Characterization of Rab45/RASEF containing EF-hand domain and a coiled-coil motif as a self-associating GTPase

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Abstract

Rab-family GTPases function as key regulators for membrane traffic. Among them, Rab45/RASEF is an atypical GTPase in that it contains a coiled-coil motif at the mid region and a distinct N-terminal EF-hand domain with C-terminal Rab-homology domain. Here, we provide the initial biochemical characterization and intracellular localization of human Rab45. Rab45 bound guanine nucleotide tri- and di-phosphates through the C-terminal Rab domain. Rab45 was capable of self-interacting, and the self-interaction required the mid region containing the coiled-coil motif. Rab45 expressed in HeLa cells was localized in a small patch in the perinuclear area of the cell, and the localization was regulated by the guanine nucleotide-bound states of Rab45. Interestingly, the mid region, together with Rab domain, appeared to be essential for the characteristic perinuclear localization of Rab45, indicating that the self-interaction may be involved in the intracellular localization of Rab45.

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Rab-family GTPases play pivotal roles in the regulation of membrane traffic [1,2]. Given that the presence of many different intracellular compartments requires a high order regulation of membrane traffic to ensure delivery and fusion of transport vesicles at the appropriate site, it is not surprising that over 60 mammalian Rab proteins have so far been identified. Some of Rab proteins have been extensively analyzed and their roles in membrane traffic are relatively well understood; however, there are still many Rab proteins, of which exact functions remain to be elucidated. Characterization and functional analysis of these Rab proteins, together with identification of their regulators and effectors, would help to unravel a complex network of membrane traffic.

In this study, we investigated the biochemical properties and subcellular localization of a novel Rab-family protein, Rab45/RASEF. It has been reported that the expression of Rab45 is decreased in cutaneous malignant melanoma [3], however, the physiological role(s) of Rab45 is unknown. Rab45 has a unique structure consisting of a N-terminal EF-hand domain, a mid region containing a coiled-coil motif, and a C-terminal Rab-homology domain (Rab domain). We found that the Rab domain of Rab45 exhibits guanine-nucleotide binding ability and that Rab45 is capable of self-interacting through the mid region. Furthermore, we found that Rab45 expressed in HeLa cells is localized to a small patch in the perinuclear area of the cell and that the mid region of Rab45 besides Rab domain is responsible for the intracellular localization of Rab45. We discuss a possible role of the self-associating Rab45 in membrane traffic.

Materials and methods

Cell culture and fluorescence microscopy. HeLa and HEK293T cells were maintained as described previously [4] and transfected with plasmid...
constructs using LipofectAMINE 2000 (Invitrogen). For fluorescence microscopy, the cells grown on a glass base dish were viewed with a Carl Zeiss LSM-510 confocal microscope 20–24 h after transfection. SYTO59 (Molecular Probes) was used to label cell nuclei.

**Plasmid construction.** A plasmid DNA containing human Rab45 cDNA (GenBank Accession No. BC023560) was obtained from ATCC (cat No. 7515966). cDNAs of Rab45 mutants were made by PCR and subcloned in modified pCMV5 plasmids (pFLAG-CMV5 and pMyc-CMV5), which place the FLAG- and Myc-tag at its amino terminus, respectively. For yeast two-hybrid assay, a cDNA fragment of Rab45 corresponding to the amino acid sequence 98–533 was subcloned in pGBT9 as a bait plasmid, and cDNA fragments corresponding to the amino acid sequences 98–332, 333–527, 528–740, and 1–97 were subcloned in pGAD424 as prey plasmids. pEGFP-C2 (Clontech) and pDsRed-Monomer (Clontech) were used for expression of GFP and DsRed-Monomer fusion proteins, respectively. A cDNA fragment of the Rab domain of Rab45 (the amino acid sequence 527–740) was subcloned in pGEX-6P-1 to prepare Escherichia coli recombinant proteins.

