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Varp interacts with Rab38 and functions as its potential effector

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ABSTRACT

Varp, a novel protein containing a VPS9 domain and ankyrin repeats, can function as a guanine nucleotide exchange factor (GEF) of Rab21. We previously reported that Varp plays an important role in the regulation of endosome dynamics. To further investigate the function of Varp, a yeast two-hybrid screen was performed and Rab38 was identified as a Varp-associated protein. We demonstrate that Varp physically interacts with Rab38, and preferentially binds to the active GTP-bound form of Rab38 both *in vitro* and *in vivo*. Furthermore, Varp was shown to be recruited to Rab38-positive organelles in an ankyrin-repeat 1 (ANK1)-dependent manner. Our data demonstrate that Varp is a potential effector of Rab38. Together with our previous study, we propose Varp serves as both an effector and a GEF by interacting with different Rabs in mammalian cells.

The Rab GTPases belong to the superfamily of small GTPases and have emerged as central regulator of cell functions, influencing numerous cellular processes such as cell growth/differentiation, cytoskeletal configuration, intracellular vesicle budding, motility and fusion [1–4]. To date, more than 60 members of the human Rab family have been identified [5]. Each Rab protein shows a characteristic subcellular distribution and may thus represent an important determinant of organelle identity [6–8]. The Rab GTPases function as molecular switches by cycling between an inactive (GDP-bound) and an active (GTP-bound) form, with different conformations [9,10]. The GDP-bound Rabs interact with Rab escort protein (REP) and GDP dissociation inhibitor (GDI) [11,12], and are maintained in the cytosol [13]. Upon targeting to a specific membrane, Rab proteins are dissociated from GDI by a GDI displacement factor (GDF) [14] and GTP binds to the Rab in a reaction catalyzed by a guanine nucleotide exchange factor (GEF) [6,15]. The active membrane-bound Rabs are then able to recruit their specific effectors and fulfill their various functions in membrane trafficking [4,16,17]. Rab effectors are recruited by Rab proteins, and at the same time contribute to the localization of Rabs, thus they play crucial roles in translating the signal from Rabs to several diverse aspects of membrane transport. Numerous GEFs and effectors of Rab GTPases have been identified.

Rab38 is a new member of the Rab small G protein family. Overexpressed Rab38 mainly co-localizes with the endoplasmic reticulum-resident and partly with intermittent vesicles between the endoplasmic reticulum and the Golgi complex [18]. Analysis of the mouse coat color phenotype "chocolate" (*cht*) confirms that a G146T mutation occurs in the conversed GTP binding domain of Rab38, which causes a melanocyte defect and decreases the efficiency of targeting Tyrp1 (tyrosinase-related protein 1) to end-stage melanosomes [19]. Another study suggests that Rab38 and Rab32, a close homolog of Rab38, regulate a critical step in the trafficking of melanogenic enzymes, in particular, tyrosinase, from the trans-Golgi network (TGN) to melanosomes [20]. To date, the GEF or effector of Rab38 has not been reported, and the role of Rab38 remains poorly characterized.

Varp is a novel protein that contains a vacuolar protein sorting 9 (VPS9) domain and ankyrin repeats. Varp is widely expressed in various phases of mouse embryo development and in a variety of adult tissues, and is also expressed in many human cell lines. We previously showed that Varp functions as a Rab21 GEF and has specific functions in regulating endosome dynamics *in vivo* [21]. To further investigate the function of Varp, a full-length of Varp was used as bait for a yeast two-hybrid screen and Rab38 was identified as a Varp-interacting protein. In this study we report that Varp functions as a Rab38 effector.

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Materials and methods

Plasmids. Plasmid pcDNA-DEST53/GFP-mRab38 was kindly supplied by Dr. Willian J. Pavan (Genetic Disease Research Branch, National Human Genome Research Institute, National Institutes of Health) [21]. Point mutations in Rab38 were generated with the Takara mutanBEST kit (Code No. D401). The cDNA sequences of wild-type Rab38 (Rab38 WT) and mutant Rab38 (Rab38T23N, Rab38Q69L) were inserted into pEFneo/HA expression vector, respectively. cDNA encoding Flag-tagged full-length Varp (Flag-Varp), Myc-tagged full-length Varp (VarpF), N-terminal of Varp (VarpN), the ankyrin-repeat 1 (ANK1) or ankyrin-repeat 2 (ANK2) of Varp was inserted into the pEFneo vector for protein expression. Rab38 cDNA sequence was also subcloned into the pGEX4T2 vector to generate a GST fusion protein.

