cloning of the Rab5-binding protein RIN3

To identify proteins that interact with GTP-bound form of Rab5, a human leukocyte cDNA library was screened with the GTPase-deficient mutant Rab5b/Q79L in the yeast two-hybrid system. Screening of 5×10^7 transformants yielded five positive clones that interacted strongly with Rab5b/Q79L. Three of these were the same as a clone encoding Rab5c, indicating the biochemical properties of Rab5 dimerization (Daitoku et al., 2001), and one contained a clone coding for the human homologue of Rabaptin-5β (Gournier et al., 1998). The remaining clone, termed HK281, consisted of a 1575-bp cDNA encoding 525 amino acids. An additional 0.7-kb fragment, which contained an in-frame stop codon and the poly(A) tail, was produced by the 3′-RACE method. The upstream 5′-end was obtained by 5′-RACE, which gave an additional 1.5-kb fragment with an initiation codon based on the Kozak consensus sequence. Analysis of the total cDNA revealed an open reading frame that encoded a 985-amino acid protein in which the original HK281 corresponded to amino acids 435-959.

The isolated cDNA has a sequence similar to two independent clones, Ras-interaction/interference 1 (RIN1) and RIN2 (see supplementary Fig. S1, http:// jcs.biologists.org/supplemental/). Therefore, this protein was designated RIN3. Although the present RIN3 and previous RIN2 (Saito et al., 2002) were isolated as Rab5b-binding proteins, they also interact with other members of Rab5 (Rab5a and Rab5c) but not with Rab4, Rab7 or Rab11 in the yeast two-hybrid system (data not shown). Thus, the RIN family could be characterized as a binding partner at least specific to Rab5 group of the Rab small GTPases. The RIN family shared a Src homology 2 (SH2) domain, a RIN-homology (RH) domain (Saito et al., 2002), a Vps9 domain conserved in the catalytic domains of the GEFs Vps9p and Rabex-5, and a Ras-association (RA)

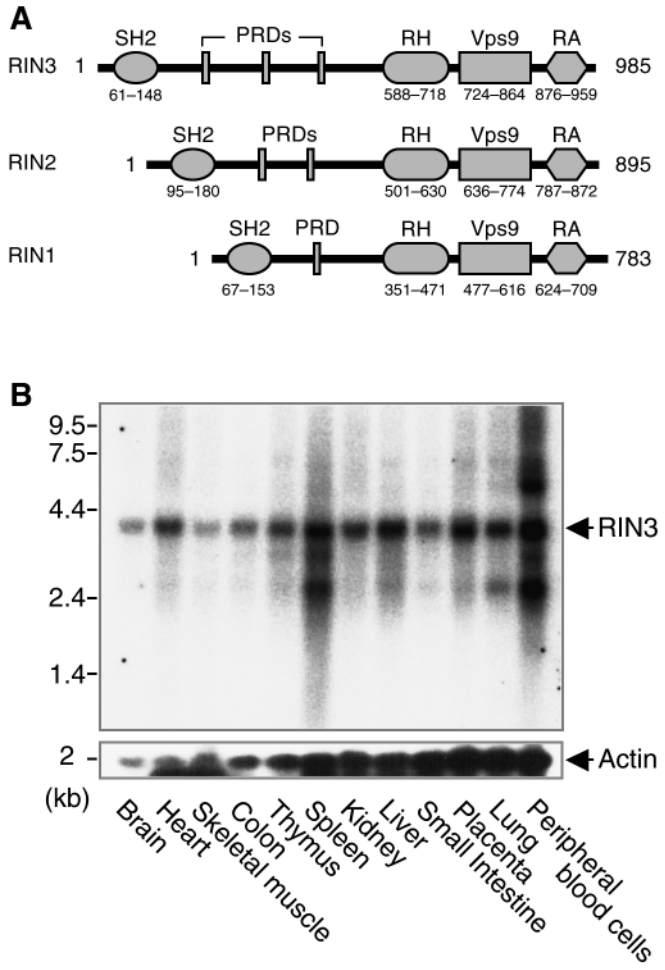

Fig. 1. The domain structure of the RIN members and northern blot analysis of *RIN3* mRNA in human tissues. (A) Diagram of the structural features of the RIN-family members. The numbers at the bottom represent the amino acid residues. (B, top) The probe corresponding to the 729-base RIN3 cDNA was labelled by random priming and hybridized to a human multiple tissue RNA blot containing 2 μg lane^{-1} of poly(A) mRNA from various human tissues. (B, bottom) The northern blot was also performed with actin cDNA as a control.

domain from their N termini (Fig. 1A). RIN1 has a proline-rich domain (PRD) that binds to the c-Abl SH3 domain (Afar et al., 1997; Han et al., 1997), and RIN2 and RIN3 further contain one and two PRDs (the class II motif of PXXPPR; Yu et al., 1994), respectively, which are lacking in RIN1.

Expression of RIN3 mRNA in human tissues

To investigate the expression pattern of RIN3 in human tissues, northern blot analysis was performed. Poly(A)-selected RNAs from several human tissues were hybridized with a radiolabelled probe containing the 729-base coding sequence of RIN3. A 4-kb transcript was detected in a variety of tissues, with the highest expression level in the peripheral blood cells (Fig. 1B). This expression pattern is slightly different from those of other RIN members, because *RIN1* and *RIN2* mRNAs have been reported to be abundant in brain (Han et al., 1997) and in heart, kidney and lung (Saito et al., 2002), respectively, although they are widely expressed. Furthermore, the 4-kb transcript was also detected in human cell lines including THP-1 and Jurkat cells (data not shown).