**Expression and purification of recombinant Rab45 proteins.** HEK293T cells cultured in a 100-mm dish for 2 days after transfection with 5 μg of pFLAG-CMV5-Rab45 plasmids were lysed with 1 ml of an ice-cold buffer (40 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl2, 1 mM DTT, 1% (w/v) CHAPS, and 0.5 mM Pefabloc SC). After centrifugation, the supernatants were preclreated with Sepharose 4B resin, followed by immunoprecipitation with Anti-FLAG M2 Affinity Gel (Sigma). After washing the resin with an ice-cold wash buffer (40 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl2, and 1% (w/v) CHAPS), FLAG-tagged Rab45 was eluted from the resin with the wash buffer containing 100 μg/ml of FLAG-peptide. Purification of GST-fusion proteins was performed as described previously [4]. The Rab domain of Rab45 was prepared using PreScission protease (GE Healthcare) as suggested by the manufacturer.

**Nucleotide binding assays.** [35S]GTPγS binding assay was performed as described previously [4]. Purified proteins (0.7 pmol) were incubated at 30 °C in a total volume of 40 μl of reaction mixture consisting of 40 mM Tris–HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl2, 1 mM DTT, 0% (w/v) CHAPS, and 2.5 μM [35S]GTPγS (0.17 KBq/pmol). After incubation for the indicated times, samples were diluted with 1 ml of an ice-cold wash buffer (20 mM Tris–HCl (pH 7.5), 20 mM MgCl2, and 100 mM NaCl) and filtered through a nitrocellulose membrane (0.45-μm pore size, ADVANTEC). The filter was extensively washed with the ice-cold wash buffer, radioactivity retained on the membrane was determined by a liquid scintillation counter.

In vivo phosphate labeling and immunoprecipitation. Guanine nucleotides bound to the GTP-binding proteins were analyzed essentially as described previously [4]. Briefly, transfected HeLa cells (~3 × 10^6 cells) were labeled for 4 h with [32P] orthophosphate in DMEM and lysed in 1 ml of an ice-cold solubilizing buffer (40 mM Tris–HCl (pH 7.5), 100 mM NaCl, 20 mM MgCl2, 1 mM DTT, 1% (w/v) Triton X-100, 2 μg/ml aprotinin, and 0.5 mM Pefabloc SC). The precleared cell lysates were incubated with the anti-FLAG M2 monoclonal antibody (2 μg) and protein G-Sepharose. After extensive washing of the immunocomplexes, associated nucleotides were separated by thin-layer chromatography and analyzed with a BAS-1800 image analyzer (Fuji Film).

Co-immunoprecipitation assay. HeLa cells cultured in a 60-mm dish for 36 h after co-transfection with the pMyc- and pFLAG-CMV5-derived plasmids were lysed with 350 μl of an ice-cold extraction buffer (40 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl2, 1 mM DTT, 1% (w/v) Triton X-100, and 0.5 mM Pefabloc SC). The precleared cell lysates were incubated with anti-Myc polyclonal antibody (1 μg) and 20 μl of Protein A-Sepharose. After washing the resin with a wash buffer (40 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl2, 1 mM DTT, and 0.2% (w/v) Triton X-100), the immunocomplexes were solubilized with 40 μl of SDS-PAGE sample buffer and subjected to SDS-PAGE, followed by immunoblotting with anti-FLAG M2 or anti-Myc 9E10 monoclonal antibody.

**Yeast two-hybrid analysis.** Yeast strain Y190 was co-transformed with pGBT9- and pGAD424-derived plasmids containing Rab45 cDNA fragments. Transformants were grown on a synthetic medium lacking leucine and tryptophan and subjected to quantitative β-galactosidase assays using 4-methylumbelliferyl-β-d-galactoside as a substrate [5].

