Varp is a Rab21 guanine nucleotide exchange factor and regulates endosome dynamics

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Summary

The small GTPases Rab5 and Rab21 are closely related, and play essential roles in endocytic trafficking. Rab5 is regulated by VPS9-domain-containing guanine nucleotide exchange factors. Here, we describe a new VPS9-domain protein with ankyrin repeats, the VPS9-ankyrin-repeat protein (Varp). Varp interacts preferentially with GDP-bound Rab21 and has a much stronger guanine nucleotide exchange activity towards Rab21 than Rab5. Furthermore, RNAi-mediated depletion of endogenous Varp significantly disrupts the activity of Rab21 in HeLa cells. Ectopically expressed Varp mainly localizes to early endosomes and causes enlargement of early endosomes and giant late endosomes. Both the VPS9 domain and ankyrin-repeats are required for the endosomal localization and the activity of Varp in vivo. These results suggest that Varp is a potential Rab21 guanine nucleotide exchange factor and might regulate endosome dynamics in vivo.

Key words: Ankyrin repeat, Endosome, Guanine nucleotide exchange factor, Rab5, Rab21, VPS9 domain

Introduction

The Rab family of small GTPases plays central roles in intracellular membrane trafficking. To date, more than 40 Rab proteins have been identified and reported to coordinate, with their regulators and effectors, the processes of the membrane transport (Olkkonen and Stenmark, 1997; Segev, 2001; Zerial and McBride, 2001). Like other GTPases, Rab proteins function as molecular switches by cycling between an inactive (GDP-bound) and an active (GTP-bound) state. This cycle is precisely regulated to ensure temporal and spatial regulation of membrane and vesicular transport. Rab5, a key regulator of endocytic trafficking, is involved not only in the homotypic fusion process of early endosomes but also in the budding of clathrin-coated vesicles from plasma membranes and their transport to early endosomes (Barbieri et al., 1998; Bucci et al., 1992; Gorvel et al., 1991; Stenmark et al., 1994). In the inactive state, GDP-bound Rab5 forms a cytoplasmic complex with the Rab5 GDP dissociation inhibitor (GDI). Upon targeting to donor membranes, Rab5 is dissociated from GDI by a GDI dissociation factor (GDF) and interacts with a Rab5 guanine nucleotide exchange factor (Rab5GEF), which switches Rab5 to the GTP-bound state. GTP-bound Rab5 then recruits several effectors to a specific membrane domain to coordinate endosomal fusion (Zerial and McBride, 2001).

Thus Rab5GEF plays a key role in the regulation of Rab5 function. To date, at least seven Rab5GEFs, including Vps9p, Rabex5, the RIN family of Ras effectors (RIN1, 2, 3), ALS2/Alsin and RME-6, have been identified (Cormont et al., 1996; Hama et al., 1999; Horiuchi et al., 1997; Kajiho et al., 2003; Otomo et al., 2003; Saito et al., 2002; Sato et al., 2005; Tall et al., 2001). All these proteins share a conserved VPS9 domain, which is required for Rab5 binding and nucleotide exchange (Delprato et al., 2004). Domains other than VPS9 in Rab5GEFs mainly mediate protein interactions that determine the specificity and/or location of Rab5GEF activation (Barbieri et al., 2004; Barbieri et al., 2003; Kunita et al., 2004). Taken together, the diversity of Rab5GEFs partly accounts for the temporal and spatial specificity of Rab5 function.

Rab21, a close homologue of Rab5, was originally identified in canine MDCKII cells (Zerial and Huber, 1995) and reported to be ubiquitously expressed (Opdam et al., 2000). Recent studies suggest that Rab21 is mainly localized on early endosomes and regulates endosome dynamics and ligands endocytosis (Simpson et al., 2004). A more recent report revealed a function of Rab21 in regulation of phagocytosis in *Dictyostelium discoideum* (Khurana et al., 2005). However, no regulator or effector of Rab21 has been characterized.

In the present study, we report a new VPS9 domain and ankyrin-repeat-containing protein, called VPS9-ankyrin-repeat protein (Varp) (GenBank accession number AY336500). Our results suggest that Varp functions as a Rab21 guanine nucleotide exchange factor and regulates endosome dynamics.

Results

Sequence information and expression pattern of Varp

Varp was originally cloned in a yeast two-hybrid screen using the intracellular domain of human LRP6 as bait (X.Z. and Z.C., unpublished results). The full-length Varp protein, which is composed of 1050 and 1048 amino acids (aa) in human and mice, respectively, contains a VPS9 domain and eight ankyrin repeats. The Varp N-terminus shares significant homology to Rabex5, a well studied Rab5GEF (Fig. 1A), implying its function as a potential Rab5GEF. It has been reported that the four amino acids residues in Rabex5, Asp313, Pro317, Tyr354 and Thr357, which are conserved in all known Rab5GEFs, are crucial for the catalytic function on Rab5 and Rab21 (Delprato

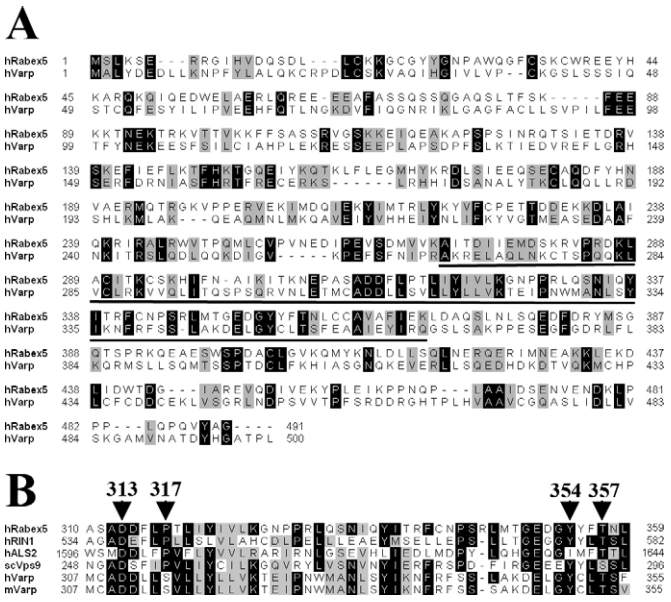

Fig. 1. The Varp protein sequence. (A) Alignment of human Rabex5 and Varp N-terminal fragment. The solid line indicates the VPS9 domain. (B) Alignment of a part of the VPS9 domains from human Rabex5 (hRabex5), human RIN1 (hRIN1), human ALS2 (hALS2), *S. cerevisiae* Vps9p (scVps9), human Varp (hVarp) and mouse Varp (mVarp) protein sequences. The shaded regions (black and gray) represent identical and conserved amino acids, respectively. Arrowheads indicate the four crucial amino acids in Rabex5 required for the GEF activity.

et al., 2004). In the sequence of Varp proteins, the aa residue corresponding to Pro317 is Ser314, whereas the other three residues are conserved (Fig. 1B). This substitution may affect the catalytic efficiency and/or specificity of Varp on Rab5 and/or Rab21. Varp is widely expressed in mouse embryos from embryonic day (E) 7 to E15, and in a variety of adult mouse tissues (Fig. 2A,B). Varp is also expressed in most human cell lines examined (except HK-2 and GRC-1), as assayed by reverse transcription (RT) PCR (Fig. 2C).