Unique biochemical properties of RIN3

RIN3 contains Vps9 domain (amino acids 724-864), which was conserved in the RIN family and other characterized GEFs for Rab5 (Hama et al., 1999; Horiuchi et al., 1997), so we first investigated whether this molecule also functions as a Rab5-GEF. For the analysis, Flag-tagged RIN3 and RIN2 proteins were purified from baculovirus-infected Sf9 cells (Fig. 2A) and assayed for their ability to stimulate [^{3}H]GDP release from prenylated Rab5. The rate of GDP-GTP exchange on Rab5 was extremely slow at physiological concentrations (mM) of Mg^{2+} (Fig. 2B). However, GDP release from Rab5 was markedly accelerated by the simultaneous addition of RIN3 or RIN2. We also examined the effects of RIN3 and RIN2 on [^{35}S]GTPγS binding to Rab5. As expected, RIN3 and RIN2 markedly enhanced the GTPγS binding to Rab5 (Fig. 2C), although RIN3 and RIN2 themselves had no detectable GTPγS-binding activity. These results clearly indicate that both RIN3 and RIN2 act as GEFs for Rab5.

We next investigated whether the nucleotide-bound state of Rab5 exerts its influence on the interaction with RIN3 by an in vitro binding assay. Flag-tagged RIN3 immobilized to resins was incubated with GDP- or GTPγS-bound form of Rab5, and the Rab5 binding was estimated by immunoblotting with an anti-Rab5 antibody. As had been observed in RIN2 (Saito et al., 2002), RIN3 associated more tightly with GTPγS-bound Rab5 than its GDP-bound form (Fig. 2D). Thus, RIN3, like RIN2, appears to act biochemically not only as a GEF for Rab5 but also as a stabilizer for GTP-Rab5.

Intracellular localization of RIN3 in transfected HeLa cells

We next examined the intracellular localization of RIN3 in mammalian cells. HeLa cells transiently expressing RFP-RIN3 were immunostained with anti-Rab5 and anti-EEA1 antibodies, and their localization analysed by confocal microscopy. RIN3 localized to punctate vesicles scattered around the cytoplasm (Fig. 3A). We found that the RIN3-positive vesicles also contained endogenous Rab5 in some degree (Fig. 3A, top), although most Rab5 localized to perinuclear early endosomes, as previously described (Chavrier et al., 1990). The co-localization of RIN3 and Rab5 was more evident when both proteins were expressed in HeLa cells (Fig. 3B). However, the RIN3 fluorescence did not overlap with the early endosomal marker EEA1 at all (Fig. 3A, bottom), indicating that the RIN3-positive vesicles are different from early endosomes. When the N-terminal region (amino acids 1-586) of RIN3, which lacks the Rab5-binding site (Saito et al., 2002), was expressed in HeLa cells, it distributed over the cytoplasm (Fig. 3C, middle). However, the C-terminal region (amino acids 587-985) of RIN3 exhibited its vesicular localization (Fig. 3C, right) similar to the full-length form. These results suggest that the translocation of RIN3 into the vesicles requires its association with Rab5. Essentially the same results were obtained in A431 cells, a human epidermal

