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Rab5-activating protein 6, a novel endosomal protein with a role in endocytosis ☆

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Abstract

Rab GTPases are regulators of membrane trafficking that cycle between active (GTP-bound) and inactive (GDP-bound) states. In this study, we report the identification of a new human Rab5 guanine nucleotide exchange factor (GEF), which we have named RAP6 (Rab5-activating protein 6). RAP6 contains a Rab5 GEF and a Ras GAP domain. We show that the Vps9 domain is sufficient for the interaction of RAP6 with GDP-bound Rab5 and that RAP6 stimulates Rab5 guanine nucleotide exchange. We also find that the Ras GAP domain of RAP6 shows GAP activity for Ras. Immunofluorescence experiments reveal that RAP6 is associated with plasma membrane and small intracellular vesicles that also contain Rab5. Additionally, the overexpression of RAP6 affects both fluid phase and receptor-mediated endocytosis. This study is the first to show that RAP6 is a novel regulator of endocytosis that exhibits GEF activity specific for Rab5 and GAP activity specific for Ras.

Keywords: Rab5; Receptor-mediated endocytosis; Small GTPases; Membrane trafficking

Endocytosis occurs in most eukaryotic cells and is essential for uptake of nutrients, downregulation of cell surface receptors, and maintenance of cell homeostasis [1]. Generally there are two types of endocytosis: receptor-mediated endocytosis and fluid-phase endocytosis.

Receptor-mediated endocytosis can be either a constitutive process (i.e., the process mediated by transferrin receptor) or a ligand-induced event (i.e., the downregulation of activated platelet-derived growth factor receptor). The internalization of cell surface receptors occurs at specific areas (coated pits) that are coated with clathrin molecules [2].

Fluid-phase endocytosis is a constitutive cellular process that internalizes liquids and solutes from extracellular environments. After internalization, fusion between endosomal vesicles occurs with delivery of endocytosed contents into a population of early endosomes where they are further sorted to other intracellular destinations. It is thought that receptor-mediated endocytosis and fluid-phase endocytosis share a common machinery at the early endosome fusion step. In the past several years, there has been an explosive discovery of molecules that control distinct membrane trafficking steps [3–5].

For example, the well-characterized small GTPase Rab5 regulates membrane fusion between early endosomes that can be derived from either receptor-mediated or fluid-phase endocytosis [4–6]. In addition, Rab5 is thought to be involved in microtubule-based motility of early endosomes and also in the organization of subdomains within the early endosomal membranes by the recruitment of specific multiple factors. The activity of Rab5 is tightly regulated at the level of nucleotide status by either the guanine nucleotide exchange (GEF) or GTP-activating protein (GAP) factors.

☆ *Abbreviations:* RAP6, Rab5-activating protein 6; Rin1, Ras interference 1; GEF, guanine nucleotide exchange factor; GFP, green fluorescence protein; GAP, GTP hydrolysis-activating protein; RGD, Ras GAP domain; Vps9, vacuolar protein sorting 9; TR, transferrin receptor; IR, insulin receptor.

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The newest additions to this group of factors include Rin1, Rin2, Rin3, Rabex5, and Alsin GEFs that specifically regulate Rab5 activity [7–13]. Rin1 and Rabex5, but not Alsin, have been shown to interact with GTP-bound Ras [13,14]. Furthermore, Ras oncogene is well known to regulate both signaling pathways [15] as well as fluid endocytosis [16,17]. Interestingly, a *Caenorhabditis elegans* RME-6 gene also shows a Rab5 GEF domain [18]. Lastly, a new Rab5 GAP has just been described [19].

We report here the cloning, characterization, and functional study of the human RAP6 (Rab5-activating protein 6) gene. Our results show that RAP6 protein is localized on the plasma membrane and on endosomes. RAP6 binds to Rab5 and Ras through the Vps9 and RGD domains, respectively. In summary, RAP6 plays an important role in the regulation of receptor-mediated endocytosis and has a significant effect on endosome morphology.