Varp catalyzes the guanine nucleotide exchange reaction for Rab5 and Rab21 in vitro

Since Varp contains a VPS9 domain that is conserved in other previously characterized Rab5GEFs, we addressed whether Varp has a GEF activity for Rab5. To examine this possibility, we performed in vitro GDP-GTP exchange assays with Flag-tagged Varp and the Varp mutant VarpN, which does not contains the ankyrin repeats (Fig. 3A). His_6-tagged Rab5a protein was used as the substrate and Rabex5 as positive control. These proteins were purified from transfected HEK293T cells and from *Escerichia coli*, respectively (Fig. 3B). Our data showed that VarpN was relatively weak in stimulating the release of [^{3}H]GDP from, and the binding of [γ-^{35}S]GTP to, Rab5a (Fig. 3C,D). The full-length Varp protein showed no obvious activity (Fig. 3C,D). Rabex5 showed strong activity in these two assays (Fig. 3C,D). These in vitro results suggest that the VPS9 domain of Varp has a low Rab5GEF activity, and this activity might be regulated by its C-terminal fragment containing the ankyrin repeats.

Since VarpN showed a relatively weak activity on Rab5 in vitro, we next examined the GEF activity of Varp towards other endosome-related Rab proteins. We used purified VarpN protein and different GST-Rab proteins (including Rab4, 5a, 7, 15, 21 and 22) to perform the in vitro [γ-^{35}S]GTP-binding assays. Interestingly, whereas its GEF activity towards Rab5 is weak, we observed relatively strong activity of VarpN towards Rab21 but little or no activity towards Rab4, 7, 15 or 22 (Fig. 3E). Rab5 and Rab21 phylogenetically belong to the same subfamily (Pereira-Leal and Seabra, 2000; Pereira-Leal and Seabra, 2001) and have similar functions in cells (Simpson et al., 2004). A previous study demonstrated that, the $Rabex5_{132\text{-}391}$ fragment exhibits equal GEF activities towards Rab5 and Rab21 in vitro, and $Rabex5_{132\text{-}391}$P317A (in which Pro317 was replaced by Ala) in comparison shows a relatively stronger activity towards Rab21 than Rab5, although its activities on both Rab5 and Rab21 were severely impaired (Delprato et al., 2004). Thus, the behavior of VarpN protein on Rab5 and Rab21 is similar to that of the $Rabex5_{132\text{-}391}$P317A mutant. As previously mentioned, the residue corresponding to Pro317 of Rabex5 is a Ser in the Varp protein. These results suggest that Varp as a GEF has a preference towards Rab21, and that Pro317 in Rabex5 as well as Ser314 in Varp might contribute to the substrate selectivity of the VPS9 domain.

Varp preferentially interacts with GDP-bound forms of Rab21 and Rab5

We next examined the interaction of Varp and Rab5 or Rab21 using coimmunoprecipitation assays in transfected HEK293T cells. Myc-tagged Varp and EGFP-tagged Rab5a or Rab21, or their respective mutant forms, were coexpressed in HEK293T cells. Whole-cell lysates were immunoprecipitated with anti-Myc antibody and the precipitates were probed with anti-GFP antibody on western blots. The data showed that Varp coimmunoprecipitated with all three forms of EGFP-Rab5a and Rab21 (Fig. 4). Consistent with the results from other

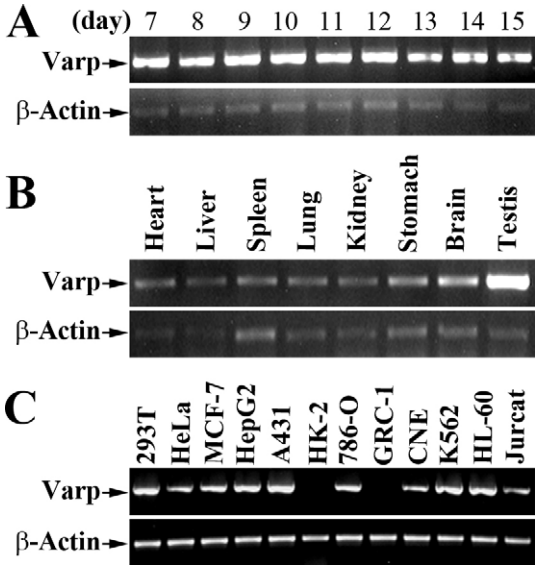

Fig. 2. Expression of Varp mRNA during (A) mouse embryogenesis, in (B) adult mouse tissues and in (C) human cell lines. A pair of specific primers for the mouse or human Varp gene was used for the RT-PCR analysis; β-actin was used as an internal control.

Rab5GEFs (Otomo et al., 2003; Tall et al., 2001), we observed that Varp preferentially interacts with EGFP-Rab5a S34N and EGFP-Rab21 T33N, mutant forms of Rab5a and Rab21 and expected to be locked in the GDP-bound conformation. Furthermore, the interaction of Varp and Rab21 T33N is much stronger than that of Varp and Rab5 S34N (as shown in Fig. 4). These results are consistent with the notion that Varp might function as a GEF for Rab21 and Rab5, however, with a strong preference for Rab21.

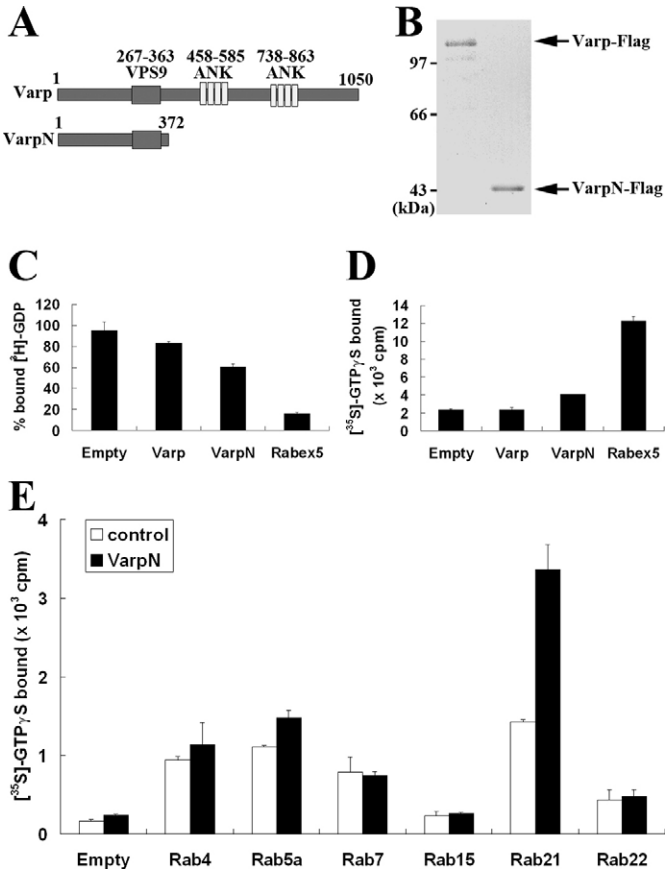

Fig. 3. The VPS9 domain of Varp catalyzes Rab21 guanine nucleotide exchange reaction in vitro. (A) A schematic diagram of the full-length Varp and VarpN protein. VPS9, vacuolar protein sorting 9 domain; ANK, ankyrin repeat. (B) Flag-tagged Varp and VarpN proteins purified from transfected HEK293T cells were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. (C) [^{3}H]GDP-loaded His$_6$-Rab5a was incubated with the purified Flag-tagged Varp, VarpN and Rabex5. The percentage of [^{3}H]GDP remained bound to Rab5a after 60 minutes is presented. Each value represents the mean and standard deviation of at least two independent assays. (D) His$_6$-Rab5a was incubated with [γ-^{35}S]GTP at 30°C in the presence of Flag-tagged Varp, VarpN or Rabex5 for 60 minutes. Counts of the bound [γ-^{35}S]GTP on Rab5a are presented. Each value represents the mean and standard deviation of at least two independent assays. (E) A total of six Rab GTPases, including Rab4, Rab5a, Rab7, Rab15, Rab21 and Rab22, were subjected to the in vitro [γ-^{35}S]GTP-binding assay. Each GST-fused Rab GTPase was incubated with [γ-^{35}S]GTP at 30°C in the presence or absence of Flag-tagged VarpN for 60 minutes. Each value represents the mean and standard deviation of at least two independent assays. Empty, no Rab protein.