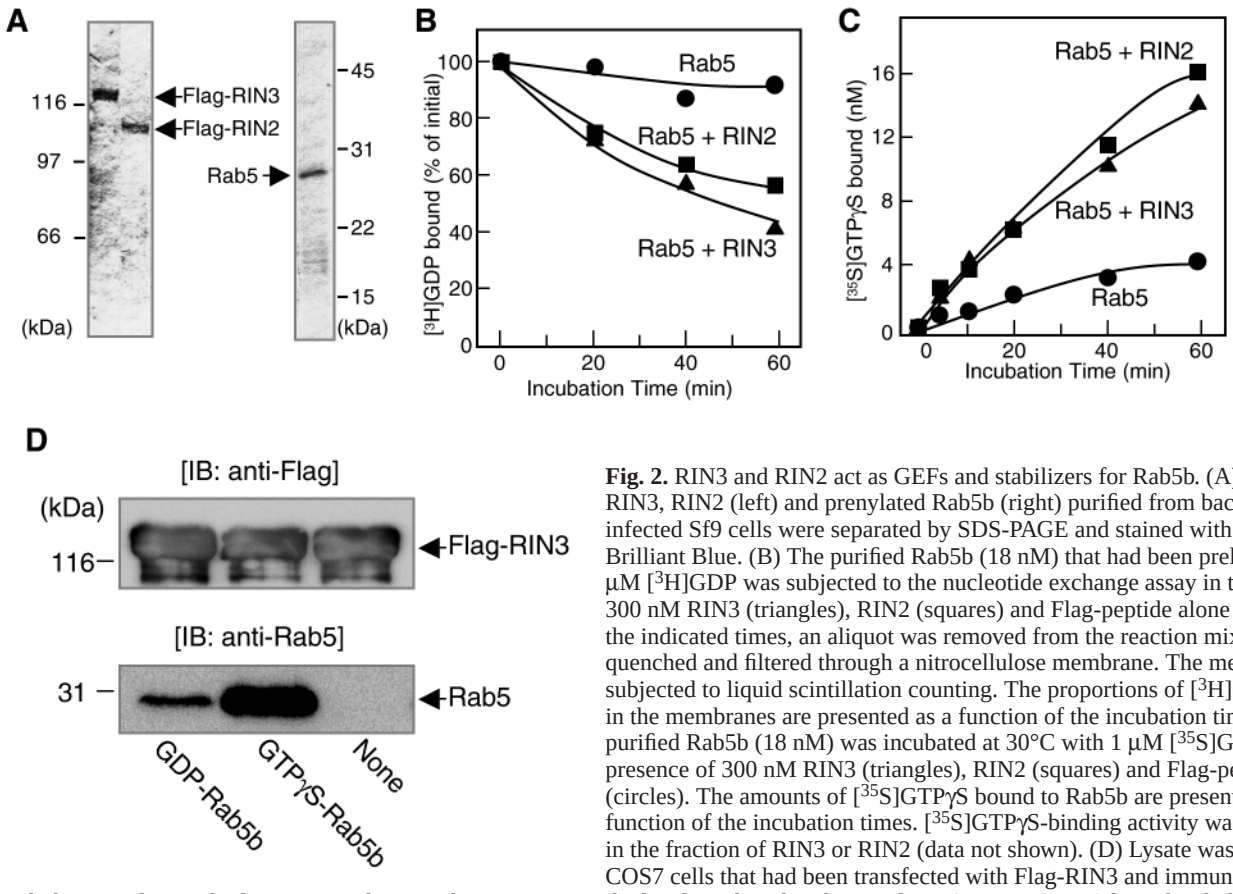

Fig. 2. RIN3 and RIN2 act as GEFs and stabilizers for Rab5b. (A) Flag-tagged RIN3, RIN2 (left) and prenylated Rab5b (right) purified from baculovirus-infected Sf9 cells were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. (B) The purified Rab5b (18 nM) that had been preloaded with 5 μM [^{3}H]GDP was subjected to the nucleotide exchange assay in the presence of 300 nM RIN3 (triangles), RIN2 (squares) and Flag-peptide alone (circles). At the indicated times, an aliquot was removed from the reaction mixture, quenched and filtered through a nitrocellulose membrane. The membranes were subjected to liquid scintillation counting. The proportions of [^{3}H]GDP retained in the membranes are presented as a function of the incubation times. (C) The purified Rab5b (18 nM) was incubated at 30°C with 1 μM [^{35}S]GTPγS in the presence of 300 nM RIN3 (triangles), RIN2 (squares) and Flag-peptide alone (circles). The amounts of [^{35}S]GTPγS bound to Rab5b are presented as a function of the incubation times. [^{35}S]GTPγS-binding activity was not detected in the fraction of RIN3 or RIN2 (data not shown). (D) Lysate was prepared from COS7 cells that had been transfected with Flag-RIN3 and immunoprecipitated with the anti-Flag antibody-conjugated resin. The resin was washed and incubated with or without GDP- or GTPγS-bound Rab5b. Proteins bound to the resin were separated by SDS-PAGE and immunoblotted (IB) with the anti-Flag (top) and anti-Rab5 (bottom) antibodies.

carcinoma cell line, upon the expression of RFP-RIN3 (data not shown).

It has been reported that the overexpression of RIN1 in a stable CHO cell line expressing Rab5a causes the enlargement of Rab5a-positive endosomes and that a proportion of RIN1 localizes with the endosomes (Tall et al., 2001). Therefore, intracellular localization of other RIN members was also investigated under the same conditions. When RFP-RIN2 was transiently expressed in HeLa cells, it localized to punctate vesicles, as observed with RIN3 (data not shown). However, RIN1 was distributed over the cytoplasm of HeLa cells (Fig. 3D). We confirmed that RFP-RIN1 and RIN3 fusion proteins were certainly produced in HeLa cells, using western blot analysis with an anti-RFP antibody (Fig. 3E).

To identify the entity of the RIN3-positive vesicles, we monitored the vesicular trafficking of transferrin in HeLa cells transiently expressing RIN3. HeLa cells that had been transfected with RFP-RIN3 were pulse-chased with Alexa-transferrin and further incubated at 25°C to allow its internalization. The fluorescently labelled transferrin was first observed in the plasma membrane (Fig. 4A), and it partly moved to the RIN3-positive vesicles 4-10 minutes after its internalization (Fig. 4B,C). However, the fluorescence of transferrin was no longer observed in the RIN3-positive vesicles at 15 minutes and it localized mostly to perinuclear EEA1-positive early endosomes (Fig. 4D). There was no significant difference between the time-dependent trafficking of Alexa-transferrin in the mock-transfected control and RIN3-expressing HeLa cells, suggesting that the RIN3-positive vesicles are not artefacts of transfection. These results strongly suggest that RIN3 is specifically involved in a transport pathway from plasma membranes to early endosomes.

Identification of amphiphysin II as a RIN3-binding protein

To uncover the function of RIN3, we further searched for RIN3-binding proteins by using the yeast two-hybrid system. A human leukocyte cDNA library was screened with full-length RIN3 as bait. Screening of 5×10^6 transformants yielded seven positive clones that strongly interacted with RIN3. Two and four of them contained clones coding for Rab5b and Rab5c, respectively. The remaining one was composed of a cDNA encoding the partial sequence of amphiphysin II. Amphiphysin I and II are nerve-terminal-enriched proteins containing SH3 domains that interact with dynamin and synaptojanin. The amphiphysins not only function in synaptic vesicle endocytosis by targeting dynamin and synaptojanin to endocytic buds through their interactions with clathrin and AP2, but also contribute to T-tubule organization and function (David et al., 1996; Lee et al., 2002; Leprince et al., 1997; Ramjaun et al., 1997; Wigge et al., 1997). Among the multiple amphiphysin II-splicing variants, amphiphysin II/BIN1 (whose exons 10, 12 and 13 are alternatively spliced) has been reported to be ubiquitously expressed (Wechsler-Reya et al., 1997). This amphiphysin II

