Vps4-A (vacuolar protein sorting 4-A) is a binding partner for a novel Rho family GTPase, Rnd2

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Rho family GTPases are implicated in a variety of biological activities, including endocytic vesicle trafficking. Rnd2 is a new member of Rho family GTPases, but its biological functions are not known. In the present study, we have performed a yeast two-hybrid screening using Rnd2 as bait and revealed that Rnd2 binds specifically to Vps4-A (where Vsp4-A is vacuolar protein sorting 4-A), a member of the AAA ATPase family and a central regulator for early endosome trafficking. This interaction was determined by the yeast two-hybrid system, *in vitro* binding and co-immunoprecipitation studies. Vps4-A associated with both guanosine 5′-[β-thio]triphosphate-bound active and guanosine 5′-[β-thio]diphosphate-bound inactive forms of Rnd2. An ATPase-defective Vps4-A mutant, Vps4-A^{E228Q}, expressed in HeLa cells was accumulated in the early endosomes. When Rnd2 was co-expressed with Vps4-A^{E228Q}, Rnd2 was recruited to the Vps4-A-bound early endosomes. These results suggest that Rnd2 is involved in the regulation of endosomal trafficking via direct binding to Vps4-A.

Key words: GTP-binding protein, small G-protein, vesicle trafficking.

INTRODUCTION

Vesicle trafficking provides protein transport through the secretory and endocytic pathways involved in biosynthesis, degradation and internalization. Within this membrane-defined network, endosomes function as intermediate sorting sites that link trafficking among the *trans*-Golgi, lysosomes and plasma membranes [1]. Endosomes are functionally classified into two types, early endosomes and late endosomes. After endocytosis, internalized macromolecules are first delivered to early endosomes and then sorted either back to cell surface or to lysosomes via late endosomes. Class E vacuolar protein sorting (Vps) proteins play important roles in appropriate sorting of materials bound for the lysosomes away from proteins that cycle through the endocytic system. Among class E Vps proteins, yeast Vps4p has been shown to play an important role in the morphological and functional organization of the endocytic pathway and to be required for efficient transport from early to late endosomes [2,3]. Vps4p is a member of the AAA ATPase family and ATP binding regulates the association of Vps4p with an endosomal compartment [4]. Vps4p catalyses the release of an endosomal membrane-associated class E protein complex(es) required for sorting activity of the endosome [4].

Rho family GTPases are implicated in a variety of biological activities, including regulation of cell morphology, transcription control and endocytic vesicle trafficking [5]. Like other GTPases of the Ras superfamily, they serve as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound state and, once activated, they can interact with their specific effectors, leading to a variety of biological functions. Activation of the Rho family proteins requires GDP–GTP exchange, catalysed by various guanine nucleotide exchange factors, and their activation is regulated by GTPase-activating proteins, which stimulate the intrinsic GTPase activities of the G proteins. Presently, at least 14 mammalian Rho family proteins have been identified: Rho (A, B and C), Rac (1, 2 and 3), Cdc42, RhoD, RhoH/TTF, TC10 and Rnd (1, 2 and 3). Among them, the functions of Rho, Rac and Cdc42 have been characterized extensively. With respect to endocytosis, it has been reported that constitutively active Rho and Rac inhibit transferrin-receptor-mediated endocytosis in HeLa cells [6], and that dominant negative Cdc42 abrogates endocytosis in dendritic cells [7]. In addition, RhoD has been shown to govern early endosome motility and distribution [8]. Therefore several Rho family GTPases participate in the regulation of endocytosis, but the contribution of other Rho family GTPases and the molecular mechanisms remain elusive.

Rnd proteins, Rnd1, Rnd2 and Rnd3, comprise a distinct branch of Rho family GTPases [9–11]. Expression of Rnd1 and Rnd3 in fibroblasts results in the loss of Rho-mediated actin stress fibres and focal adhesions, indicating the antagonistic effect on the Rho-regulated signalling pathway [11]. In contrast with Rnd1 and Rnd3, the biological functions of Rnd2 remain to be elucidated. In the present study, we show the interaction of Rnd2 with Vps4-A as determined by the yeast two-hybrid system, *in vitro* binding and co-immunoprecipitation studies. Furthermore, we show that Rnd2 is targeted to the Vps4-A-bound early endosome. Our results suggest a new role for Rnd2 in vesicular sorting.

