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Novel interaction of Rab13 and Rab8 with endospanins

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ABSTRACT

Rab GTPases regulate vesicular traffic in eukaryotic cells by cycling between the active GTP-bound and inactive GDP-bound states. Their functions are modulated by the diverse selection of effector proteins that bind to specific Rabs in their activated state. We previously described the expression of Rab13 in bone cells. To search for novel Rab13 interaction partners, we screened a newborn rat bone marrow cDNA library for Rab13 effectors with a bacterial two-hybrid system. We found that Rab13 binds to the C-terminus of Endospanin-2, a small transmembrane protein. In addition to Rab13 also Rab8 bound to Endospanin-2, while no binding of Rab7, Rab10, Rab11 or Rab32 was observed. Rab13 and Rab8 also interacted with Endospanin-1, a close homolog of Endospanin-2. Rab13 and Endospanin-2 colocalised in perinuclear vesicular structures in Cos1 cells suggesting direct binding also *in vivo*. Endospanin-2 is implicated in the regulation of the cell surface growth hormone receptor (GHR), but the inhibition of Rab13 expression did not affect GHR cell surface expression. This suggests that the Rab13–Endospanin-2 interaction may have functions other than GHR regulation. In conclusion, we have identified a novel interaction for Rab13 and Rab8 with Endospanin-2 and Endospanin-1. The role of this interaction in cell physiology, however, remains to be elucidated.

1. Introduction

Vesicular trafficking is precisely monitored in eukaryotic cells to maintain specific features of intracellular organelles and membrane domains. Rab proteins constitute the largest subfamily of Ras small GTPases and more than 60 Rabs in humans and 11 in yeast [1] have been identified. They are important regulators of all steps of vesicular trafficking, including vesicle formation, movement along cytoskeletal tracks, tethering and vesicle fusion to the target membrane [2].

Rab proteins cycle between their membrane-associated activated GTP-bound and cytosolic inactivated GDP-bound conformations. They bind to several types of proteins that regulate their activity or membrane association and further increase the specificity of their function. Rab effectors are functionally defined as molecules that selectively bind to specific Rabs in their GTP-bound state and modulate their downstream effects [2]. Diverse functions of Rabs in different cell types or in distinct vesicular pathways within cells are most likely defined by the unique set of effectors present.

Rab13 is a small GTPase with an array of functions in different cell types, from regulation of tight junctions to neuronal plasticity, cell migration and glucose transporter trafficking [3–6]. We have previously described the expression of Rab13 in bone cells including osteoclasts. In bone-resorbing osteoclasts Rab13 is located in small vesicular structures between the trans Golgi network and the non-bone facing plasma membrane. It is not involved in bone degradation process, nor associates with early or recycling endosomes. The vesicle type and the cargo of Rab13-positive vesicles in osteoclasts remain still to be identified [7]. In this study, we screened for novel Rab13 effectors in rat bone marrow and describe the interaction of Rab13 with Endospanin-2, a small transmembrane protein.

2. Materials and methods

2.1. Antibodies

Goat polyclonal GST and mouse monoclonal β-actin antibodies were purchased from Sigma–Aldrich. The BD living colours Full-length A.v. rabbit polyclonal antibody used to detect GFP-Rab fusion proteins was from Clontech Takara Bio. The mouse monoclonal Ha.11

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; GHR, growth hormone receptor; GST, glutathione-S-transferase; HA, human influenza hemagglutinin; MBP, maltose binding protein; OB-R, leptin receptor; VPS55, vacuolar protein sorting 55.

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antibody (clone 16B12) was obtained from Covance. Polyclonal rabbit Rab13 antibody was purchased from Atlas antibodies, and the mouse anti-rat β_3-integrin monoclonal antibody was a generous gift from Dr. M.A. Horton, University College London, UK. Mouse monoclonal antibodies for the growth hormone receptors; mAb263 and Mab5 were purchased from Abcam and Santa Cruz, respectively. HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Dako and the HRP-conjugated anti-goat antibody was purchased from Zymed Laboratories. Alexa Fluor 488- or 546-conjugated secondary antibodies were purchased from Molecular Probes.

2.2. Cell lines and transfections

Easily transfectable Cos1 cells were used in immunoprecipitation and colocalisation studies, and HeLa cells were used in siRNA silencing studies. LNCaP cells (an androgen sensitive prostate adenocarcinoma cell line) were used in growth hormone receptor studies [8]. Cos1 and HeLa cells were cultured in DMEM (Gibco) supplemented with 10% inactivated foetal calf serum (iFCS, Gibco), Glutamax (Gibco) and penicillin/streptomycin (Gibco) at 37 °C under a 5% CO_2 atmosphere. LNCaP cells were cultured in RPMI (Lonza) supplemented with 15% iFCS, Glutamax, Hepes (Lonza), sodium pyruvate (Lonza) and penicillin/streptomycin. Cos1 cells were transfected using Fugene 6 (Roche Diagnostics), HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) and LNCaP cells were transfected using Amaxa Nucleofector (Lonza) with Amaxa Cell Line Nucleofector Kit R.