Intracellular localization and function of Varp in HeLa cells

We investigated the cellular localization of the Varp protein with a purified rabbit anti-Varp polyclonal antibody, generated against a part of the Varp N-terminus. The immunostaining data showed that, in HeLa cells, expressed Varp protein was localized in the cytoplasm with a specific dot pattern and distributed mainly in the perinuclear region, similar to endosome-like structures (Fig. 5A,a,d,g). Therefore, we co-stained the Varp protein with EEA1, a specific marker for early endosomes (Mu et al., 1995). We found that Varp and EEA1 colocalized considerately on the early endosome in the cytoplasm (Fig. 5A,a-c). Since Rab5 is a well studied key regulator of the endosome system, we further examined whether Varp and Rab5 colocalized on the early endosome. By coexpression of Myc-tagged Varp and EGFP-Rab5a in HeLa cells, we observed that Varp and EGFP-Rab5a partially colocalized on endosome-like structures (Fig. 5A,d-f). Furthermore, Varp partially colocalized with internalized Alexa-Fluor-594-conjugated transferrin, a marker for early and recycling endosomes (Bucci et al., 1992) (Fig. 5A,g-i). These results suggest that Varp is mainly localized on the early endosomes.

Interestingly, we observed that in HeLa cells, the ectopically expressed Varp protein caused an enlargement of early endosomes (Fig. 5B,b,f). The data also showed that EEA1 and EGFP-Rab5a colocalized with Varp on these enlarged early endosomes (Fig. 5B,e-h, arrows). Since Varp preferentially interacts with Rab21 and catalyzes its guanine nucleotide exchange reaction, we also studied the colocalization of Varp and Rab21 in HeLa cells and found that Varp and EGFP-Rab21 proteins colocalized on the enlarged EEA1-positive early endosomes (Fig. 5C,e-h, arrows). We further observed that the late endosomal marker Rab7 was redistributed to the enlarged EEA1-positive early endosomes in HeLa cells that expressing Varp protein (Fig. 5D,e-h, arrows). The data also show that a small amount of Varp protein colocalized with Rab7 on EEA1-negative endosomal structures (Fig. 5D,e-h, arrowheads). In wild-type HeLa cells, Rab7 did not colocalize much with EEA1 (Fig. 5D,a-d). Taken together, these results suggest that Varp promotes early endosome fusion and regulates membrane trafficking between the early and late endosomes.

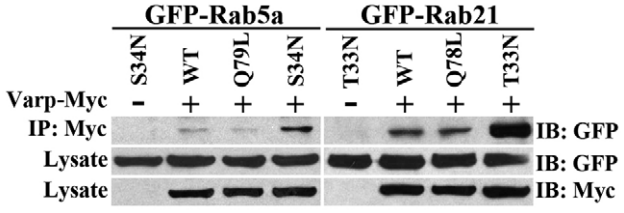

Fig. 4. Varp interacts with Rab21 and Rab5a. Coimmunoprecipitation of the full-length Varp protein and Rab5a or Rab21, or their mutant forms. Myc-tagged Varp (Varp-Myc) and each of the GFP-tagged Rabs were coexpressed in HEK293T cells. Whole-cell lysates were immunoprecipitated with an anti-Myc antibody and precipitates were analyzed by western blotting with an anti-GFP antibody. Aliquots of whole-cell lysates were also examined by western blotting with anti-GFP or anti-Myc antibodies.

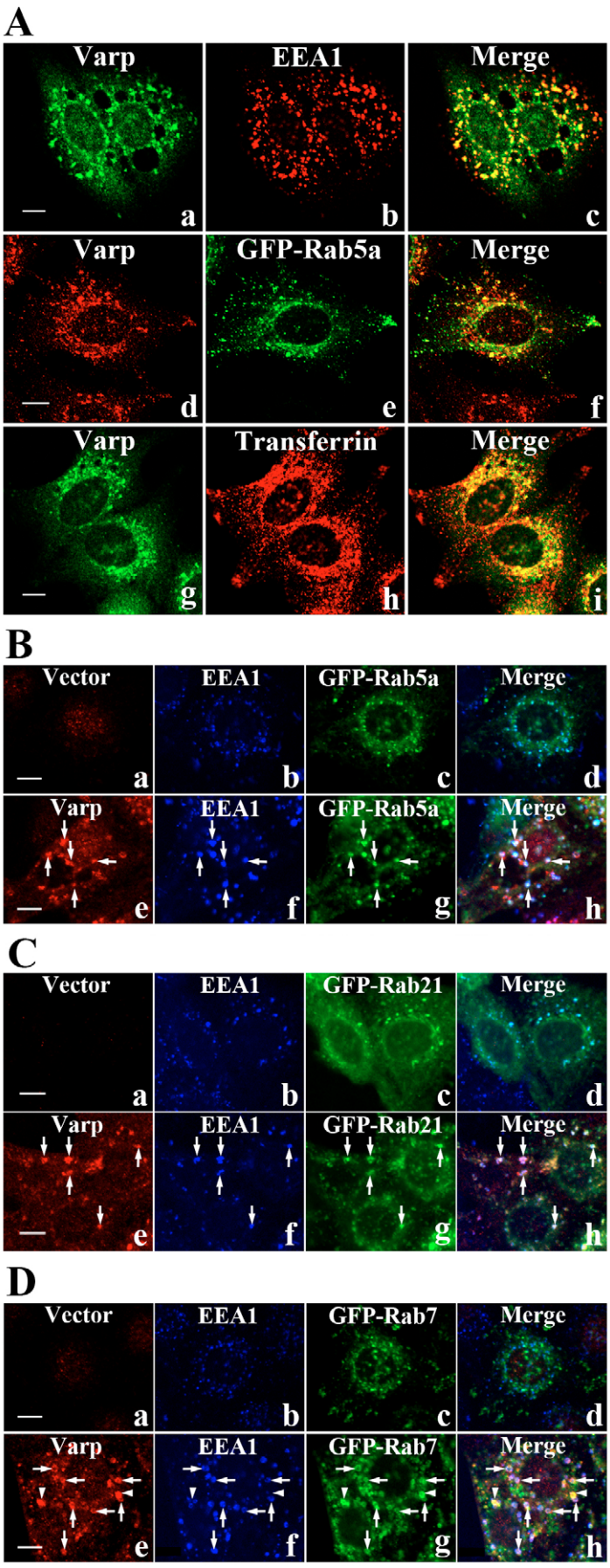

Fig. 5. Ectopically expressed Varp colocalizes with endosomal markers. (A) HeLa cells stably expressing Varp-Myc were used in these assays. (a-c) Cells were fixed and double labeled with a rabbit polyclonal anti-Varp antibody (green) and a mouse anti-EEA1 antibody (red). (d-f) Cells were transfected with an EGFP-Rab5a construct and labeled with anti-Myc antibody (red). (g-h) Cells were labeled with 25 μg/ml Alexa 594-conjugated human transferrin in DMEM at 37°C for 10 minutes and washed with ice-cold PBS, and then fixed and immunostained with an anti-Varp antibody (green). (B) Control HeLa cells or HeLa cells expressing the Varp protein were transfected with an EGFP-Rab5a construct and labeled with an anti-Varp antibody (red) and an anti-EEA1 antibody (blue). Arrows indicate the colocalization of Varp, EEA1 and Rab5. (C) Control HeLa cells or HeLa cells expressing the Varp protein were transfected with an EGFP-Rab21 construct and labeled with an anti-Varp antibody (red) and an anti-EEA1 antibody (blue). Arrows indicate the colocalization of Varp, EEA1 and Rab21. (D) Control HeLa cells or HeLa cells expressing the Varp protein were transfected with an EGFP-Rab7 construct and labeled with an anti-Varp antibody (red) and an anti-EEA1 antibody (blue). Arrows indicate colocalization of Varp, EEA1 and Rab7, and arrowheads indicate colocalization of Varp and Rab7, but not EEA1. Bars, 10 μm.