MATERIALS AND METHODS

Plasmid construction

Wild-type Rnd1, Rnd2, Rnd3, RhoA, Rac1 and Cdc42 were obtained as described previously [12–14]. For the yeast two-

Abbreviations used: DTT, dithiothreitol; EEA1, early endosome antigen 1; GDP[S], guanosine 5′-[β-thio]diphosphate; GST, glutathione S-transferase; GTP[S], guanosine 5′-[γ-thio]triphosphate; HA, haemagglutinin; Vps, vacuolar protein sorting.

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hybrid construct, the CAAX motif (where C represents cysteine, A represents an aliphatic amino acid and X represents any amino acid) of Rnd2 was inactivated by a substitution of the cysteine residue with serine ($Rnd2^{C224S}$), introduced by PCR, and was fused to the GAL4 DNA-binding domain ('GAL4-BD') of the pAS2-1 vector (Clontech). For purification of recombinant proteins, cDNAs encoding wild-type RhoA, Rac1 and Cdc42 and full-length Vps4-A and its deletion mutants, synthesized by PCR, were subcloned into pGEX-4T-2 (Amersham Biosciences), whereas Rnd2 cDNA was subcloned into pAcG2T (PharMingen). For expression in mammalian cells, cDNAs encoding Vps4-A, Vps4-A^{E228Q}, Rnd1, Rnd2 and Rnd3 were fused in-frame with a sequence in pcDNA3 (Invitrogen) encoding an initiating methionine residue followed by a Myc epitope or haemagglutinin (HA) tag sequence at the N-terminus.

Yeast two-hybrid screening

A rat brain cDNA library fused to the GAL4 activation domain ('GAL4-AD') of the pACT2 vector (Clontech) was screened using pAS2-1-$Rnd2^{C224S}$ as a bait in the yeast strain Y190, according to the manufacturer's instructions. Interaction between the bait and library proteins activated transcription of the reporter gene *HIS3* or *lacZ*. Approx. 1.5×10^7 yeast colonies were screened for their ability to grow on selective medium lacking histidine, leucine, tryptophan and uracil, and containing 10 mM 3-aminotriazole. Colonies that grew successfully were replated and screened using the β-galactosidase assay. From the positive yeast colonies, prey plasmids were isolated. The yeast Y190 was then co-transformed with these cDNAs and pAS2-1-$Rnd2^{C224S}$ to confirm the interaction. Positive clones were sequenced using an ABI PRISM 310 DNA sequencer.

Recombinant proteins

Glutathione S-transferase (GST)-fused GTPases and Vps4-A and its deletion mutant proteins were purified from *Escherichia coli* as described previously [13]. GST–wild-type Rnd2 protein was purified from Sf9 cells as described previously [13]. Protein concentration was determined by comparing with BSA standards after SDS/PAGE and staining with Coomassie Brilliant Blue. For *in vitro* binding assays, recombinant GTPases were loaded with guanine nucleotides by incubation with 200 μM guanosine 5′-[γ-thio]triphosphate (GTP[S]) or 2 mM guanosine 5′-[β-thio] diphosphate (GDP[S]) in loading buffer [20 mM Tris/HCl (pH 7.4), 1 mM dithiothreitol (DTT) and 4 mM EDTA] at 30 °C for 30 min. The reaction was stopped by the addition of $MgCl_2$ to a final concentration of 20 mM.

Immunoblotting

Proteins were separated by SDS/PAGE [10 % (w/v) gel] and electrophoretically transferred on to a PVDF membrane (Millipore Corporation). The membrane was blocked with 5 % (w/v) low-fat milk in Tris-buffered saline, and then incubated with primary antibodies. The primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (DAKO) and the ECL® detection kit (Amersham Biosciences). Mouse monoclonal anti-Myc (9E10; Santa Cruz Biotechnology) and anti-HA antibodies (12CA5; Boehringer Mannheim) were used at 1:100 and 1:200 dilutions respectively.