2.3. Plasmid constructs and siRNA molecules

Rat Rab13 and Rab7 reading frames were cloned into a pBT vector to generate the bait plasmids for the bacterial two-hybrid screen. The construction and cloning of the rat trabecular bone marrow cDNA library into a pTRG plasmid is described elsewhere [9]. Reading frames of rat Rab7, Rab13, Rab11 and Rab32 were cloned into a pGEX-4T-1 plasmid (Amersham Biosciences) to produce glutathione-S-transferase (GST)-Rab fusion proteins. The last 20 C-terminal amino acids of rat Endospanin-2 or Endospanin-1 were cloned into pMAL-c2e plasmid (New England Biolabs) to produce Maltose Binding Protein (MBP)-fusion proteins.

Human Rab13 and Rab32 reading frames were cloned into a pEGFP-Actin plasmid (Clontech Laboratories, Inc.) to produce GFP-tagged Rab eukaryotic overexpression vectors. A eukaryotic expression vector for full-length rat Endospanin-2 was generated by cloning the Endospanin-2 reading frame into a pcDNA3.1(Neo)(+)-plasmid (Invitrogen) with a human influenza hemagglutinin (HA)-tag (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) inserted into its first extracellular loop. Rab13 mutants defective in GTP hydrolysis (Rab13Q67L) or in GDP-to-GTP exchange (Rab13T22N) were generated with QuickChange Site-Directed Mutagenesis Kit (Stratagene). GFP-Rab8 and GFP-Rab10 eukaryotic expression vectors were from Yi Sun (Program in Cell Biology, The Hospital for Sick Children, Toronto, ON, Canada). The human Rab13 siRNA molecule (target sequence: cagggcaaacataaatgtaaa) and non-related negative control siRNA molecule were purchased from Qiagen.

2.4. Bacterial two-hybrid screen

pBT-Rab13Q67L was used as bait to screen the bone marrow cDNA library [9] in the Bacteriomatch II Two-Hybrid System with Electrocompetent Cells (Stratagene). Positive interactions were demonstrated by bacterial growth in the selective medium lacking histidine and in the presence of 5 mM 3-amino-1,2,4-triazole (Sigma). Positive interactions were verified in a secondary screen and validated by individual retransformation of purified target plasmids with the bait plasmids.

2.5. In vitro pull-down experiments

MBP- or GST-fusion proteins were produced by bacterial expression and cellular pellets were collected. Cells were suspended in binding buffer (50 mM Tris, 300 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100 and protease inhibitors (Roche), pH 7.2) and lysed by sonication. Equimolar amounts of MBP-bait proteins (~10 μg) were incubated with GST-target proteins (~2 μg) for 2 h at 4 °C together with amylose resin beads (New England BioLabs). The beads were washed, and bound proteins were eluted in 10 mM maltose and processed for western blotting.

MBP-fusion proteins were bound to amylose resin beads and incubated with HeLa or Cos1 cell lysates in binding buffer for 2 h at 4 °C. The beads were washed, and bound proteins were eluted and processed for western blot analysis.

2.6. Immunoprecipitation

Cos1 cells co-transfected with pCDNA-Endospanin-2HA and pEGFP constructs were lysed in binding buffer (50 mM Tris, 150 mM NaCl, phenylmethylsulfonyl fluoride, 0.5% Triton X-100 and protease inhibitors). Equal amounts of cell lysate were incubated with GFP antibody and protein A magnetic beads (Invitrogen) for 2 h at 4 °C. After being washed, the bound proteins were eluted in Laemmli buffer and subjected to immunoblotting.

LNCaP protein lysate was incubated with or without the mAb263 anti-growth hormone receptor (GHR) antibody together with protein G magnetic beads (Invitrogen) in binding buffer for 2 h at 4 °C. After a washing step, the bound proteins were eluted in Laemmli buffer and subjected to western blotting with an Mab5 anti-GHR antibody.

2.7. Immunofluorescence

Forty-eight hours post-transfection, Cos1 cells were rinsed with PBS and fixed in 3% paraformaldehyde. Free aldehyde groups were quenched in 50 mM NH_4Cl and cells were permeabilised in 0.1% Triton-PBS on ice. Nonspecific binding was blocked with 2% BSA before incubation with an Ha.11 antibody. After washing steps, primary antibody binding was visualised using Alexa Fluor 488- or 546-labelled secondary antibodies. The cells were observed with a Leica TCS-SP confocal laser scanning microscope equipped with an Argon–Krypton laser (Leica Microsystems). Confocal images were acquired by sequential scanning.

2.8. Flow cytometry

Twenty-four hours after siRNA transfection, LNCaP cells were serum-starved for 20 h and detached using non-enzymatic Cell Dissociation Solution (Sigma–Aldrich). Cells were washed in ice-cold PBS containing 0.5% BSA and 2% normal goat serum and incubated with an anti-GHR antibody (mAb263) for 90 min on ice in the same buffer. The cells were washed three times, and treated with Alexa Fluor 488-conjugated anti-mouse antibodies. After being washed, 10^5 cells were analysed with a FACScan flow cytometer (Becton-Dickinson) and Cell Quest data acquisition and analysis software. Background fluorescence was excluded from the analysis by gating.

2.9. Osteoclast isolation, RNA purification and PCR

All animal experiments were carried out in accordance with EU Directive 2010/63/EU for animal experiments. Osteoclasts were isolated from the bone marrow of newborn rats with anti-integrin β_3-coated magnetic beads as previously described [10]. RNA was purified using a Total RNA Isolation Kit (Qiagen), and mRNAs were reverse transcribed with Superscript III Reverse Transcriptase (Invitrogen). PCR reactions were incubated in a thermal cycler (Eppendorf) with