Cell culture and transfection. HEK293T and COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were transfected using LipofectAMINE 2000 (Invitrogen).

Co-immunoprecipitation assay and Western blot. HEK293T cells grown in 6-well plates were transfected with indicated expression plasmids and were lysed in cell lysis buffer (50 mM Tris–Cl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.5% NP40, 10% glycerol, 1 mM DTT, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Whole-cell lysates were incubated with 2 μg of indicated antibodies and 30 μl of protein G-Sepharose beads (Santa Cruz Biotechnology) for 8 h at 4 °C. Beads were washed with cell lysis buffer 6 times and bound proteins were eluted with 2× SDS–PAGE loading buffer and analyzed by Western blot with indicated antibodies.

Pull-down assay. The pull-down assay was performed according to a published protocol with minor modifications [22]. The GST-Rab38 protein, purified from *Escherichia coli* strain BL21 by affinity chromatography and immobilized on Glutathione-Sepharose (GE Healthcare) beads, was incubated with 1 mM GTPγS or GDP in PBS buffer containing 1 mg/ml BSA, 1 mM EDTA, 1 mM $MgCl_2$, and 1 mM DTT for 1 h at room temperature. HEK293T cells transfected with indicated expression plasmids were lysed in cell lysis buffer (20 mM Tris–Cl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 10 mM $MgCl_2$, 0.5% NP40, 10% glycerol, 1 mM DTT, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). The lysates were then incubated with GTPγS- or GDP-bound GST-Rab38-containing beads (about 750 μg of protein) for 1 h at room temperature. After six washes with the lysis buffer, the bound proteins were eluted with 2× SDS–PAGE loading buffer and analyzed by Western blotting.

Antibodies. Anti-HA (both monoclonal and polyclonal) and monoclonal anti-Myc (9E10) antibodies were purchased from Santa Cruz Biotechnology. Monoclonal anti-Flag (M2) antibody was purchased from Sigma. TRITC-conjugated goat anti-rabbit and FITC-conjugated goat anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

Immunostaining and confocal microscopy. COS7 cells grown on cover slips were transfected with the indicated expression plasmids. 24 h later, the cells were washed with PBS, fixed for 20 min at room temperature with 4% paraformaldehyde in PBS, and permeabilized for 10 min with 0.2% Triton X-100 in PBS. The cells were then blocked with 10% normal goat serum for 45 min at room temperature. Primary antibodies, diluted in PBS with 3% bovine serum albumin, were allowed to bind by incubating for 2 h at room temperature. Bound antibodies were detected with TRITC- or FITC-conjugated goat anti-rabbit or anti-mouse immunoglobulin G (IgG), with the stained cells analyzed using an Olympus scanning confocal microscopy.

Results

Varp associates with Rab38

A yeast two-hybrid screen using Varp as bait identified Rab38 as a Varp-interacting protein. To confirm this interaction, a co-immunoprecipitation assay was performed by co-expressing Flag-Varp and GFP-Rab38 in HEK293T cells. The results of this assay show that GFP-Rab38 is only detectable in the complex immunoprecipitated with an anti-Flag antibody when both Flag-Varp and GFP-Rab38 were co-expressed (Fig. 1A). This result indicates that Varp associates with Rab38 in mammalian cells *in vivo*.

In order to elucidate which region of Varp binds to Rab38, a Myc-tagged full-length Varp (VarpF) and various deletion mutants of Varp, including the N-terminal (VarpN), ankyrin-repeat 1 (ANK1) and ankyrin-repeat 2 (ANK2) were constructed (Fig. 1B). A co-immunoprecipitation analysis indicates that GFP-Rab38 is precipitated down by an anti-Myc antibody only when it is co-expressed with Myc-VarpF or Myc-ANK1 (Fig. 1C, lanes 2 and 4 on the top panel). These results suggest that Rab38 interacts with VarpF and ANK1, but not with VarpN or ANK2, implying that ANK1 is critical in mediating Varp–Rab38 interaction.