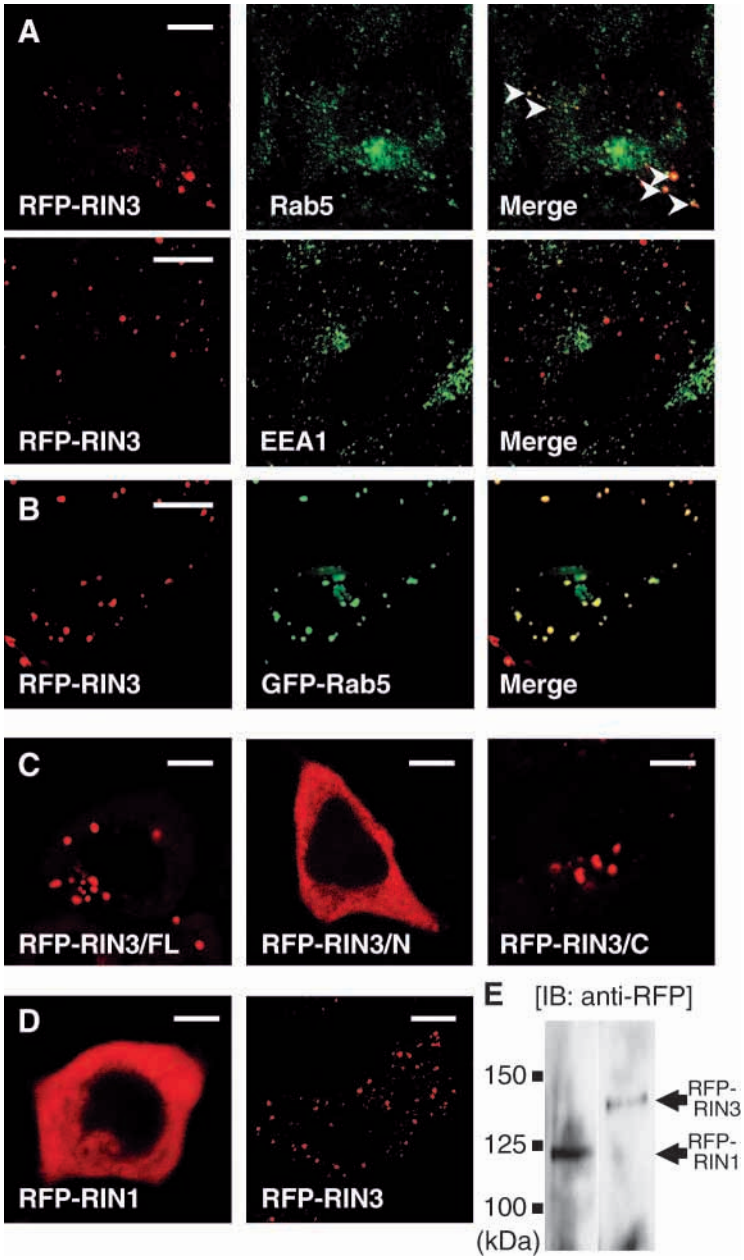

Fig. 3. RIN3 transfected into HeLa cells co-localizes with Rab5 but not EEA1. (A) HeLa cells were transiently transfected with RFP-RIN3 and cultured for 48 hours. The cells were fixed and labelled with antibodies to Rab5 (top) or EEA1 (bottom). The fluorescence of RFP-RIN3 (left) and Alexa-488 secondary antibody (middle) was visualized by confocal microscopy, and merged images of the two signals are displayed in yellow (right). Arrowheads indicate the co-localization of RIN3 and Rab5. (B) HeLa cells transiently expressing RFP-RIN3 and GFP-Rab5b were subjected to confocal microscopy, and the fluorescence of RFP-RIN3 (left) and GFP-Rab5b (middle) was visualized by confocal microscopy as described in (A). (C) HeLa cells transiently expressing the full-length (FL, left), N-terminal (N, middle) or C-terminal (C, right) form of RFP-RIN3 were subjected to confocal microscopy. (D) HeLa cells transiently expressing RFP-RIN1 (left) or RFP-RIN3 (right) were subjected to confocal microscopy. (E) Lysates from the transfected cells (RFP-RIN1, left; RFP-RIN3, right) were separated by SDS-PAGE and immunoblotted (IB) with an anti-RFP antibody. Scale bars, 10 μm.

variant is a 409-amino-acid protein and the isolated clone as a RIN3-binding protein corresponded to amino acids 28–409.

N-terminal region of RIN3 interacts specifically with the SH3 domain of amphiphysin II

To identify the interacting regions between amphiphysin II and RIN3, the full-length and SH3 domain of amphiphysin II were purified as GST-fused proteins and subjected to GST pull-down assays. The full-length and SH3 domain of amphiphysin II were capable of binding to RIN3 (Fig. 5A). The importance of the SH3 domain was also investigated in HeLa cells expressing the full-length and SH3-deleted form (ΔSH3) of Flag-amphiphysin II. When the cell lysate was immunoprecipitated with the anti-Flag antibody, the full-length form but not amphiphysin II/ΔSH3 co-precipitated RIN3 (Fig. 5B). Various forms of RIN3 were also expressed in HeLa cells as Flag-tagged proteins and were subjected to a GST pull-down assay with GST-fused amphiphysin II. Amphiphysin II appeared to interact with the full length of RIN3 and its N-terminal region but not with its C-terminal region (Fig. 5C). These results indicate that the SH3 domain of amphiphysin II and the N-terminal region of RIN3 that contains PRDs are sufficient for their direct interaction.