In vitro binding assays

COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in a humidified atmosphere containing 5 % CO_2. Transient transfections were carried out using LIPOFECTAMINE 2000 (Life Technologies) according to the manufacturer's instructions. Cells transfected with HA-tagged G-proteins or Myc-tagged Vps4-A were rinsed once with PBS and lysed with ice-cold cell lysis buffer [20 mM Tris/HCl (pH 7.4), 2 mM $MgCl_2$, 1 mM DTT, 0.2 % Triton X-100, 1 mM PMSF, 2 μg/ml aprotinin and 2 μg/ml leupeptin]. Cell lysates were then centrifuged at 18000 ***g*** for 10 min at 4 °C. The supernatants were incubated for 1 h at 4 °C with 2 μg of GST-fusion proteins and subsequently incubated with glutathione–Sepharose beads for 2 h at 4 °C. After the beads were washed with ice-cold lysis buffer, the bound proteins were eluted in Laemmli sample buffer and analysed by SDS/PAGE and immunoblotting with anti-HA or anti-Myc monoclonal antibodies.

To examine the direct interaction between Rnd2 and Vps4-A, an overlay assay was performed as described by the modified method of Manser et al. [15]. GST–Vps4-A and its deletion mutant proteins (2 μg) were subjected to SDS/PAGE and transferred on to a nitrocellulose membrane. The membrane was soaked for 5 min in 6 M guanidinium hydrochloride dissolved in buffer A [25 mM Hepes/NaOH (pH 7.0), 0.5 mM $MgCl_2$ and 0.05 % Triton X-100] at 4 °C. The buffer was diluted with an equal volume of buffer A and agitated for a further 3 min. This process was repeated five times. The membrane was then agitated five times for 10 min each in buffer A, transferred to PBS containing 1 % (w/v) BSA, 0.1 % Triton X-100, 0.5 mM $MgCl_2$ and 5 mM DTT, and incubated in buffer B [25 mM Hepes/NaOH (pH 7.0), 5 mM $MgCl_2$, 0.05 % Triton X-100, 2.5 mM DTT and 100 μM GTP] containing [γ-^{32}P]GTP (6000 Ci/mmol; NEN Life Science Products)-loaded GST–Rnd2. After washing with buffer B without GTP and DTT, the membrane was dried and bound radioactivity was visualized using an FLA-3000 image analyser (Fuji). GST–Rnd2 (1 μg) was loaded with [γ-^{32}P]GTP for 1 h as described above.

Immunoprecipitation

COS-7 cells co-transfected with Myc-tagged Vps4-A and HA-tagged Rnd proteins were lysed with ice-cold cell lysis buffer. After centrifugation, the supernatants were incubated with an anti-HA polyclonal antibody (MBL) for 1 h and then with Protein A–Sepharose (Amersham Biosciences) for 1 h. The beads were washed with the lysis buffer and bound proteins were analysed by SDS/PAGE and immunoblotting.

Immunofluorescence microscopy

HeLa cells were cultured under the same condition as COS-7 cells described above. Cells were seeded on to round 13-mm glass coverslips in 24-well plates at a density of 2×10^4 cells/well. Transient transfections were carried out using SuperFect (Qiagen) according to the manufacturer's instructions. All steps for immunofluorescence microscopy were carried out at 20 °C, and the cells were rinsed with PBS between each step. Cells on coverslips were fixed with PBS containing 3.7 % formaldehyde for 15 min. After residual formaldehyde had been quenched with PBS containing 50 mM NH_4Cl for 10 min, cells were permeabilized with PBS containing 0.2 % Triton X-100 for 10 min and incubated with PBS containing 10 % (v/v) fetal bovine serum for 30 min to block non-specific antibody binding. For detection of Myc-tagged Vps4-A^{E228Q}, cells were incubated with a rabbit anti-Myc polyclonal antibody (1:500 dilution; MBL) diluted in PBS for 1 h, followed by incubation with either rhodamine-conjugated or FITC-conjugated donkey anti-(rabbit