Varp binds preferentially to the activated, GTP-bound form of Rab38

As Rab38 has GDP- and GTP-bound forms, we attempted to determine the guanine nucleotide specificity of the interaction between Varp and Rab38 via a glutathione *S*-transferase (GST) pull-down experiment. The purified GST-Rab38 was bound to GST-Sepharose beads to incubate with cell lysates containing overexpressed VarpF, VarpN or ANK1 in the presence of either GTPγS or GDP. Analysis on the pull-down complex demonstrates that both VarpF and ANK1 preferentially associate with Rab38 in the presence of GTPγS while VarpN has no ability to interact with either Rab38-GTP or Rab38-GDP (Fig. 2A). This result indicates that Varp preferentially interacts with GTP-bound Rab38.

Our previous study had indicated that Varp is a guanine nucleotide exchange factor (GEF) for Rab21 [18]. Combined with our current results, we propose that Varp might play a dual role by switching as either a GEF or an effector during different Rab-mediated signalings. To examine this hypothesis, we simultaneously tested the binding-affinities of Varp to the different states of Rab21 and Rab38. The pull-down analysis shows that Varp binds more efficiently to the GDP but not GTP form of Rab21, whereas for Rab38, Varp preferentially interacts with the GTP form of Rab38 (Fig. 2B).

To further test whether Varp could preferentially bind to the activated form of Rab38 *in vivo*, we constructed a Rab38 mutant (Rab38Q69L, a GTP-hydrolysis-deficient form only allowing the GTP-bound state) as well as a Rab38 mutant (Rab38T23N, which is expected to be frozen into a GDP-bound conformation) (Fig. 2C). Our immunoprecipitation assays demonstrate that VarpF and ANK1 have clear interactions with both Rab38Q69L and wild-type Rab38 (Rab38 WT) but not with Rab38T23N, however, no association of VarpN with either the wild-type or mutant derivatives of Rab38 was detected (Fig. 2D). Taken together, our data indicate that Varp preferentially binds to the GTP-bound form of Rab38, and thus probably acts as a novel effector of Rab38.

Recruitment of Varp to Rab38

In order to reveal the function of Varp as a Rab38 effector, we investigated the intracellular localization of Varp and Rab38. When expressed alone in COS7 cells, Rab38 and its mutant derivatives demonstrate a punctuated distribution in the cytoplasm (Fig. 3A),