Ectopically expressed Varp protein induces giant late endosome-like structures in HeLa cells

In addition to inducing the enlargement of early endosomes, we also observed that the ectopic expression of the full-length Varp protein in HeLa cells caused the formation of dramatically enlarged vacuolar structures around the nucleus (Fig. 6B,a). These structures were similar to the giant endosomes caused by Rab5Q79L, a constitutively active form of Rab5 (Stenmark et al., 1994) (Fig. 6B,d). The number and size of the vacuoles induced by expression of Varp protein were varied among individual cells (Fig. 6B,a). Some vacuoles were relatively small and numerous, whereas in other cases one or two big vacuoles were observed. This possible conversion between small and large vacuoles suggests that these vacuoles undergo dynamic fusion events. No large vacuolar structures were observed in HeLa cells expressing VarpN (Fig. 6B,b), indicating that the C-terminus containing the ankyrin repeats is required for vacuole formation. In contrast to Varp, Rabex5 did not cause the vacuolar structures in HeLa cells (Fig. 6B,c), suggesting a functional distinction between Varp and Rabex5.

To further characterize the giant vacuolar structures, we double-labeled Varp protein and several well-characterized organelle markers in HeLa cells expressing the Varp protein. We found that the early endosomal marker EEA1 showed little localization on the giant vacuoles (marked * in Fig. 6C,b). On the contrary, the late endosomal marker Rab7 (Bottger et al., 1996; Bucci et al., 2000) (Fig. 6C,e) and the late endosomal and lysosomal marker lysosome-associated membrane protein 3 (LAMP3) (Escola et al., 1998; Fukuda, 1991) (Fig. 6C,h) were predominantly localized on the border (membrane) of the giant vacuoles caused by Varp expression, suggesting that these giant vacuoles are late endosomes. Furthermore, we observed that, in HeLa cells, some of the ectopically expressed Varp protein colocalized with EGFP-Rab7 and LAMP3 on the

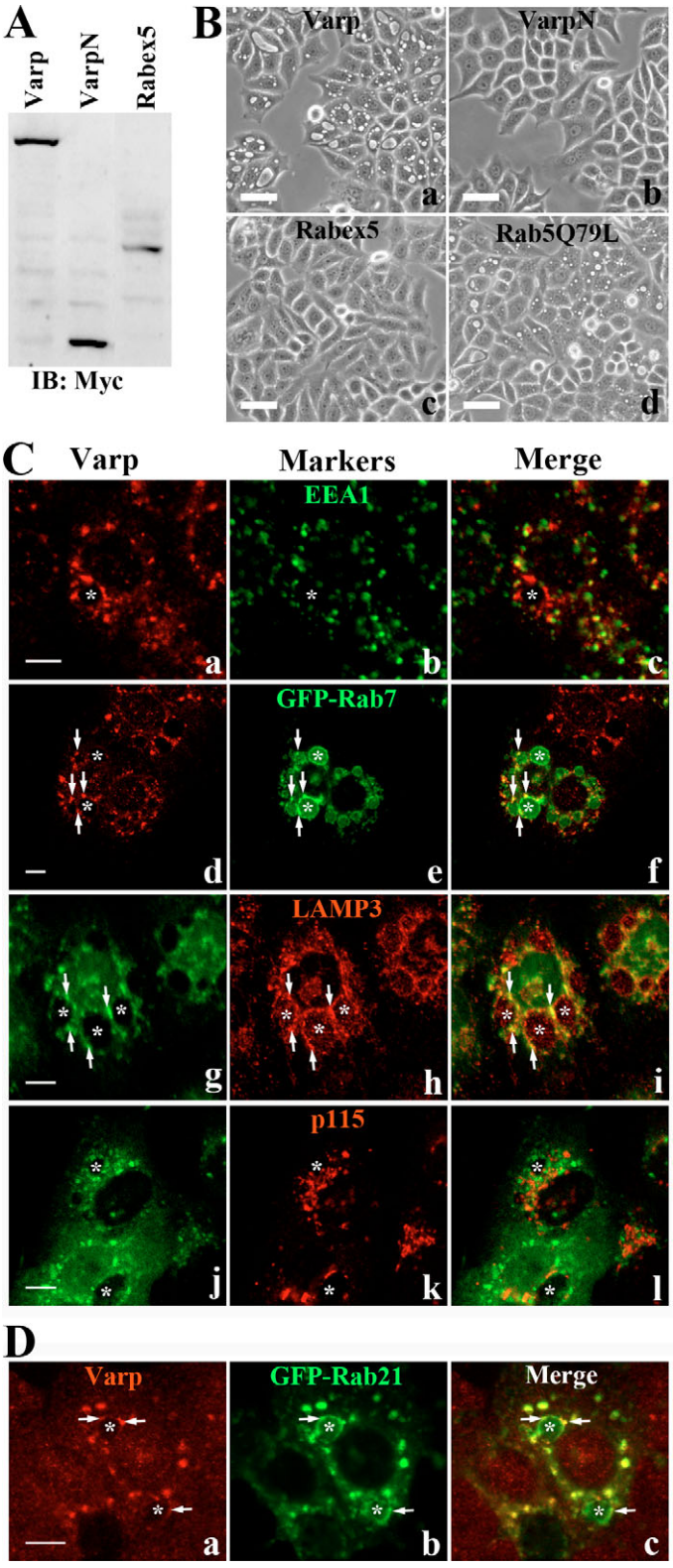

Fig. 6. Ectopic expression of Varp induces giant vacuoles in HeLa cells. (A) Lysates from HeLa cells stably expressing Myc-tagged Varp, VarpN, or Rabex5 were separated by SDS-PAGE and immunoblotted (IB) with an anti-Myc antibody. (B) Phase-contrast micrographs of living HeLa cells stably expressing Varp (a), VarpN (b), Rabex5 (c), or transiently expressing EGFP-Rab5Q79L (d). Bars, 50 μm. (C) Late endosomal and/or lysosomal markers are localized on the giant vacuoles in HeLa cells expressing the Varp protein. HeLa cells expressing Varp were labeled with (a-c) anti-Varp (green) and anti-EEA1 antibodies (red), or (g-i) anti-LAMP3 antibody (red). (d-f) HeLa cells expressing Varp were transfected with an EGFP-Rab7 construct and labeled with an anti-Varp antibody (red). (j-l) HeLa cells expressing EGFP-tagged Varp were labeled with a rabbit anti-p115 antibody (red). Bars, 10 μm. (D) HeLa cells expressing Varp were transfected with an EGFP-Rab21 construct and labeled with an anti-Varp antibody (red). Colocalization of Varp with EGFP-Rab7, LAMP3 or EGFP-Rab21 on the giant endosomes is also shown (arrows). *, giant endosomes induced by Varp. Bars, 10 μm.

membrane of the giant late endosomes in a dot pattern (Fig. 6C,d-f,g-i, arrows). The cis-Golgi marker, p115 (Seemann et al., 2000), showed little or no colocalization with Varp or the giant late endosomes (Fig. 6C,j-l), suggesting that these structures are not be related to the Golgi network. Intriguingly, we observed a significant colocalization of EGFP-Rab21 with Varp on the giant late endosomes (Fig. 6D, arrows), suggesting that Varp recruits Rab21 to the late endosomes. These results suggest that Varp plays an important role in the endosome dynamics in vivo.