IgG) antibodies (1:500 dilution; Chemicon International) diluted in PBS for 1 h. For detection of HA-tagged Rnd2, cells were incubated with a rat anti-HA monoclonal antibody (3F10; 1:250 dilution; Roche) diluted in PBS for 1 h, followed by incubation with a rhodamine-conjugated goat anti-(rat IgG) antibody (1:500 dilution; Chemicon International) diluted in PBS for 1 h. Early endosome antigen 1 (EEA1) was visualized with a mouse anti-EEA1 monoclonal antibody (1:100 dilution; Transduction Laboratories), followed by a FITC-conjugated donkey anti-(mouse IgG) antibody (1:500 dilution; Chemicon International). Cells on coverslips were mounted in 90% (v/v) glycerol containing 0.1% *p*-phenylenediamine dihydrochloride in PBS. Confocal microscopy was performed using an MRC-1024 laser scanning confocal imaging system (Bio-Rad Laboratories) equipped with a Nikon Eclipse E800 microscope and a Nikon Plan Apo 40×1.0 or 60×1.4 oil-immersion objective.

RESULTS

Interaction between Rnd2 and Vps4-A

To isolate Rnd2-interacting proteins, we employed a yeast two-hybrid screening of a rat brain cDNA library using Rnd2^{C224S} as a bait. Several positive clones were isolated, and sequence analyses revealed that one of these encoded the full-length of rat Vps4-A. The interaction between Rnd2 and Vps4-A was specific, since neither the Rnd2^{C224S} nor Vps4-A clone alone activated transcription of *HIS3* and *lacZ* in yeast (results not shown). Furthermore, among the three Rnd subfamily GTPases and the well-studied Rho family GTPases, RhoA, Rac1 and Cdc42, Vps4-A only specifically interacted with Rnd2 (results not shown).

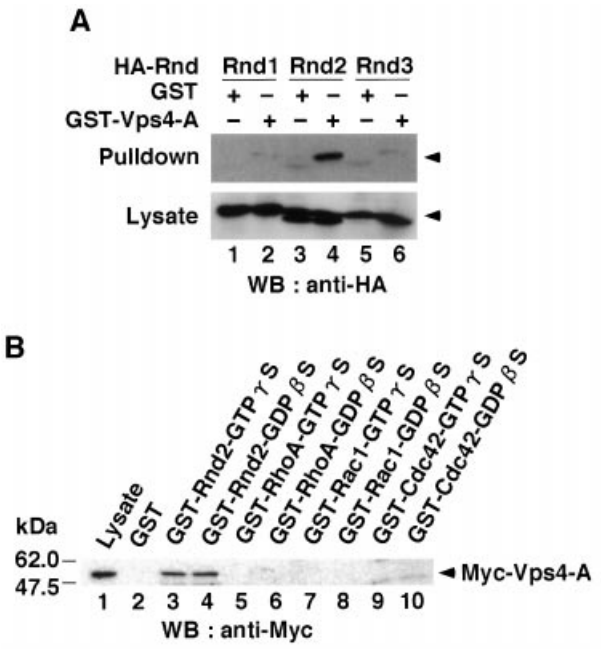


Figure 1 ***In vitro*** **interaction of Rnd2 with Vps4-A**

(**A**) COS-7 cells were transiently transfected with an expression vector encoding HA-tagged Rnd1 (lanes 1 and 2), Rnd2 (lanes 3 and 4) or Rnd3 (lanes 5 and 6) and the cells were lysed. The cell lysates were incubated with either GST (lanes 1, 3 and 5) or GST–Vps4-A (lanes 2, 4 and 6) and then immobilized by incubation with glutathione–Sepharose beads. Bound G-proteins (pulldown; upper panel) and untreated lysate from transfected cells (lower panel) were analysed by immunoblotting (WB) using an anti-HA monoclonal antibody. (**B**) COS-7 cells were transiently transfected with an expression vector encoding Myc-tagged Vps4-A and the cells were lysed. The cell lysates were then incubated with GST alone (lane 2), GST–Rnd2 (lanes 3 and 4), GST–RhoA (lanes 5 and 6), GST–Rac1 (lanes 7 and 8) or GST–Cdc42 (lanes 9 and 10) preloaded with 200 μM GTP[S] (lanes 3, 5, 7 and 9) or 2 mM GDP[S] (lanes 4, 6, 8 and 10), and then immobilized on glutathione–Sepharose beads. Bound Vps4-A and an untreated lysate from transfected cells (lane 1) were analysed by immunoblotting using an anti-Myc monoclonal antibody.