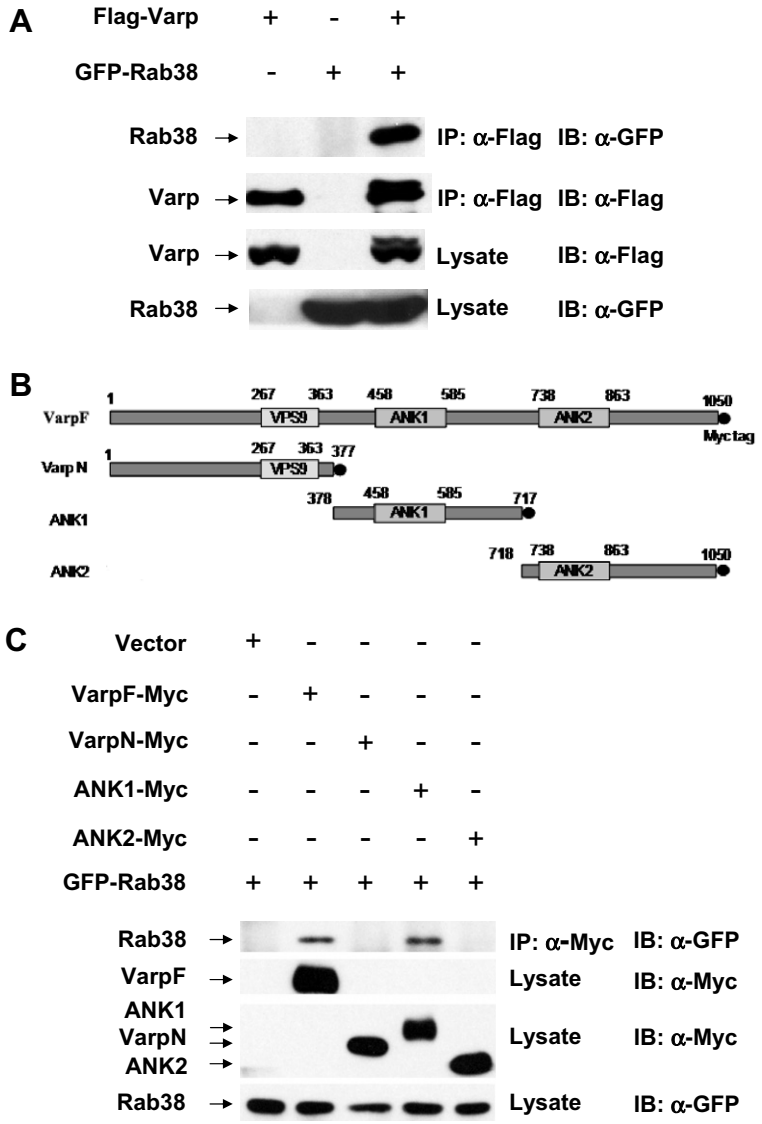

Fig. 1. Association of Varp with Rab38. (A) Co-immunoprecipitation of Varp and Rab38 *in vivo*. Flag-Varp and GFP-Rab38 were transfected into HEK293T cells, respectively, or simultaneously. Cell lysates were immunoprecipitated with an anti-Flag antibody conjugated to protein G-Sepharose beads. The precipitation was subsequently analyzed by Western blot with anti-GFP antibodies. The protein levels of Flag-Varp and GFP-Rab38 loaded in the reactions were also evaluated. (B) A schematic diagram shows the protein structures of VarpF and its truncated mutants VarpN, ANK1 and ANK2. (C) ANK1 is required for the interaction of Rab38 and VarpF. GFP-Rab38 was co-transfected with Myc-tagged VarpF, VarpN, ANK1 or ANK2 into HEK293T cells. The whole-cell lysates were first immunoprecipitated with an anti-Myc antibody conjugated to protein G-Sepharose beads. Then the reaction product was analyzed by Western blot with anti-GFP antibodies. The protein levels of GFP-Rab38, Flag-Varp and Varp deletion mutants loaded in the reactions were also evaluated.

while VarpF (Fig. 3B, b), VarpN or ANK1 (data not shown) are diffusely distributed in the cytosol. It appears that Rab38T23N has a smaller and Rab38Q69L has a larger punctuated distribution (Fig. 3A, b and c). When co-expressed with Rab38 WT (Fig. 3B, d–f) or Rab38Q69L (Fig. 3B, j–l), VarpF is recruited to the same punctuated region where Rab38 is localized (Fig. 3B, f and l, arrows). In contrast, co-expression of Rab38T23N with VarpF did not change the cytosolic distribution of VarpF (Fig. 3B, h), indicating that the GTP-bound form of Rab38 actively recruits Varp protein to its subcellular compartment.

To further test the role of GTP in Rab38-mediated Varp recruitment, Rab38Q69L was co-expressed with VarpF, VarpN or ANK1 in COS7 cells. Consistent with our earlier observations, VarpF and ANK1 co-localize with Rab38Q69L in a punctuated expression pattern (Fig. 3C, c and i, arrows). However, the VarpN protein remains to be distributed diffusely throughout the cytoplasm (Fig. 3C, f). Interestingly, the Rab38-positive vesicle-like structures are shrunken and Rab38Q69L does not co-localize with VarpN when VarpN (which has lost the ability to interact with Rab38) is overexpressed. All of our immunostaining data suggest that Varp is assembled into a Rab38-positive structure and that ANK1 is required for this recruitment.