Materials and methods

Materials and cell lines. HeLa and HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD). CHO cells expressing human transferrin receptor (CHO-Tf) and 293T cells were a generous gift of Dr. P.D. Stahl (Washington University, MO). A polyclonal anti-GST and anti-Rab5 antibodies were obtained from BD Biosciences. Anti-RAP6 antibodies were prepared in rabbit against synthetic peptides corresponding to the C terminus of the proteins (CFLKVQIAEAINLQD KNL), coupled to keyhole limpet hemocyanin (KLH), and affinity purified with the same peptide according to the manufacturer's instructions (Pierce). All other reagents were from Sigma unless otherwise noted.

RAP6 identification by MALDI mass spectrometry. Silver-stained bands were cut out of the gels for in-gel digestion as previously described [26]. Briefly, the gel pieces were equilibrated in ammonium bicarbonate-acetonitrile buffer. The supernatant was decanted and the gel pieces were dried, rehydrated for digestion with porcine trypsin (Promega). The reaction was stopped and the peptides were analyzed by matrix-assisted laser desorption ionization (MALDI) with time-of-flight detection mass spectrometry on a Perseptive Biosystems (Foster City, CA) Voyager DE-Pro mass spectrometry workstation at the Protein and Nucleic Acid Chemistry Laboratory (PNACL) at Washington University. This analysis gave an unequivocal protein identification based on a clear distinction of the first MOWSE score relative to all other unrelated ranked proteins.

Cloning of RAP6 gene. Total HeLa cell RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RAP6 cDNA was amplified from total HeLa cell RNA by PCR and subcloned into the pGEM-T Easy vector (Promega, Madison, WI) using the following oligonucleotides (all were made by Integrated DNA Technologies, Coralville, IA): 5′-AGGATCCGCCGCCACCATG GAACAA AAACTCATCTCAGAAGAGGATCTGAATGCCCATCATCATCAC CATCATGCCGCAGTGAAACTAGATATTCATACTCTG and 3′-AC GGGCGGCCGCTCAC TTTCG GTCATCGATGG TTTTAAT. The construct was digested with *Bam*HI and *Not*I, and the RAP6-FL cDNA was cloned into the pcDNA3.1+ (Invitrogen) vector at the same sites. All ligations were done using the Rapid DNA Ligation Kit (Roche). RAP6:Vps9 fragment was cloned in-frame into the pGEX-4T-1 (Amersham Biosciences, Piscataway, NJ) vector at the *Bam*HI and *Not*I sites. RAP6-RGD fragment was cloned into pGEX-4T-1 at the *Eco*RI and *Not*I sites. RAP6:RGD was cloned at the *Eco*RI and *Xho*I sites, and RAP6:Vps9 was cloned at the *Xho*I site in-frame into the pB42AD vector. RAP6:RGD and RAP6:Vps9 fragments were cloned in-frame into the pEGFP-C1 vector at the *Xho*I and *Bam*HI sites. The final four amino acids were removed from the carboxy terminus of Rab5A:WT and Rab5A:S34N were cloned in-frame into the pLexA vector at the *Eco*RI and *Bam*HI sites as described earlier [7]. Rab5A:WT was cloned in-frame into another mammalian expression vector (peRFP) using *Bgl*II and *Bam*HI sites. peRFP vector was generated by replacing GFP gene in the peGFP-C1 vector by the monomeric red fluorescence (mRFP) using the *Eco*47III and *Bsp*EI sites.

Transfection of HeLa, HepG2, 293T, and CHO-Tf cells. Cells were transfected with the FUGENE6 method as described by the manufacturer (Life Technology). Under our experimental conditions we were able to transfect more than 95% of the cells.