Wortmannin treatment disrupts the early endosomal localization of Varp protein

It has been reported that Rabex5 forms a stable complex with Rabaptin-5 in the cytosol and that Rabex5 may be targeted to endosome membrane by Rabaptin-5 (Horiuchi et al., 1997). Since the N-terminus of Varp shares significant homology with Rabex5, we wondered whether Varp uses the same mechanism to target to endosomes. We thus examined whether Varp can interact with Rabaptin-5. We coexpressed Flag-tagged Rabaptin-5 and Myc-tagged Varp or Rabex5 in HEK293T cells and used the whole-cell lysates to perform the immunoprecipitation assay with anti-Myc antibody. The data showed that Varp did not interact with Rabaptin-5, whereas Rabex5 showed a strong interaction with Rabaptin-5 (Fig. 7). This result suggests that Varp and Rebex5 are probably targeted to endosomes through distinct mechanisms.

It has been reported that some endosome proteins such as EEA1 are targeted to early endosomes by binding to phosphatidylinositol 3-phosphate [PtdIns(3)*P*] that is highly enriched on early endosomes (Birkeland and Stenmark, 2004; Simonsen et al., 2001). We examined whether the early endosomal localization of Varp uses a similar mechanism. We used the common phosphoinositide 3-kinase (PI 3-kinase) inhibitor wortmannin to treat HeLa cells expressing Varp protein. As shown in Fig. 8, in DMSO-treated (control) cells, Varp protein colocalized with EEA1 on the early endosomes. However, in wortmannin-treated cells, the early endosomal localization of EEA1 was severely impaired (Fig. 8e), consistent with a previous report (Patki et al., 1997). Importantly, we observed that the early endosomal localization of Varp was also disrupted by wortmannin treatment (Fig. 8,d). These results suggest that the early endosome targeting of Varp protein depends on PI 3-kinase activity and that Varp protein is targeted to the early endosomes through a direct or an indirect interaction with PtdIns(3)*P*.

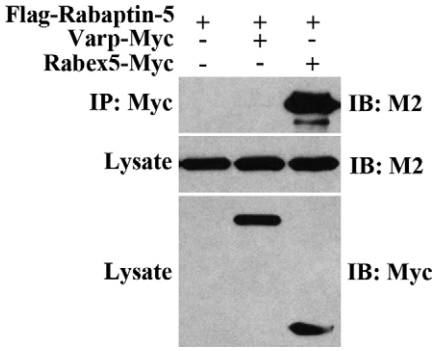

Fig. 7. Varp does not associate with Rabaptin-5. Flag-tagged Rabaptin-5 (Flag-Rabaptin-5) and Myc-tagged Varp (Varp-Myc), or Myc-tagged Rabex5 were coexpressed in HEK293T cells. Whole-cell lysates were immunoprecipitated with an anti-Myc antibody and precipitates were analyzed by western blotting with an anti-Flag M2 antibody. Aliquots of whole-cell lysates were also examined by western blotting with anti-Flag or anti-Myc antibodies.

The activation of Rab21 in cells requires Varp protein

To address the question whether the endogenous Varp protein is relevant for regulating the activation of Rab21 and endosomal morphology, we used a DNA-vector-based small interference RNA (siRNA) method to knock-down the endogenous Varp protein. Western blotting revealed that the endogenous Varp protein was efficiently depleted by a specific human Varp siRNA but not by a control siRNA in HeLa cells (Fig. 9A). In HeLa cells expressing the control siRNA, EGFP-Rab21 was distributed in a punctate pattern and exhibited significant colocalization with EEA1 (Fig. 9B,a-c), which is consistent with a previous report (Simpson et al., 2004). By contrast, in the Varp-depleted HeLa cells, EGFP-Rab21 showed a diffused cytoplasmic distribution and was concentrated in a compact perinuclear pattern (Fig. 9B,d), which is similar to the cellular distribution of EGFP-Rab21 T33N, the inactive GDP-bound mutant (Fig. 9B,g). These results suggest that the endogenous Varp function is required for the activation and endosomal localization of Rab21. We did not observe any overt morphological changes of early endosomes in Varp-depleted HeLa cells (Fig. 9B,b,e).

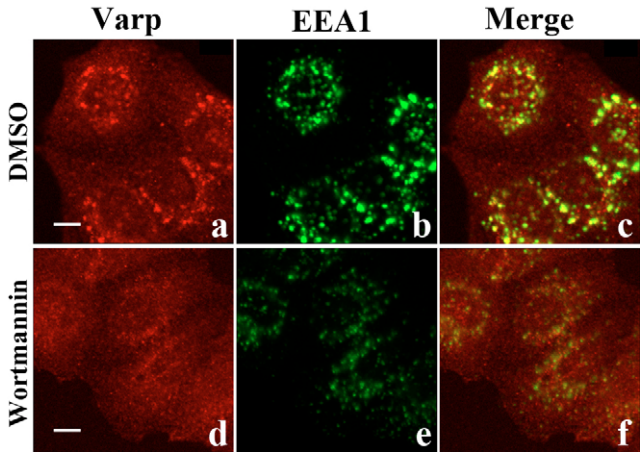

Fig. 8. Wortmannin disrupts the association of Varp protein with the early endosomes. HeLa cells expressing Varp were treated with DMSO or 100 nM wortmannin for 30 minutes at 37°C. The cells were fixed and stained with anti-Varp (red) and anti-EEA1 (green) antibodies. Bars, 10 μm.

Analysis of domains on the localization and function of Varp in cells

To further examine whether the specific domains of the Varp protein are responsible for its subcellular distribution, we generated constructs expressing EGFP-fused Varp and four truncated mutants. In the latter, the C-terminal ankyrin repeats (VarpN), VPS9 domain (Varp/ΔVPS), ankyrin repeats (Varp/ΔANK) or both VPS9 domain and ankyrin repeats (Varp/ΔVPSANK) were deleted (Fig. 10A). We first confirmed the expression of these constructs in cells, by transfecting them into HEK293T cells and detecting their expression by western blotting with anti-GFP or anti-Varp antibodies (Fig. 10B). We transfected each of these constructs into HeLa cells and labeled