**Results**

**Biochemical characterization of Rab45 as a guanine-nucleotide binding protein.** To examine the biochemical properties of Rab45, we expressed it in HEK293T cells as a FLAG-tagged protein and purified it using Anti-FLAG M2 Affinity Gel. Fig. 1A shows that the purified Rab45 bound GTPγS in a time-dependent manner, with maximum binding occurring at about 20 min. The GTPγS-binding to Rab45 was selectively competed by GTP or GDP, but not by ATP (not shown), indicating that Rab45 specifically binds guanine nucleoside tri- and di-phosphates. Besides the C-terminal Rab domain, Rab45 has an additional N-terminal region composed of an EF-hand domain and a coiled-coil motif. We thus investigated whether other regions besides Rab domain are critical for guanine-nucleotide binding. Fig. 1B shows that the purified Rab domain protein bound GTPγS in a similar time-dependent manner to that observed for FLAG-Rab45, demonstrating that Rab domain is responsible for the guanine-nucleotide binding properties of Rab45.

We next examined the nucleotide forms associated with Rab45 in living cells. To analyze the nucleotide forms of Rab45 in living cells, FLAG-Rab45 proteins expressed in HeLa cells were immunoprecipitated after in vivo phosphate labeling of the cells. The guanine nucleotides associated with the immunocomplex were then analyzed by thin-layer chromatography. We found that wild-type Rab45 predominantly existed as a GDP-bound form as shown in Fig. 1C (WT in upper panel). Rab45/Q600L and Rab45/S555N are mutants with substitution of Gln to Leu at position 600 and Ser to Asn at position 555 located in Rab domain, respectively. Fig. 1C shows that Rab45/Q600L was predominantly a GTP-bound form, while most of Rab45/S555N existed as a nucleotide-free form, as expected from the previous studies of the equivalent mutants in other small GTPases [6]. Fig. 1D shows that the Rab domain of Rab45 bound guanine nucleotides and the effects of point mutations on the states of guanine-nucleotide binding were the same as that observed for full-length Rab45. These results suggest that the EF-hand domain and the mid region of Rab45 are not essential for guanine-nucleotide binding to Rab45, though we cannot rule out the possibility that the N-terminal region besides Rab domain is involved in the stoichiometry of guanine-nucleotide binding to Rab45.

**Self-interaction of Rab45 through a mid region containing coiled-coil motif.** In the domain architecture analysis of Rab45 using SMART database (http://smart.embl-heidelberg.de/), we
found that Rab45 has a coiled-coil motif extending from residues 170 to 362. Coiled-coil is a structural motif that mediates oligomerization of proteins, and a number of coiled-coil proteins have been shown to be engaged in membrane traffic [7,8]. Thus, we sought to determine whether Rab45 is capable of self-interacting. HeLa cells were co-transfected with FLAG-Rab45 and Myc-Rab45 expression vectors, and the cell lysates were subjected to immunoprecipitation using an anti-Myc antibody followed by Western blotting of the immunocomplexes with an anti-FLAG antibody. Fig. 2 B shows that FLAG-Rab45 was detected in the immunocomplexes containing Myc-Rab45/WT (WT in Fig. 2 B), indicating that FLAG-Rab45 can interact with Myc-Rab45 either directly or indirectly. The interaction between FLAG-Rab45 and Myc-Rab45 (Rab45 self-interaction) was independent on the guanine-nucleotide form of Rab45: FLAG-Rab45 was equivalently co-immunoprecipitated with Myc-Rab45/Q600L or Myc-Rab45/S555N (Q600L and S555N in Fig. 2B). To investigate which regions of Rab45 are responsible for the Rab45 self-interaction, a series of deletion mutants of Myc-Rab45 was co-expressed with FLAG-Rab45. Fig. 2 illustrates a schematic representation of the Rab45 mutants used in this study. Fig. 2 A shows FLAG-Rab45 was co-immunoprecipitated with a deletion mutant lacking EF-hand domain or Rab domain (ΔEF and ΔRab in Fig. 2 B). In contrast, FLAG-Rab45 was no longer co-immunoprecipitated with a deletion mutant lacking the mid region (ΔMid in Fig. 2B). It has been shown that some Rab proteins can interact with themselves [9,10], however, the Myc-tagged Rab domain of Rab45 did not interact with FLAG-Rab45 in the co-immunoprecipitation analysis (Rab in Fig. 2 B).