We further investigated whether the interaction of amphiphysin II is specific for RIN3, because other RIN members (RIN1 and RIN2) have also contained PRDs between the SH2 and RH domains (Fig. 1A). Each member of the RIN family was purified from Sf9 cells and incubated with GST-fused amphiphysin II that had been immobilized on glutathione Sepharose beads. Amphiphysin II associated with RIN3 (and RIN2, data not shown) but not with RIN1 (Fig. 5D). RIN3 and RIN2 were also capable of binding to the SH3 domain derived from amphiphysin II in vitro (data not shown). Thus, the binding of amphiphysin II appeared to be rather specific for a certain type (probably the class II) of PRDs present in RIN3 and RIN2 but not in RIN1.

Translocation of cytoplasmic amphiphysin II into RIN3-positive vesicles in transfected HeLa cells

To investigate how the interaction between amphiphysin II and RIN3 exerts its influence on the distribution of their proteins in intact cells, HeLa cells were transfected with GFP-amphiphysin II and/or RFP-RIN3 and subjected to confocal microscopic analysis (Fig. 6A). As previously observed (Lee et al., 2002), amphiphysin II exhibited a diffused pattern of cytoplasmic distribution, regardless of whether RFP-mock was co-expressed or not. By contrast, amphiphysin II was entirely targeted to and concentrated in the RIN3-positive vesicles and not present in the cytoplasm upon co-expression with RIN3. The same translocation of amphiphysin II into RIN-positive vesicles was observed with the expression of RIN2 but not with RIN1 (data not shown). We also investigated whether this targeting depends on the SH3 domain of amphiphysin II, because its deletion form failed to interact with RIN3 (Fig. 5B).

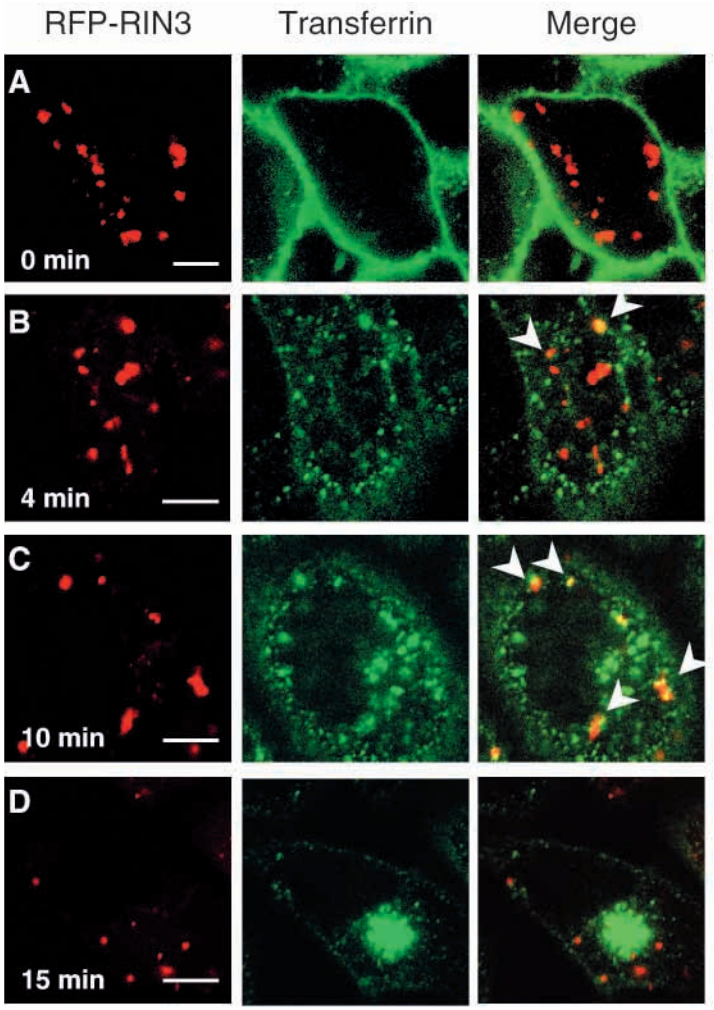

Fig. 4. Endocytic transferrin is transported through RIN3-positive vesicles to early endosomes in HeLa cells. HeLa cells expressing RFP-RIN3 were pulse-chased with Alexa-488/transferrin at 4°C, shifted to 25°C to allow its internalization and further incubated for the indicated times. The fluorescence of RFP-RIN3 (left) and transferrin (middle) was visualized by confocal microscopy, and merged images of the two signals are displayed in yellow (right). Arrowheads indicate the co-localization of RIN3 and transferrin. Scale bars, 10 μm.

Amphiphysin II/ΔSH3 distributed in the cytoplasm (Fig. 6B). This distribution was not altered by the co-expression with RIN3, although the formation of RIN3-positive vesicles was certainly observed. These data indicate that the targeting of amphiphysin II to the RIN3-positive vesicles depends on its SH3 domain. We finally investigated whether the ternary complex RIN3/Rab5/amphiphysin II might be observed in HeLa cells by means of the co-expression of RFP-RIN3, GFP-amphiphysin II and YFP-Rab5. Both amphiphysin II and Rab5 thoroughly localized in the RIN3-positive vesicles (Fig. 6C), and this localization was not markedly altered by the nucleotide-bound forms of Rab5 (data not shown). RIN3, Rab5 and amphiphysin II form a complex, but Rab5 does not directly interact with amphiphysin II in vitro (data not shown). Collectively, these data suggest that the ternary complex RIN3/Rab5/amphiphysin II is involved in the early endocytic transport pathway.