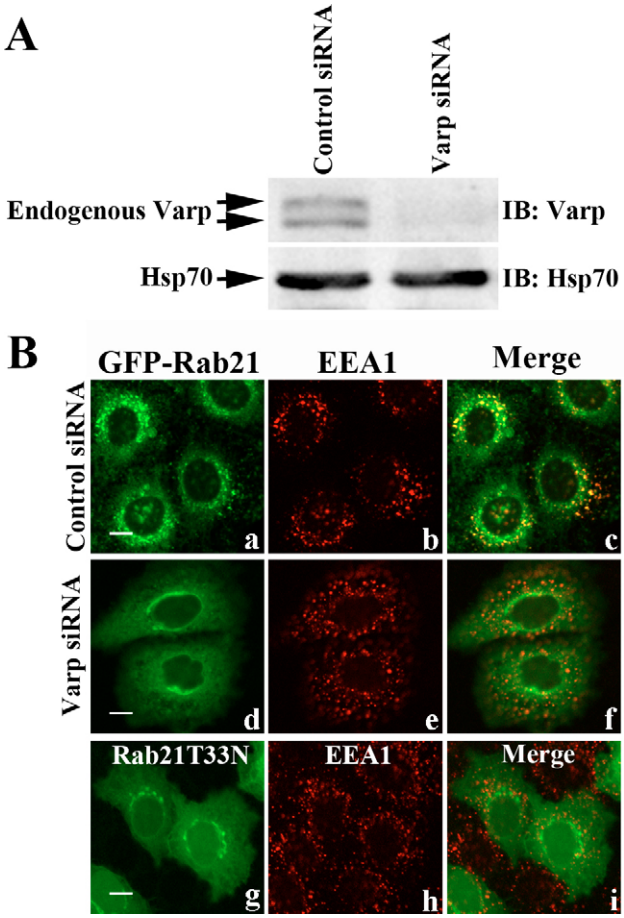

Fig. 9. The activation and endosomal localization of Rab21 depends on the endogenous Varp protein. (A) HeLa cells were transfected with a Varp-siRNA- or control siRNA-expressing construct and the cell lysates were analyzed by western blotting with anti-Varp and anti-Hsp70 antibodies 72 hours post transfection. (B) HeLa cells were transfected with (a-f) indicated siRNA- and EGFP-Rab21 WT-expressing plasmids for 72 hours, and were fixed and labeled with an anti-EEA1 antibody (red). (g-i) HeLa cells expressing EGFP-Rab21 T33N were labeled with an anti-EEA1 antibody. Bars, 10 μm.

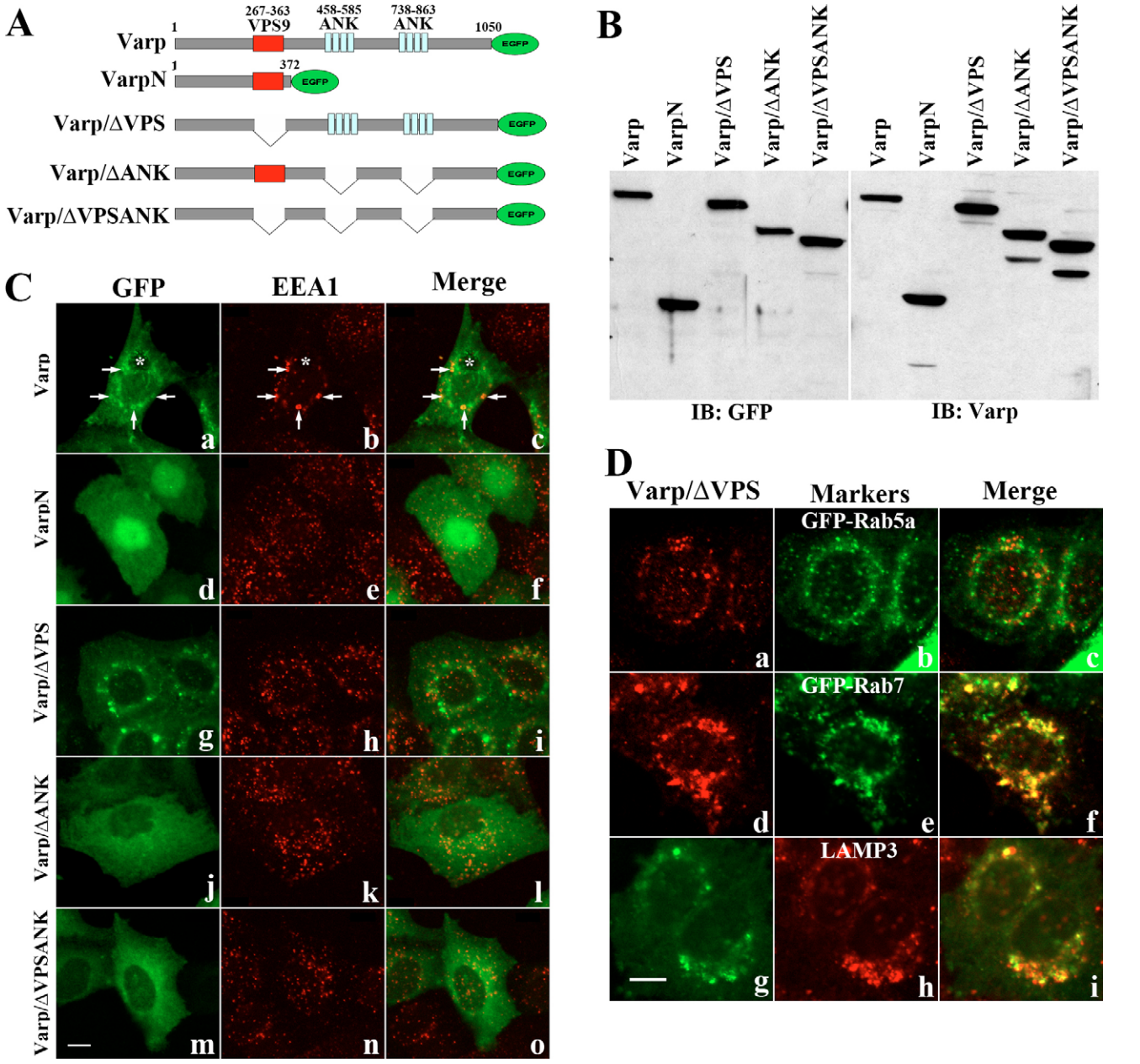

Fig. 10. The VPS9 domain and ankyrin repeats are required for the subcellular localization and activity of Varp in HeLa cells. (A) Schematic diagram of the Varp protein and its mutants used in this experiment. (B) Cell lysates from cells expressing EGFP-fused Varp proteins were analyzed by western blotting with anti-GFP and anti-Varp antibodies. (C) Distribution of Varp and its mutants. HeLa cells ectopically expressing EGFP-fused proteins were fixed and labeled with an anti-EEA1 antibody (red); (c,f,i,l,o) show the co-staining of Varp or mutants with the early endosomal marker EEA1. The effect of the full-length or Varp mutants on the size of early endosomes was also analyzed (b,e,h,k,n). Bars, 10 μm. Arrows indicate the colocalization of Varp and EEA1 on the enlarged early endosomes; *, giant late endosome. (D) Colocalization of Varp/ΔVPS and late endosomal markers. (a-c) HeLa cells transfected with Myc-tagged Varp/ΔVPS and EGFP-tagged Rab5 were stained with an anti-Myc antibody (red). (d-f) HeLa cells transfected with Myc-tagged Varp/ΔVPS and EGFP-tagged Rab7 were stained with anti-Myc antibody (red). (g-i) HeLa cells expressing EGFP-tagged Varp/ΔVPS were stained with an anti-LAMP3 antibody (red). Bars, 10 μm.

the cells with anti-EEA1 antibody. We observed that Varp-EGFP exhibited an early endosomal localization (Fig. 10C,a-c), which is consistent with the previous result from expression of Myc-tagged Varp (Fig. 5A,a-c). Interestingly, we found that none of the four truncated mutant forms of Varp colocalized with EEA1 (Fig. 10C,f,i,l,o). Instead, VarpN showed a diffused distribution in cytoplasm but localized mainly in the nucleus (Fig. 10C,d); Varp/ΔVPS was located on vesicle-like but EEA1-negative structures (Fig. 10C,g) (see below); Varp/ΔANK and Varp/ΔVPSANK showed a diffused distribution in cytoplasm (Fig. 10C,j,m). We also observed that Varp-EGFP induced enlarged early endosomes and giant late

endosomes (Fig. 10C,a-c, arrows indicate the enlarged early endosomes, * represents giant late endosomes), which is consistent with the aforementioned results. However, none of the truncated Varp mutants could induce the enlarged early endosomes and giant late endosomes. Together, these results suggest that the VPS9 domain and ankyrin repeats are both required for the early endosomal localization and function of Varp.