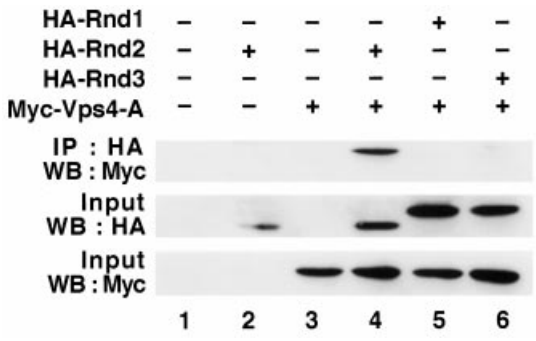


Figure 2 ***In vivo*** **interaction of Rnd2 with Vps4-A**

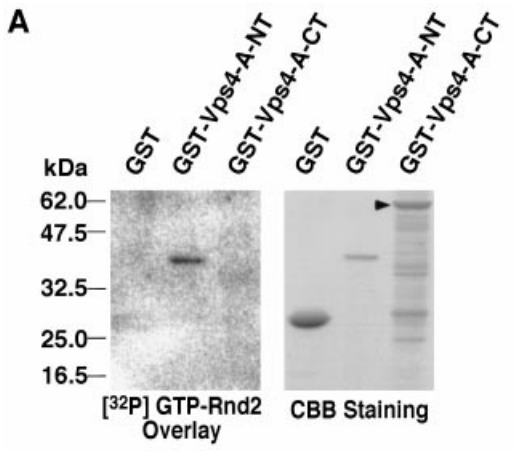
COS-7 cells were transiently transfected with an empty vector (lane 1), expression vectors encoding either HA-tagged Rnd2 (lane 2) or Myc-tagged Vps4-A (lane 3) alone, or co-transfected with Myc-tagged Vps4-A and either HA-tagged Rnd1 (lane 5), Rnd2 (lane 4) or Rnd3 (lane 6). Following transfection for 48 h, cell lysates were immunoprecipitated (IP) with an anti-HA polyclonal antibody. Myc-tagged Vps4-A in the immunoprecipitates (top panel) or the untreated lysate from transfected cells (input; bottom panel) were analysed by immunoblotting (WB) using an anti-Myc antibody. HA-tagged G-proteins in the untreated lysates from transfected cells (input; middle panel) were analysed by immunoblotting with an anti-HA monoclonal antibody.

To verify the interaction between Rnd2 and Vps4-A *in vitro*, HA-tagged wild-type Rnd1, Rnd2 and Rnd3 were expressed in COS-7 cells and pull-down assays were performed using purified GST–Vps4-A. As shown in Figure 1(A), wild-type Rnd2 strongly interacted with Vps4-A, whereas neither Rnd1 nor Rnd3 bound to Vps4-A. We examined further the interaction of Vps4-A with active and inactive forms of wild-type Rnd2 and both forms of other well-studied Rho family GTPases, including RhoA, Rac1 and Cdc42. GST–GTPases preloaded with GTP[S] or GDP[S] were incubated with lysates of COS-7 cells expressing Myc-tagged Vps4-A. Vps4-A interacted with both the GTP[S]-loaded active and GDP[S]-loaded inactive forms of Rnd2, whereas Vps4-A did not interact with any forms of RhoA, Rac1 or Cdc42 (Figure 1B). We performed further immunoprecipitation assays to determine the *in vivo* interaction between Vps4-A and Rnd2. Consistent with the result of the *in vitro* pull-down assay, Rnd2, but not Rnd1 and Rnd3, co-immunoprecipitated with Vps4-A (Figure 2).

To determine the direct interaction between Vps4-A and Rnd2 and the Rnd2-interaction domain in Vps4-A, an overlay assay was performed using Rnd2 preloaded with [γ-^{32}P]GTP as a probe. As shown in Figure 3(A), [γ-^{32}P]GTP-loaded Rnd2 showed strong binding to the N-terminal region (amino acids 2–127), but not to the C-terminal ATPase region (amino acids 128–437). This interaction of Rnd2 with the N-terminal region was also confirmed by the yeast two-hybrid analysis (Figure 3B). Therefore Rnd2 binds directly to Vps4-A and this binding domain is located in the N-terminal region of Vps4-A.