Discussion

In the present study, we have identified a novel Rab5-binding protein, RIN3, which contains SH2, proline-rich, RH, Vps9 and RA domains from the N terminus. This domain structure is conserved in other RIN members, RIN1 (Han et al., 1997) and RIN2 (Saito et al., 2002). RIN3 stimulates guanine nucleotide exchange reaction on Rab5 (Fig. 2B,C) but preferentially interacts with the GTP-bound form of Rab5 (Fig. 2D). The same properties are observed in RIN1 and RIN2, except for the preferential binding of RIN1 to GDP-bound Rab5 (Saito et al., 2002; Tall et al., 2001). These biochemical properties are equivalent to the combined actions of Rabex-5 and Rabaptin-5, which form a complex in early endosomes (Horiuchi et al., 1997; Lippe et al., 2001). Rabex-5 is a Rab5 GEF involved in the homotypic fusion of early endosomes, and Rabaptin-5 specifically associates with the GTP-bound form of Rab5 to stabilize its nucleotide form (Stenmark et al., 1995). Thus, RIN3, together with RIN2, appears to function as both the stimulator and the stabilizer for Rab5 in an endocytic transport pathway just as the Rabex-5/Rabaptin-5 complex does in the homotypic fusion of early endosomes.

Although RIN1, RIN2 and RIN3 appear to belong to the same family in terms of their domain structure and biochemical properties for Rab5, several differences are highlighted from the present study. First, the distributions of mRNAs are different among the RIN family in spite of their wide expression: *RIN1*, *RIN2* and *RIN3* mRNAs are abundant in brain (Han et al., 1997), in heart, kidney and lung (Saito et al., 2002), and in peripheral blood cells (Fig. 1B), respectively. Second, the intracellular localizations of the three RFP-RIN members transiently expressed in HeLa cells are not identical to one another (Fig. 3): RIN3 and RIN2 localize to endocytic vesicles, whereas RIN1 exhibits a cytoplasmic distribution. RIN3 (and RIN2) appears to localize with Rab5 in the vesicles, especially when both proteins were expressed in the cells (Fig. 3A,B). However, it has been reported that RIN1 could partially co-localize with Rab5-positive vesicles upon their co-expression, and stimulates epidermal-growth-factor-receptor-mediated endocytosis (Tall et al., 2001). The different localizations of the RIN members might be derived from the types of cells used, but it is likely that RIN1 also localizes to endocytic vesicles if the cells are stimulated by membrane receptors. Third, there is selectivity in the association of RINs with amphiphysin II: RIN3 and RIN2, but not RIN1, can interact with amphiphysin II (Fig. 5D). The N terminus of RIN3 that contains PRDs directly associates with the SH3 domain of amphiphysin II (Fig. 5A-C). The class-II PRD present in both RIN3 and RIN2 but not in RIN1 appears to be responsible for the association, because a mutation of proline in the class-II PRD failed to interact with amphiphysin II (data not shown).

Another unique feature of the RIN family is the existence of an RA domain in their C termini, which was initially identified as a region interacting with H-Ras (Hofer et al., 1994; Ponting and Benjamin, 1996). This suggests that RINs are capable of