Discussion

Varp is a newly identified protein with a VPS9 domain and ankyrin repeats. The VPS9 domain of Varp is present in all identified GEFs of Rab5 [23], which promoted us to suspect that Varp may be a GEF for Rabs. Consistent with this assumption, our previous results had shown that Varp protein indeed functions as a Rab21 guanine nucleotide exchange factor (GEF) [21], however, the function of Varp still remains largely unknown. In this study, we show that Varp interacts with Rab38 specifically. The *in vivo* interaction was initially uncovered by a yeast two-hybrid approach and subsequently confirmed by immunoprecipitation analysis. Mapping of Varp showed that ANK1 but not the VPS9 domain plays

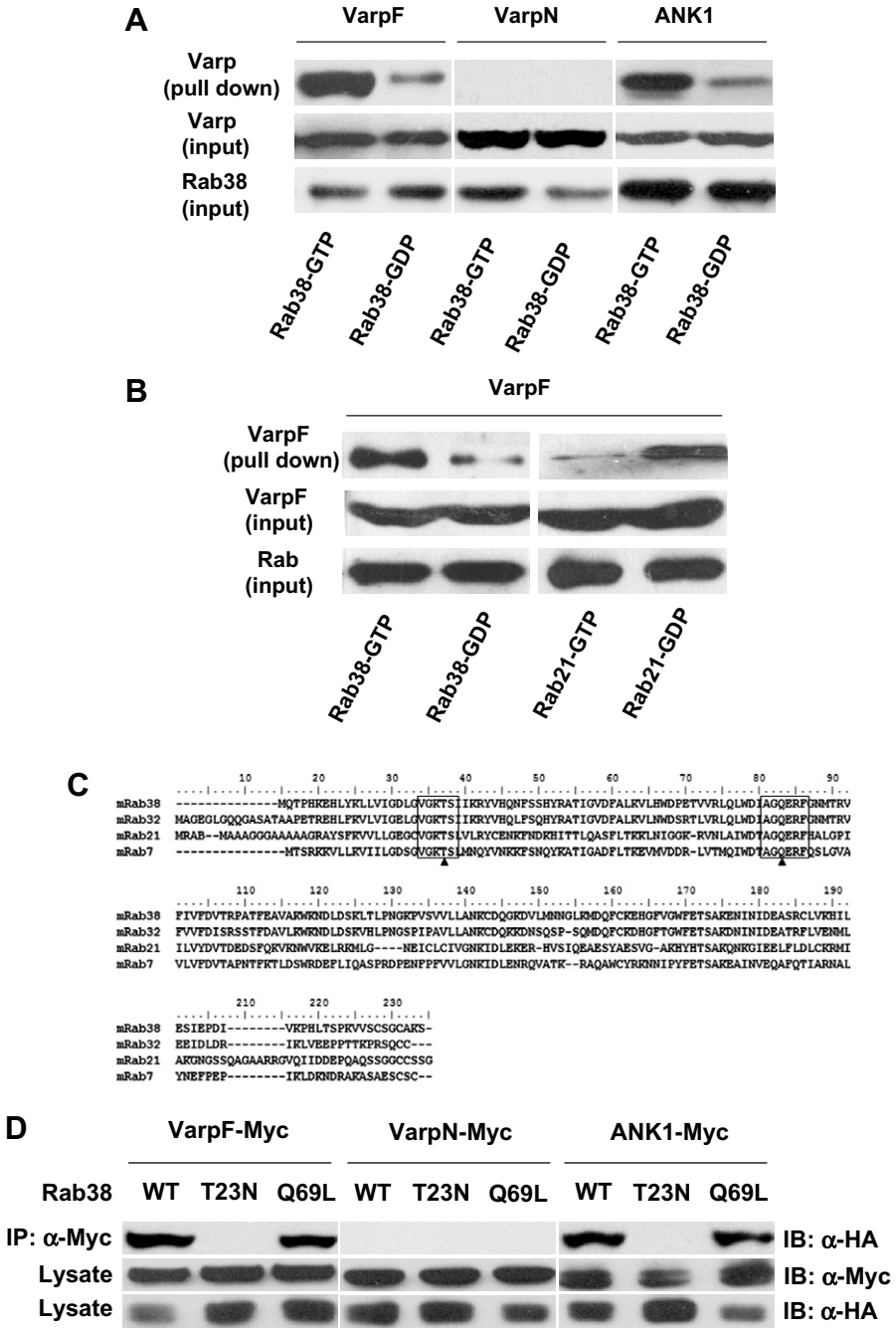

Fig. 2. Varp preferentially binds to the GTP-bound form of Rab38. (A) Varp preferentially interacts with Rab38-GTP. A pull-down experiment was performed to show the potential interaction between Varp or its mutant derivatives and the GTPγS or GDP form of GST-Rab38. Total Varp proteins and purified GST-Rabs loaded were also analyzed by Western blot. (B) Varp has a dual effect to associate with Rab38 and Rab21. A pull-down experiment was performed to demonstrate the preferential interaction of Varp with Rab38-GTP and Rab21-GDP. The GTP or GDP form of GST-Rab38 or GST-Rab21 was individually incubated with cell lysates from HEK293T cells expressing VarpF. The eluted products were analyzed by Western blot analysis. Protein loading controls were also evaluated. (C) Identification of the conserved motifs critical for GDP or GTP binding in Rabs. Sequence alignment of