Localization of Rnd2 and Vps4-A to early endosomes

Rnd2 binds to Vps4-A. Vps4p is involved in the invagination of multivesicular bodies and sorting of proteins into these invaginations [1]. We then examined the subcellular localization of Rnd2



B

GAL4-BD/	GAL4-AD/	β-Gal Assay
$Rnd2^{C224S}$	Vps4-A-FL	+
$Rnd2^{C224S}$	Vps4-A-NT	+
$Rnd2^{C224S}$	Vps4-A-CT	−
$Rnd2^{C224S}$	None	−

Figure 3 Direct binding of Rnd2 to the N-terminal region of Vps4-A

(**A**) GST, GST fused to the N-terminal region of Vps4-A (Vps4-A-NT; amino acids 2–127) and GST fused to the C-terminal region of Vps4-A (Vps4-A-CT; amino acids 128–437) were subjected to SDS/PAGE, and the proteins were transferred on to nitrocellulose membranes. Membranes were then probed with [γ-^{32}P]GTP-labelled GST–Rnd2 (Overlay; left-hand panel) as described in the Materials and methods section. GST and GST-fused N- and C-terminal regions of Vps4-A used in this experiment were shown by staining with Coomassie Brilliant Blue (CBB; right-hand panel). The arrow indicates the product of the GST-fused C-terminal region of Vps4-A. Molecular-mass markers (in kDa) are indicated on the left. (**B**) The yeast two-hybrid interaction assay between Rnd2 and Vps4-A was performed as described in the Materials and methods section. The interaction was determined by β-galactosidase (β-Gal) filter lift assay and the results are shown as positive (+) or negative (−) interactions. GAL4-BD, GAL4-binding domain; GAL4-AD, GAL4 activating domain.

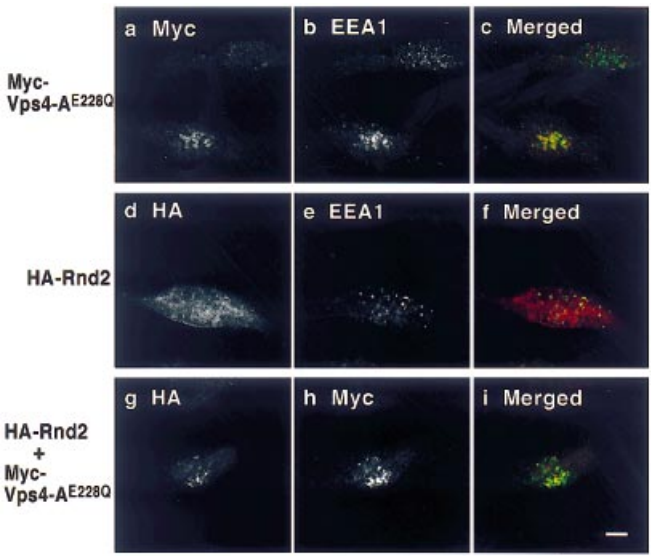


Figure 4 Co-localization of Rnd2 and Vps4-A to early endosomes

HeLa cells were transiently transfected with an expression vector encoding Myc-tagged Vps4-A^{E228Q} (**a**–**c**), HA-tagged Rnd2 (**d**–**f**) or both (**g**–**i**). Following transfection for 24 h, cells were fixed and co-stained with an anti-EEA1 monoclonal antibody (**b** and **e**; green staining in **c** and **f**) and either an anti-Myc polyclonal antibody (**a**; red staining in **c**) or an anti-HA monoclonal antibody (**d**; red staining in **f**). Cells co-transfected with Myc-tagged Vps4-A^{E228Q} and HA-tagged Rnd2 were co-stained with anti-HA monoclonal (**g**; red staining in **i**) and anti-Myc polyclonal (**h**; green staining in **i**) antibodies. Note that red/green overlap leads to yellow in merged images. The scale bar represents 20 μm.