Fluorescence microscopy. Cells grown on glass coverslips were examined by confocal microscopy as described previously [6]. Briefly, cells expressing myc-RAP6:WT and constructs were grown on coverslips. The cells were fixed for 30 min in phosphate-buffer saline (PBS) containing 3% (w/v) paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA), washed twice with PBS, and then incubated for 20 min in PBS/50 mM NH_4Cl. After treatment with 10% serum (in PBS) for 30 min at room temperature, the cells were incubated with anti-myc antibody (Invitrogen, Carlsbad, CA) solutions (diluted in PBS containing 10% goat serum) for 1 h at room temperature. Endogenous RAP6 was visualized by indirect immunofluorescence using anti-RAP6 polyclonal antibodies. As a control we used irrelevant IgG rabbit antibodies. Bound primary antibodies were detected with Alexa 488-conjugated (green) secondary antibodies (Molecular Probes, Eugene, OR). GFP-RAP6:Vps9 and GFP-RAP6:RGD domains were detected by viewing the protein's intrinsic fluorescence. Co-localization of RAP6 with RFP-Rab5 was investigated using mRFP tagged to Rab5:WT protein. Cells were co-transfected with both cDNAs and were then viewed using a confocal scanning beam fluorescent microscope at excitation wavelengths of 476 and 543 nm. Confocal microscopy was carried out on a Leica SP2 confocal microscope.

Yeast two-hybrid system. The matchmaker LexA two-hybrid system (Clontech) was used according to the manufacturer's instructions. RAP6 domains were cloned into the pB42AD vector, while Rab5 constructs were cloned into the plexA vector. These plasmids were transformed into the reporter strain EGY48, using the yeast transformation Kit (Sigma). To assess the expression of the LEU2 reporter, transformants were grown on plates that lack leucine, histidine, tryptophan, and uracil, containing 2% galactose/1% raffinose at 30 °C for 5 days. Expression of the lacZ reported gene was assessed using the yeast β-galactosidase assay kit according to the manufacturer's instructions (Pierce, Rockford, IL). Relative units are expressed as the β-galactosidase activity/yeast OD_{600}.

Pull-down assay. HeLa cell lines were solubilized at 4 °C in 20 mM Tris–Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and a mixture of protease inhibitors (Sigma). After a 15-min centrifugation at 15,000*g*, each supernatant was incubated for 1 h with 50 μg of immobilized GST fusion proteins at 4 °C. After washing three times, bound protein was eluted by SDS-sample buffer. Solubilized protein was separated by SDS–PAGE, silver-stained, and analyzed by mass spectrometry. Alternatively, the solubilized protein was transferred to nitrocellulose filters and probed with specific antibodies.

Rab5 Guanine nucleotide exchange assays. Rab5 guanine nucleotide release assays were performed by monitoring the time-dependent release of [^{3}H]GDP (Amersham) from 185 pmol of His6-Rab5:WT in the presence of 300 pmol GST alone, GST-RAP6:RGD or GST-RAP6:Vps9 as previously described [7]. The amount of phosphate released was measured by scintillation counting. His6 and GST fusion proteins were purified using either Ni–NTA–agarose (Qiagen) or GSH–agarose (Sigma) according to the manufacturer's protocol, respectively.

Ras GTPase stimulating assays. The GAP activity each purified GST fusion protein (GST alone, GST-*RAP6*:*RGD* and *GST-RAP6*:*Vps9*). Eighty micorliters of [γ-^{32}P]GTP charged H-Ras protein (0.1 nM Ras-GTP final concentration) was mixed with 377.5 μl of 2 × reaction buffer (40 mM Na Hepes, 2 mM $MgCl_2$), 80 μl BSA (10 mg/ml), and 200 μl of double-distilled H_2O. Serial 1:2 dilutions of each GST fusion proteins were made and kept on ice. Sixty microliters of Ras mix was prewarmed at 25 °C for 1 min followed by the addition of 15 μl of diluted GAP proteins. The reactions were incubated at 25 °C for 10 min, quenched, and then stopped at the appropriate time point as previously described [20]. The amount of phosphate released was measured by scintillation counting.