Rab38, Rab32, Rab21 and Rab7 was performed. The amino acids that are conserved in these Rabs and crucial for GDP/GTP-bound are marked by panes. The arrow heads show the positions of point mutants. (D) Varp interacts with Rab38Q69L. HA-tagged Rab38 WT, Rab38T23N or Rab38Q69L was co-transfected with Myc-tagged VarpF, VarpN or ANK1 in HEK293T cells. Whole-cell lysates were immunoprecipitated with an anti-Myc antibody conjugated to protein G-Sepharose beads. The precipitation was analyzed by Western blot with an anti-HA antibody. The levels of Varp and Rab38 proteins in the whole-cell lysates were also evaluated.

a major role during this process. This is interesting, as we have previously shown that the VPS9 domain is critical for the activation of Rab21. We also provide evidence that Varp preferentially interacts with the GTP-bound form of Rab38 both *in vitro* and *in vivo* and that the recruitment of Varp to Rab38-positive structures occurs in a GTP- and ANK-dependent manner. Therefore, we identified that Varp functions as a potential effector for Rab38 although it also functions as a GEF for Rab21 [21].

Rab proteins participate in almost every step in intracellular vesicle transport and experience conversions in cascade. In this process, an effector of an upstream Rab protein serves as a GEF of downstream Rab GTPase [9]. Ypt31/32p and Sec4p, which are involved in several trafficking events in yeast, are functionally linked in a regulatory Rab cascade through Sec2p. Therefore Sec2p is both a GEF for Sec4p and an effector for Ypt31/32p that acts just upstream [22,24–26]. In analogy, our results demonstrated that Varp