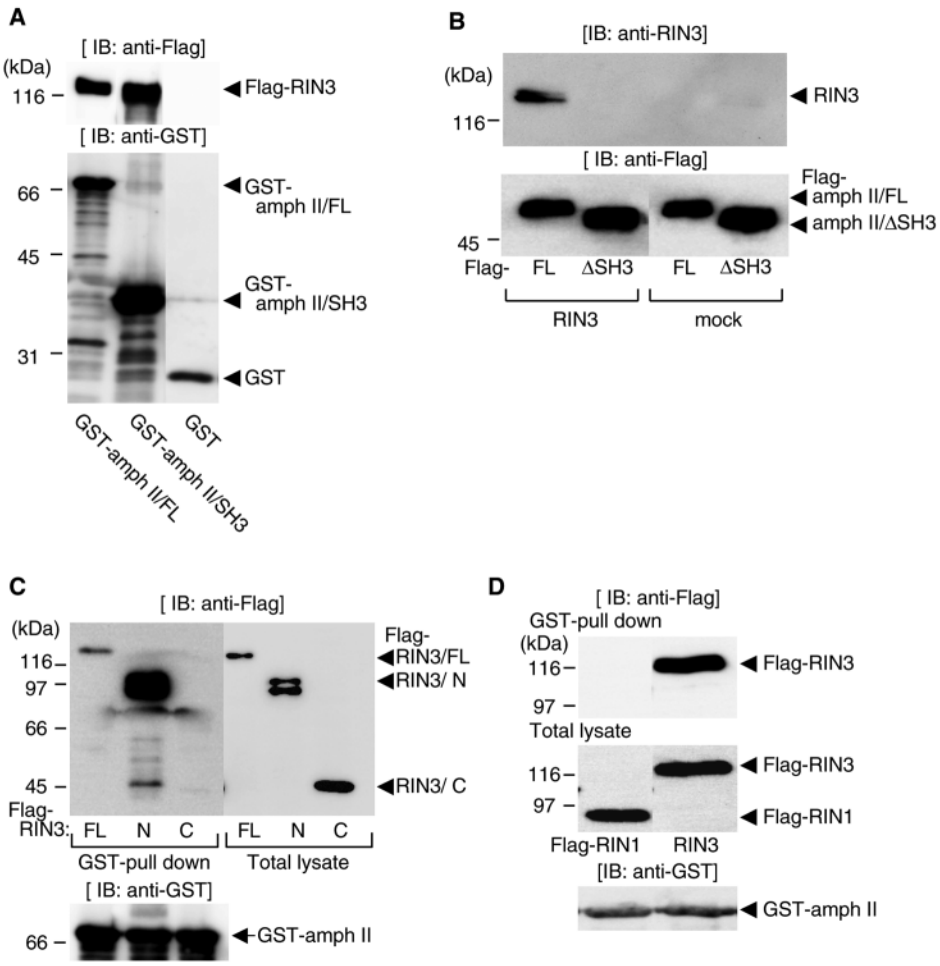

Fig. 5. The N-terminal region of RIN3 containing PRDs specifically interacts with the SH3 domain of amphiphysin II. (A) The full-length (FL) or SH3 domain (SH3) of GST-fused amphiphysin II (GST-amph II) or GST alone was incubated with Flag-RIN3 purified from baculovirus-infected Sf9 cells and glutathione resin. Proteins bound to the resin were separated by SDS-PAGE and immunoblotted (IB) with anti-Flag (top) and anti-GST (bottom) antibodies. (B) Lysates were prepared from HeLa cells expressing RIN3 and the full length (FL) or SH3-domain-deleted form (ΔSH3) of Flag-tagged amphiphysin II (Flag-amph II) and incubated with glutathione resin. Proteins bound to the resin were separated by SDS-PAGE and immunoblotted with anti-RIN3 (top) and anti-Flag (bottom) antibodies. (C) Lysates were prepared from HeLa cells expressing the full length (FL), N-terminal (N) or C-terminal (C) form of Flag-tagged RIN3 (Flag-RIN3) and incubated with GST-fused amphiphysin II (GST-amph II) and glutathione resin. Proteins bound to the resin (GST-pull down) and the total lysate were separated by SDS-PAGE and immunoblotted with anti-Flag (top) and anti-GST (bottom) antibodies. (D) Flag-RIN1 and RIN3 were purified from baculovirus-infected Sf9 cells, and GST pull-down assay was performed as described in (A). Proteins bound to the resin (GST pull-down) and the total lysate were separated by SDS-PAGE and immunoblotted with anti-Flag (top) and anti-GST (bottom) antibodies.

binding not only to Rab5 but also to the Ras-family GTPases. In this regard, there is an interesting report showing that the GEF activity of RIN1 for Rab5 is enhanced by its interaction with Ha-Ras (Han and Colicelli, 1995; Tall et al., 2001). However, Ha-Ras failed to interact with RIN3 or RIN2 to stimulate their GEF activities in conditions under which it certainly binds to RIN1. Instead, several Ras members other than Ha-Ras could interact with RIN2 (K.S., unpublished). These results suggest that each RIN interacts with certain types of Ras members. In addition, the RIN-family members also contain an SH2 domain in their N-terminal regions, suggesting that receptor-linked tyrosine kinases might regulate the functions of RINs through their interactions with tyrosine-phosphorylated receptors and/or adaptors. We are currently investigating how the Ras members and/or receptor stimulation exert their influences on the GEF activities of RINs.

One of the important findings of this study is the identification of amphiphysin II/BIN1 as another binding partner for RIN3. Amphiphysin II forms heterodimer with amphiphysin I (Wigge et al., 1997) and they are involved in endocytosis, particularly in synaptic vesicle recycling (Wigge and McMahon, 1998). Many amphiphysin II isoforms appear to arise through alternative splicing of a single gene (Butler et al., 1997; Kadlec and Pendergast, 1997; Leprince et al., 1997; Ramjaun et al., 1997; Sakamuro et al., 1996; Sparks et al., 1996; Tsutsui et al., 1997). Among them, the isoform that was cloned to interact with RIN3 in the present study lacks exon 10 (encoding the nuclear localization signal), exon 12 (which mediates interactions with clathrin and AP-2) (David et al., 1996; Ramjaun and McPherson, 1998) and exon 13 (a part of the *c-myc* binding domain). This form, also called BIN1-10-13, is ubiquitously expressed (Wechsler-Reya et al., 1997), although its exact role is unclear. In the present study, we showed that the C-terminal SH3 domain of amphiphysin II is necessary and sufficient for the interaction with RIN3 (Fig. 6), and that this domain is present in all the isoforms. Thus, it is very likely that RIN3 associates with all spliced forms of amphiphysin II, including the one that can interact with clathrin and AP-2.