We next investigate whether the mid region of Rab45 can directly interact with itself using yeast two-hybrid assay. We constructed a pGBT9 vector containing the cDNA fragment of the mid region of Rab45 as a bait.
Materials and methods.

C DNA fragments of the full-length, the mid region, the Rab domain, and the EF-hand domain of Rab45 were subcloned in pGAD424 vector. Fig. 2C shows that the mid region of Rab45 specifically interacted with the full-length or the mid-region of Rab45, but not with the Rab domain or the EF-hand domain. These results indicate that the mid region including coiled-coil motif can directly interact with itself, which may be responsible for the Rab45 self-interaction.

Analysis of the intracellular localization of Rab45 in HeLa cells

We next examined the intracellular localization of Rab45 in HeLa cells. When GFP-tagged Rab45 was transiently expressed in HeLa cells, the fusion proteins were predominantly observed in a small patch near the perinuclear region of the cells (Fig. 3A). We sought to determine which region(s) of Rab45 is responsible for the characteristic localization. For this purpose, we transfected a set of plasmid constructs of GFP-Rab45 mutants into HeLa cells to observe their intracellular localization. Fig. 3B shows that GFP-Rab45/ΔEF exhibited a small patch localization similar to that observed for GFP-Rab45/WT (a in Fig. 3B). In contrast, GFP-Rab45/ΔRab was dispersed throughout the cytoplasm (b in Fig. 3B), indicating Rab domain is essential for the small patch localization of Rab45. The localization pattern of GFP-Rab45/ΔMid was also different from that of GFP-Rab45/WT: GFP-Rab45/ΔMid was observed in both cytosol and dispersed vesicular structures near the perinuclear region (c in Fig. 3B). In addition, GFP-Rab45/Rab showed a similar localization pattern to that observed for GFP-Rab45/ΔMid (d in Fig. 3B). These results indicate that the mid region besides Rab domain is required for the small patch localization of Rab45. Both GFP-Rab45/Mid and GFP-Rab45/EF were dispersed throughout the cells (e and f in Fig. 3B).

Generally, GTP-bound active Rab proteins can associate with specific membrane compartments [2]. Indeed, GFP-Rab45/Q600L was localized to the perinuclear compartment, while GFP-Rab45/S555N was dispersed throughout the cells (g and h in Fig. 3B). These observations implied that the perinuclear localization of Rab45 is regulated by its guanine nucleotide-bound states.

Interestingly, when GFP-Rab45/ΔRab was co-expressed with DsRed-Monomer-Rab45/WT (a–c in Fig. 3C), it was redistributed to a small patch near the nuclear region, where it was remarkably colocalized with Rab45/WT (compare b in Fig. 3C with b in Fig. 3B). In addition, GFP-Rab45/Mid was also colocalized with DsRed-Monomer-Rab45/WT in the perinuclear region when co-expressed (compare e in Fig. 3C with e in Fig. 3B), while GFP-Rab45/EF was still dispersed even in the presence of DsRed-Monomer-Rab45/WT (compare h in Fig. 3C with f in Fig. 3B). These results, together with the results shown in Fig. 2B and 2C, support the idea that Rab45 can interact with itself through the mid region.

Discussion

Several Rab GTPases and Rab effectors can form homodimers or multimers, and the self-interaction of these pro-
proteins appear to be important for their functions [9–21]. The Rab7-binding domain of RILP forms a coiled-coil homodimer, and the disruption of RILP dimerization abolishes its interaction with Rab7 and late endosomal/lysosomal targeting [12], suggesting that the dimerization of RILP is essential for its function. ALS2 is a Rab5-binding protein acting as a GEF (guanine nucleotide-exchange factor) for Rab5 [17]. Homo-oligomerization of ALS2 is crucial for the GEF activity and the ALS2-mediated endosome enlargement [18]. A family of Rab11-interacting proteins (FIPs) has also been shown to have ability to self-interact [19–21]. Structural analysis of the heterotetrameric com-