We further studied the vesicular localization of the Varp/ΔVPS mutant. The data showed that Varp/ΔVPS was not colocalized with EGFP-Rab5 (Fig. 10D,a-c). To our surprise, Varp/ΔVPS showed an extensive colocalization with the late endosomal markers EGFP-Rab7 and LAMP3 (Fig. 10D,d-f,g-i), suggesting that Varp/ΔVPS is mainly localized on late endosomal structures. Together with the previous result that Varp/ΔVPSANK distributed diffusedly in cytoplasm (Fig. 10C,m), we suggest that the ankyrin repeats target Varp protein to late endosomes.

Discussion

In this study, we described Varp, a new protein containing a VPS9 domain and ankyrin repeats. Because a VPS9 domain is present in all identified Rab5GEFs and is the catalytic core for GDP-GTP exchanging reaction (Delprato et al., 2004), we reasoned that Varp functions as a GEF for Rab5 and/or related Rab proteins. Indeed, in the in vitro GDP-GTP exchange assays, the N-terminal fragment of Varp containing the VPS9 domain showed a low activity towards Rab5 and a much stronger activity towards Rab21, but no activity towards other Rabs examined. Although Varp associates with both Rab5 and Rab21, Varp shows a much stronger interaction with Rab21 T33N than with Rab5 S34N. We further demonstrated that siRNA-mediated depletion of the endogenous Varp protein significantly abolishes the activity and endosomal localization of Rab21 in HeLa cells. Together, these results suggest that Varp is a specific GEF for Rab21. Rab5 and Rab21 are phylogenetically similar and have related functions (Pereira-Leal and Seabra, 2000; Pereira-Leal and Seabra, 2001; Simpson et al., 2004). Thus, we suggest that Varp preferentially catalyzes Rab21 and distinguishes Rab21 from Rab5.

Although the endocytic pathway has been studied extensively, many key questions remain to be fully understood, including the mechanisms by which cargos are transferred from early to late endosomes. Recent studies revealed that Rab5 and Rab7 were involved in this process (Rink et al., 2005; Vonderheit and Helenius, 2005). It has been reported that the activated Rab5 transiently recruits Rab7 to the early endosomes through the class C VPS-HOPS complex, which is a GEF for Rab7 and an effector of Rab5 (Rink et al., 2005). In the present study, we demonstrate that ectopically expressed Varp is mainly localized to early endosomes in the perinuclear region and induces enlargement of early endosomes and giant late endosomal structures. Varp not only colocalized with EEA1 and Rab5 on the enlarged early endosomes, but also re-distributed the late endosomal marker Rab7 to the enlarged early endosomes. Furthermore, a part of Varp protein also colocalized with Rab7 and LAMP3 on the membranes of the giant late endosomes. Together, these observations suggest that Varp promotes early-endosome fusion and regulates progression from early to late endosomes. Overexpression of the Varp protein in HeLa cells might accelerate membrane trafficking from early to late endosomes causing the accumulation of late endosomal structures, and thus induce the giant late endosomes. The function of Varp might require its GEF activity towards Rab21 (or Rab5 to a lesser degree) because the Varp/ΔVPS mutant failed to induce the enlargement of early endosomes and giant late endosomes. It has been reported that overexpression of the GTPase-deficient Rab5Q79L mutant caused formation of similar giant endosomes (D'Arrigo et al., 1997; Duclos et al., 2000; Rink et al., 2005; Rosenfeld et al., 2001; Stenmark et al., 1994). These and our studies support the notion that overexpression of Varp causes overactivation of Rab21 (or Rab5) on the early endosomes. Our data further suggest a function of Rab21 in the regulation of membrane trafficking between the early and late endosomes, and Varp can activate and recruit Rab21 to the giant late endosomes induced by Varp. This is consistent with a previous report that Rab21 T33N interfered with the transport of the endocytosed EGF ligand to the late endosomes and/or lysosomes for degradation (Simpson et al., 2004).

Endosomal membrane targeting is required for the activity of Rab5GEFs. Rabex5 is targeted to endosomes through an interaction with a Rab5 effector, Rabaptin-5 (Horiuchi et al., 1997). In our experiments, we did not detect any interaction between Varp and Rabaptin-5, suggesting that Varp is targeted to endosomes by a distinct mechanism. Interestingly, we observed that wortmannin, a PI 3-kinase inhibitor, disrupts the early endosomal localization of Varp. This observation suggests that the early endosomal localization of Varp depends on the PI 3-kinase activity, probably by direct or indirect binding of Varp to PtdIns(3)*P*.

We further demonstrate that the VPS9 domain is required for Varp targeting to the early endosomes because the Varp/ΔVPS mutant does not colocalize with early endosomal markers, suggesting that the early endosomal targeting of Varp depends on its GEF activity on Rab21 (and Rab5). The fact that Varp/ΔVPS mainly localizes on the late endosomal structures suggests that Varp can be targeted to late endosomes. Varp has a unique domain structure with eight ankyrin repeats at its C-terminal end. Ankyrin repeats have been found in a number of biologically interesting proteins with a typical function in mediating protein-protein interactions (Mosavi et al., 2004). Ankyrin repeats have been reported to interact with different membrane proteins and to mediate the assembly of multi-protein complexes (Bennett and Chen, 2001; Denker and Barber, 2002). In our experiments, we demonstrated that the ankyrin repeats of Varp are necessary for the endosomal targeting of Varp and Varp/ΔVPS. We speculate that Varp interacts with certain endosomal proteins through its ankyrin repeats. Recently, another ankyrin-repeat-containing protein, Rabankyrin-5, has been characterized as a new Rab5 effector (Schnatwinkel et al., 2004). It has been demonstrated that overexpression of Rabankyrin-5 also induces large vacuolar structures, which were identified as EEA1-positive early endosomes located in the perinuclear region and EEA1-negative macropinosomes located in the periphery of the cells. In the case of Varp, these enlarged vacuolar structures are EEA1-positive early endosomes and Rab7-positive late endosomes, and they are all located in the perinuclear region. Thus, Varp and Rabankyrin-5 might function in related but distinct pathways. In conclusion, we identified a Rab21 guanine nucleotide exchange factor, Varp, and further

characterized its specific functions in regulating endosome dynamics.

Materials and Methods

Reverse transcriptase-PCR

Total cellular RNA was prepared from various mouse tissues, embryos and human cell lines using TRIZOL reagent (Invitrogen). Of each sample, 0.5 μg total RNA was used in a reverse transcriptase (RT)-PCR reaction. The primers for mouse Varp are 5′-GGAAGTCTCTCCACGAAGAC-3′ and 5′-CTTGGGACACTGGCATAAGG-3′. The primers for human Varp are 5′-GAGTCTGAGGGATTTGGAG-3′ and 5′-GGACCGGGAAGCACTAAC-3′.