We also revealed here that amphiphysin II is recruited to RIN3-positive vesicles in the endocytic transport pathway. This translocation appears to depend on the association between the PRDs of RIN3 and the SH3 domain of amphiphysin II (Figs 5,6). Amphiphysin II has been reported to interact via its SH3 domain with dynamin and synaptojanin (Leprince et al., 1997; Ramjaun et al., 1997; Wigge et al., 1997). Dynamin functions in the fission steps of clathrin-coated vesicles from the plasma membrane. Synaptojanin is a PtdIns-5-phosphatase, which

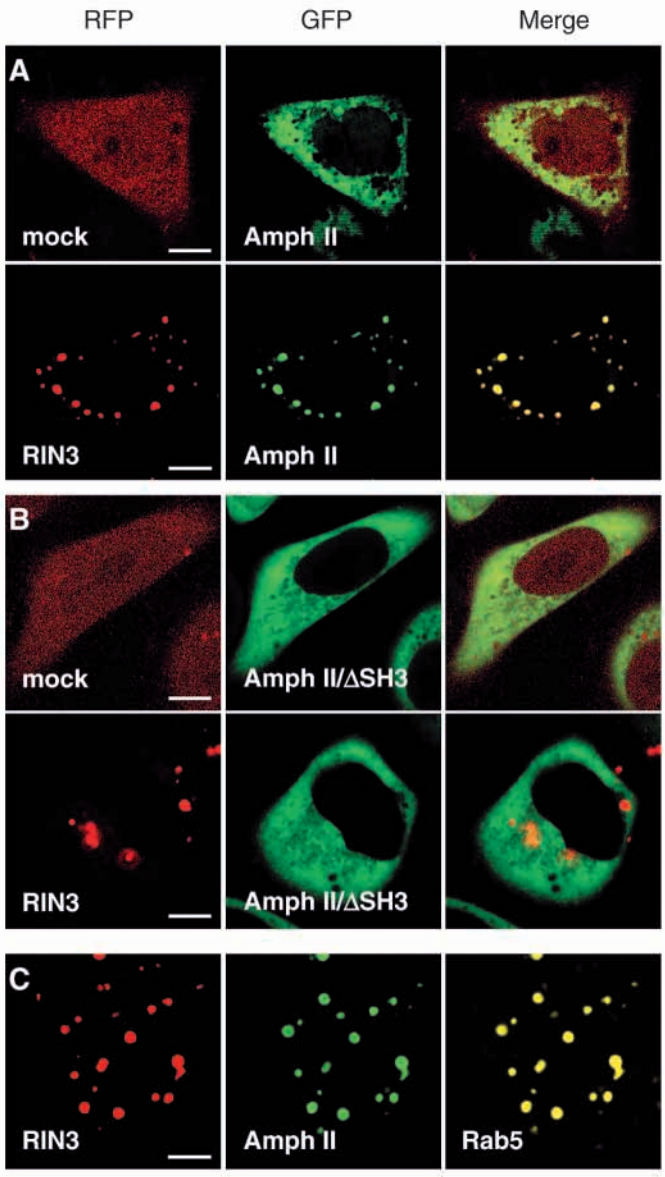

Fig. 6. Cytoplasmic amphiphysin II translocates into RIN3-positive vesicles in HeLa cells. (A,B) HeLa cells were transiently transfected with GFP-amphiphysin II (A) or GFP-amphiphysin II/ΔSH3 (B) and RFP-mock (A,B, top) or RFP-RIN3 (A,B, bottom) and further incubated for 48 hours. The fluorescence of RFP (left) and GFP (centre) was visualized by confocal microscopy, and merged images of the two signals are displayed in yellow (right). (C) HeLa cells were transiently transfected with RFP-RIN3 (left), GFP-amphiphysin II (middle) and YFP-Rab5 (right), and further incubated for 48 hours. Scale bars, 10 μm.

hydrolyses PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3 (Chung et al., 1997; McPherson et al., 1996; Woscholski et al., 1997), and is enriched in nerve terminals. This phosphatase has also been implicated in the uncoating of clathrin at a step closely related to the action site of dynamin (Cremona et al., 1999; McPherson et al., 1994). Thus, amphiphysin II appears to interact not only with dynamin and synaptojanin in the fission step of clathrin-coated vesicles but also with RIN3 after the fission in the endocytic transport pathway. In addition, we observed here that the RIN3-positive vesicles contain Rab5 but not the early endosomal marker EEA1 (Fig. 3A). Furthermore, transferrin appeared to be partly transported through the RIN3-positive vesicles to early endosomes (Fig. 4). Taken together, these results suggest that the unique vesicles identified by the co-localization of RIN3, Rab5 and amphiphysin II might participate in an intermediate process from endocytosis to early endosomes.

Although the molecular mechanism whereby Rab5 regulates the early endocytic pathway is still unclear, it is tempting to speculate that a regulatory system similar to the homotypic fusion process of early endosomes might also operate on the endocytic pathway. In this regard, it has been reported that Rab5 can interact with the class-Ia PtdIns-3(OH)-kinase p110β to stimulate the production of PtdIns(3,4,5)P_3 (Kurosu and Katada, 2001). Interestingly, this PtdIns-3(OH)-kinase subtype is markedly enriched in clathrin-coated vesicles compared with the class-II PtdIns-3(OH)-kinase hVps34, which produces PtdIns(3)*P* in the early endosomes (Christoforidis et al., 1999). Taken together, the different distributions of PtdIns-3(OH)-kinases and Rab5-GEFs might be responsible for the strict regulation of Rab5-dependent processes involved in many membrane trafficking pathways. Although further experiments are apparently necessary to elucidate the molecular mechanisms underlying the Rab5-regulated processes, our present data strongly suggest that the RIN family interacting with Rab5 and amphiphysin II is involved in the early endocytic pathway.

We are grateful to D. W. Russell for generous gifts of pCMV5 vector and J. Colicelli for the gift of the RIN1 plasmids used in this study. This work was supported in part by research grants from the 'Research for the Future' Program of the Japan Society for the Promotion of Science (JSPS-RFTF 96L00505), the Mitsubishi Foundation, and the Scientific Research Funds of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government.

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