Fig. 3. Localization of wild-type and mutant Rab45 in HeLa cells. (A) HeLa cells were transiently transfected with pEGFP-Rab45/WT and stained with SYTO59 to mark the nuclei. The fluorescence signals of GFP (green) and SYTO59 (red) were recorded in a Carl Zeiss LSM-510 confocal microscopy (a) and merged with images taken using differential interference contrast microscopy (b). (B) Localization of Rab45 mutants in HeLa cells. HeLa cells were transiently transfected with pEGFP vectors containing each of Rab45 mutants and observed using the confocal microscopy (a–h). (C) Rab45 mutants containing the mid region are redistributed to a perinuclear region by co-expression with wild-type Rab45. HeLa cells were transiently co-transfected with pDsRed-Monomer-Rab45/WT and pEGFP-Rab45/ΔRab (a–c), pEGFP-Rab45/ΔMid (d–f) or pEGFP-Rab45/ΔEF (g–i). The distribution of the fluorescence proteins was recorded by the confocal microscopy. (c, f, and i) Merged images for a and b, d and e, and g and h, respectively. Bars, 10 μm.
plex of Rab11 with the C-terminus of FIP3 showed that dimerization generates a binding surface for Rab11 extending over both chains of the FIP3 homodimer [20,21]. In this study, we have shown that Rab45 can interact with itself through the mid region. Given that the mid region contains a coiled-coil motif, the self-interaction of Rab45 is likely to be mediated by the coiled-coil motif. Further analysis of the Rab45 self-interaction using deletion and point mutants is required to determine whether the coiled-coil motif is responsible for the self-interaction of Rab45.

Coiled-coil proteins involved in membrane traffic often have functionally discrete domains at their N- or C-termini [8]. For example, a large coiled-coil protein, Rabaptin5, has a C-terminal Rab5-binding domain and a distinct N-terminal Rab4-binding domain [13]. Rab5 is involved in endocytosis, whereas Rab4 is important for recycling from early endosomes to the plasma membrane. Given that Rabaptin5 is recruited to early endosomes in a Rab5-dependent manner, Rabaptin5 appears to function to coordinate two pathways of endocytosis and recycling traffic at endosomes [13]. As described above, Rab45 has a C-terminus Rab domain and a distinct N-terminus EF-hand domain. We found that the Rab domain is involved in the perinuclear localization of Rab45 probably through its interaction with a membrane compartment. It has been shown that Rab proteins are localized to specific membrane compartments of both the endocytic and exocytic pathways, and the characteristic localization of Rab proteins is dependent on the guanine nucleotide-bound forms [2]. Indeed, we found that Rab45 was localized to a small patch in a perinuclear region, and the localization was regulated by the guanine-nucleotide forms of the Rab domain. Interestingly, in addition to the Rab domain, the mid region is also involved in the perinuclear localization of Rab45. Considering that the mid region is important for the self-interaction of Rab45, the self-interaction of Rab45 may be involved in the characteristic perinuclear localization. Rab proteins have been shown to regulate a membrane traffic step that is reflected by their characteristic localization [2]. Our preliminary results showed that Rab45 was significantly co-localized with Rab11 in a perinuclear region of HeLa cells (not shown). Future analysis of the possible involvement of Rab45 in Rab11-mediated membrane may provide insight into the physiological function of Rab45.

Functional significance of the EF-hand domain of Rab45 remains to be determined in the present study. EF-hand proteins undergo conformational changes upon binding Ca$^{2+}$, and the conformational changes are responsible for the interaction with target molecules [22]. Considering that Ca$^{2+}$ is involved in various aspects of intracellular membrane transport including exocytosis [23], it is tempting to speculate that the EF-hand domain of Rab45 binds target molecule(s) in a Ca$^{2+}$-concentration dependent manner and regulates membrane traffic in cooperation with Rab domain. Identification of the binding partner(s) for the EF-hand domain, together with that for the Rab domain, will help to analyze the Rab45-mediated membrane traffic pathway.

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References