and Vps4-A in HeLa cells. It has been shown previously that wild-type human Vps4 has a mainly cytosolic localization, but a substantial fraction of the ATPase-defective mutant $Vps4^{E228Q}$ was localized to membranes [16,17]. In cells transiently expressing Vps4-A^{E228Q}, the mutant was accumulated in punctate structures, overlapping with the fluorescent signal of EEA1 (Figures 4a–c), an established marker for early endosomes [18]. On the other hand, transiently expressed wild-type Vps4-A was distributed diffusely in the cytosol. We next examined the subcellular localization of Rnd2 in the presence or absence of Vps4-A^{E228Q}. When cells were transiently transfected with Rnd2 alone, Rnd2 was distributed diffusely throughout the cell with the EEA1-positive small punctates (Figures 4d–f). When cells were co-transfected with Rnd2 and Vps4-A^{E228Q}, both proteins were, in part, co-localized in the punctate structures (Figures 4g–i). Therefore Rnd2 was recruited to the Vps4-A^{E228Q}-bound early endosomes.

DISCUSSION

Rnd2 is a new member of Rho family GTPases, but its biological functions remain to be elucidated. In the present study, we have described the specific binding of Rnd2 to Vps4-A and the targeting of Rnd2 to the Vps4-bound early endosome.

Vps4 is a member of the AAA ATPase family. Mammalian Vps4 comprises Vps4-A and Vps4-B (also known as SKD1), displaying a high degree of sequence identity with each other (80 %) [19]. Yeast Vps4p forms a homomeric complex, whereas Vps4-A and Vps4-B have been proposed to form heteromeric complexes [4,19]. Vps4p is recruited from the cytosol to the membranes and associates with class E Vps protein complexes [4]. Vps4p then hydrolyses ATP and disassembles the class E Vps protein complexes bound to the cytoplasmic face of early endosomes, leading to invagination of multivesicular bodies [1]. When ATP hydrolysis is prevented by the introduction of a point mutation in the ATPase domain of Vps4p, the mutated Vps4p stably associates with class E Vps protein complexes on the membranes and blocks the dissociation of the complexes [4]. The interaction of class E Vps proteins with mammalian Vps4 has also been shown [20,21], and the ATPase-defective mutant of mammalian Vps4 has been reported to cause severe endosomal abnormalities and sorting defects [16,17]. Thus the ATPase-defective mutant of Vps4 acts as a dominant negative form. Consistent with a previous report [16], the expression of wild-type Vps4-A shows a cytosolic distribution, whereas the ATPase-defective mutant Vps4-A^{E228Q} is accumulated in the early endosomes and induces significant enlargement of the early endosomes in HeLa cells, indicating that Vps4-A plays an important role in the functions of endosomes and ATPase activity of Vps4-A is crucial for endosomal morphology.

In the present study, we have demonstrated that Vps4-A is a physical binding partner of Rnd2, as measured by the yeast two-hybrid system, *in vitro* binding and co-immunoprecipitation studies. Vps4-A interacted with both GTP[S]-bound active and GDP[S]-bound inactive forms of Rnd2. When Rnd2 is co-expressed with Vps4-A^{E228Q}, which is stably associated with

the early endosomes, Rnd2 is recruited to the Vps4-A^{E228Q}-bound early endosomes. Thus Vps4-A does not appear to be a downstream effector, but rather acts as a linker protein for Rnd2. Vps4 binds directly to class E Vps proteins, including Vps23, Vps28 and chromatin modifying protein 1 ('CHMP1'), and regulates assembly and disassembly of these class E protein complexes formed on early endosomes and their recycling between the cytosol and early endosomes, which are essential for the vesicular sorting from early endosomes [20,21]. Similar to the regulation of class E Vps proteins, Vps4 may recruit Rnd2 to the early endosomes and insert Rnd2 into the regulatory system for vesicular sorting from the early endosomes.

In conclusion, we have shown that Vps4-A is a binding partner for Rnd2, a novel Rho family GTPase, and Rnd2 is recruited to the Vps4-A-bound early endosomes. This is the first report for a direct link between Rho family GTPases and the endocytic machinery. However, many questions have not yet been answered concerning Rnd2, including, for example, the identification of effectors for Rnd2. Further studies focusing on the identification of Rnd2-interacting proteins will contribute to the understanding of Rnd2 signalling and regulation of endocytosis.

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