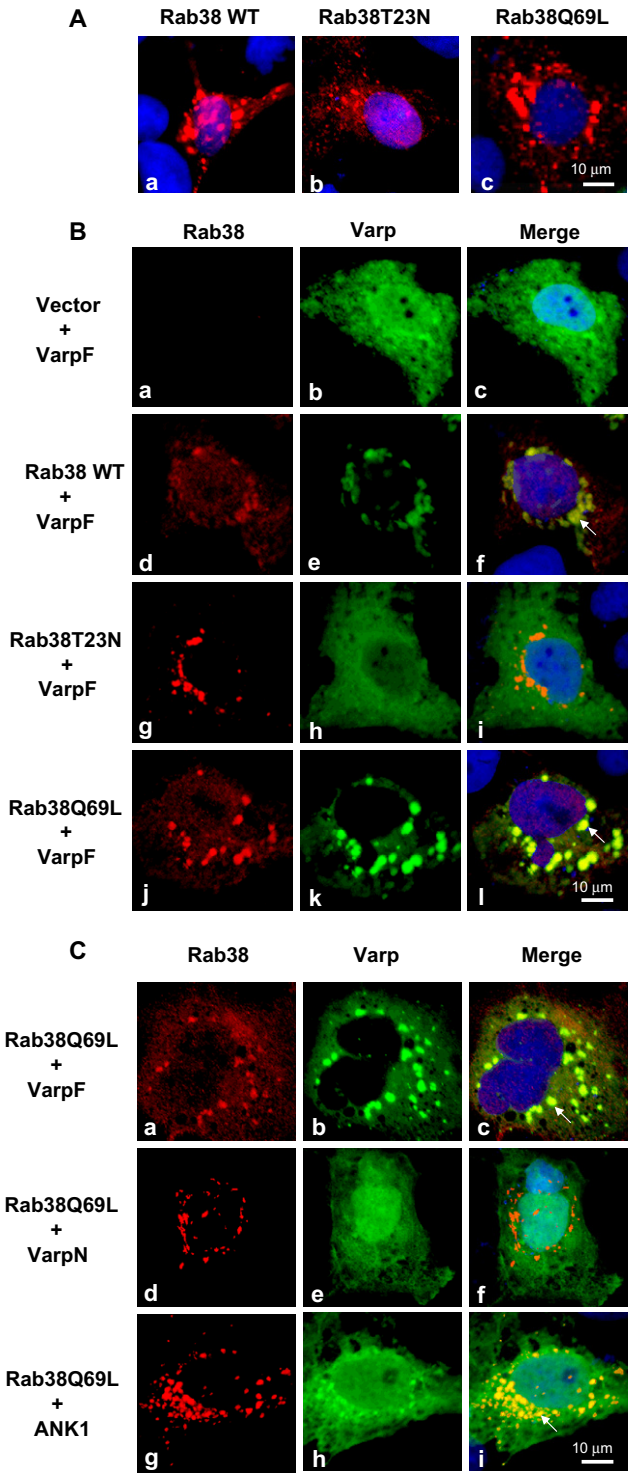

Fig. 3. Recruitment of Varp by the GTP-bound form of Rab38. (A) The localization of overexpressed Rab38 WT, Rab38T23N, or Rab38Q69L in COS7 cells. (B) Varp co-localizes with Rab38. VarpF was co-transfected with vector, Rab38 WT, Rab38T23N, or Rab38Q69L into COS7 cells. (C) Rab38Q69L co-localizes with Varp. Rab38Q69L was co-transfected with VarpF, VarpN, or ANK1 into COS7 cells. The cells were immunostained with a mouse anti-Myc antibody and rabbit anti-HA antibodies, followed by FITC-conjugated goat anti-mouse and FRITC-conjugated goat anti-rabbit secondary antibodies. The localization of Varp and Rab38 is shown in green and red, respectively. Arrows indicate the co-localization of Varp and Rab38 proteins. The blue area denotes nuclear region stained by DAPI. Cover slips were viewed with an Olympus confocal microscope. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

can serve as either a GEF or an effector by interacting with different Rab signaling molecules.

Rab21 is localized mainly to early endosomes and regulates endosome dynamics [27]. Ectopically expressed Varp in HeLa cells co-localizes with Rab21 on enlarged early endosomes, thus promoting early-endosome fusion and regulating progression from early to late endosomes [21]. Previous studies indicated that Rab38 showed a co-localization with the early endosomal marker protein EEA1 [28] and might participate in the transport of melanogenic substances at the level of the endoplasmic reticulum to the Golgi apparatus [18]. In this study, our immunostaining results show that the active form of Rab38 (Rab38Q69L) induces the enlargement of Rab38-positive vesicles (Fig. 3B, compare l and i), where Varp is recruited. Therefore, we suggest that Varp might be required for the enlargement of Rab38-positive vesicles between the endoplasmic reticulum and Golgi apparatus, and that the function of Rab21 and Rab38 might be linked by Varp.

Rab38 is predominantly expressed in melanocytes and retinal pigment epithelial cells [20]. Mutations in the Rab38 gene were demonstrated in the chocolate mouse and in Fawn-hooded and Tester-Moriyama rats [19,29]. The melanin biosynthesis related protein TYRP1 displayed a reduced level of expression in melanocytes of Rab38^{cht}/Rab38^{cht} mice compared to wild-type animals, suggesting that Rab38 might be required for the normal trafficking of TYRP1 from the trans-Golgi network (TGN) to the maturing melanosome [19]. Furthermore, Wasmeier et al. demonstrated that Rab38 and Rab32 may play a redundant role in regulating transport of tyrosinase from the TGN to melanosomes [20]. Given that Varp acts as a Rab38 effector, Varp might also be involved in targeting TYRP1 or/and tyrosinase to the melanosome for the synthesis of pigment. Therefore, it would be interesting to further elucidate the detailed functions of Varp in Rab32- and Rab38-mediated cellular processes.

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