Antibodies and other reagents

Monoclonal anti-Myc (9E10), anti-LAMP3 and rabbit polyclonal anti-p115 antibodies were purchased from Santa Cruz Biotechnology. Monoclonal anti-EEA1 antibody was from BD Biosciences Pharmingen. Anti-Varp rabbit polyclonal antibody was raised by immunizing rabbits with purified GST-VarpN (N-terminus of Varp) fusion protein and was affinity-purified by an antigen-coupled sepharose column. Fluorescent secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) were purchased from Jackson ImmunoResearch Laboratories. Alexa-Fluor-594-conjugated human transferrin was from Molecular Probes. Wortmannin and monoclonal anti-Flag (M2) antibody were purchased from Sigma.

cDNA constructs

The FLJ00040 clone containing the full-length Varp coding sequence was kindly provided by Takahiro Nagase, Kazusa DNA Research Institute, Chiba, Japan. We first constructed pcDNA3.1/Varp-Myc, pcDNA3.1/VarpN-Myc, pcDNA3.1/Varp/ΔVPS-Myc, pcDNA3.1/Varp/ΔANK-Myc, pcDNA3.1/Varp/ΔVPSANK-Myc, pcDNA3.1/Varp-Flag and pcDNA3.1/VarpN-Flag plasmids by applying a PCR-based strategy using the FLJ00040 clone. For better expression in mammalian cells, we subcloned Varp-Myc, VarpN-Myc, Varp-Flag and VarpN-Flag from pcDNA3.1 vector into pEFNeo vector. For expression of EGFP fused proteins, we subcloned Varp, VarpN, Varp/ΔVPS, Varp/ΔANK and Varp/ΔVPSANK into pEGFP-N1 vector. pEFNeo/Rabex5-Myc and pEFNeo/Rabex5-Flag plasmids were constructed using the same strategy from pCR2.1-TOPO/Rabex5 (a gift from Oliver Mueller, Max-Planck-Institut für Molekulare Physiologie, Dortmund, Germany). pEGFP-C1/EGFP-Rab5a WT, Q79L and S34N plasmids were gifts from Brian J. Knoll, University of Houston. pGEX-4T1/Rab15, Rab21 and Rab22 were gifts from David G. Lambright, University of Massachusetts Medical School, Worcester, MA. pEGFP-C1/EGFP-Rab7, pGEX-4T1/Rab4, Rab5a and Rab7 were constructed by RT-PCR based strategy. For expression and purification of His_6-Rab5a in bacteria, we subcloned Rab5a from pEGFP-C1/EGFP-Rab5a into pET30a vector. From Elena Korobko, we received the pFLAG-Rabaptin-5 plasmid to express Flag-tagged Rabaptin-5 in mammalian cells. pEGFP-C1/EGFP-Rab21 WT, Q78L and T33N were gifts from Arwyn T. Jones, Cardiff University, Cardiff, UK. For constructing Varp siRNA expressing plasmid, we first synthesized two single-strand DNA fragments: 5′-ATGTGTCACCCTCTCTGCTTCAAGAGAGCAGAGAGGGTGACACATCTTTTTTG-3′ and 5′-AATTCAAAAAAGATGTGTCACCCTCTCTGCTCTCTTGAAGCAGAGAGGGTGACACAT-3′ [The underlined sequence is the RNAi target of the human Varp gene (nt 1287-1305)]. These two DNA fragments were annealed and cloned into the *Apa*I-*Eco*RI site of pBSU6 vector (a gift from Yang Shi, Harvard University, Boston, MA). We also construct a control siRNA expressing construct (the target sequence is GAGACATATCTAATGTGCC, which has no significant sequence similarity to human and mouse gene sequences).

Cell culture and transfection

HeLa and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin and 100 mg/ml streptomycin. HeLa cells were transfected with plasmid constructs using LipofectAMINE 2000 (Invitrogen). HEK293T cells were transfected by using the calcium phosphate method. For establishing stable HeLa cell lines expressing Myc-tagged Varp, VarpN or Rabex5, cells were transfected with the respective plasmids and single clones were selected in culture medium containing 1 mg/ml neomycin (G418). Two clones of each stable cell line were picked for functional studies.

Protein purification and in vitro GDP-GTP exchange assay

GST-Rab4, 5a, 7, 15, 21, 22 and His_6-Rab5a were expressed in *E. coli* BL21 (DE3) pLys S (Novagen) and purified with glutathione-SepharoseTM 4B beads (Amersham Pharmacia) or Ni-NTA-agarose beads (Qiagen). For purification of Flag-tagged Varp, VarpN and Rabex5, transfected HEK293T cells were solubilized in cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1.4 μg/ml pepstatin A and 0.5% (v/v) NP-40) and the supernatant was immunoprecipitated with an anti-Flag M2 antibody. Flag-tagged fusion proteins were eluted with 0.5 mg/ml Flag peptide.

In vitro GDP-GTP exchange assays were performed as described previously (Kajiho et al., 2003; Otomo et al., 2003). For the [^{3}H]GDP dissociation assay, purified His_6-Rab5a (100 nM) that had been preloaded with 5 μM [^{3}H]GDP (370 Gbq/mmol; PerkinElmer) at 30°C for 30 minutes was incubated in the presence or absence of 100 nM Flag-tagged Varp, VarpN or Rabex5 in a reaction mixture (50 μl) consisting of 25 mM Tris-HCl pH 7.5, 70 mM NaCl, 0.25% (w/v) CHAPS, 1 mM unlabelled GDP, 100 μM [γ-^{35}S]GTP, 5 mM EDTA and 20 mM $MgCl_2$ at 30°C for 60 minutes. For the [γ-^{35}S]GTP binding assay, purified His_6-Rab5a or GST-Rab proteins (25 nM) was incubated with 10 nM [γ-^{35}S]GTP (37 Tbq/mmol; Amersham Pharmacia) at 30°C for 60 minutes in the presence or absence of 25 nM Flag-tagged Varp, VarpN or Rabex5 in a reaction mixture (50 μl) consisting of 25 mM Tris-HCl pH 7.5, 70 mM NaCl, 1 mM DTT, 0.25% (w/v) CHAPS, 500 nM GDP, 5 mM EDTA and 20 mM $MgCl_2$ at 30°C for 60 minutes. Reactions were terminated by adding 900 μl ice-cold STOP buffer (25 mM Tris-HCl pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$), and were filtered through BA85 nitrocellulose filters (Schleicher & Schell). The radioactivity trapped on the filters was counted.

Coimmunoprecipitation and immunoblotting

For coimmunoprecipitation assays, HEK293T cells in 60-mm dishes were transfected with the indicated plasmids and were lysed in 800 μl cell lysis buffer. 500 μl whole-cell lysates were incubated with 2 μg of indicated monoclonal antibody and 30 μl protein G Sepharose beads at 4°C for 4 hours. The beads were washed 4 times with cell lysis buffer and precipitates were eluted with 2× SDS-PAGE sample buffer and analyzed by western blotting.

Immunostaining and confocal microscopy

Cells growing on glass cover slips in 6-well clusters were washed with PBS, fixed for 20 minutes at room temperature with 4% paraformaldehyde in PBS, and permeabilized for 10 minutes with 0.2% Triton X-100 in PBS. Cells were blocked with 10% normal goat serum (NGS) for 45 minutes at room temperature. Primary antibodies diluted in 3% BSA-PBS were incubated for 2 hours at room temperature and bound antibodies were detected with FITC- or TRITC-conjugated goat anti-rabbit or anti-mouse IgG. Cover slips were mounted in a glycerol-based anti-fade mounting medium and analyzed with a laser scanning confocal microscopy with a 60× oil-immersion objective.

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