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Original Articles

EGF-stimulated activation of Rab35 regulates RUSC2–GIT2 complex formation to stabilize GIT2 during directional lung cancer cell migration

Biao Duan [a], Jie Cui [b,c], Shixiu Sun [a], Jianchao Zheng [a], Yujie Zhang [b,d], Bixing Ye [e], Yan Chen [b], Wenjie Deng [a], Jun Du [a,d], Yichao Zhu [a], Yongchang Chen [f], Luo Gu [a,b,d,*]

[a] Department of Physiology, Nanjing Medical University, Nanjing, Jiangsu 210029, China
[b] Department of Biochemistry and Molecular Biology, Nanjing Medical University, Nanjing, Jiangsu 210029, China
[c] Department of Physiology, Xuzhou Medical University, Xuzhou, Jiangsu 221004, China
[d] Jiangsu Key Lab of Cancer Biomarkers, Prevention and Treatment, Collaborative Innovation Center For Cancer Personalized Medicine, Nanjing Medical University, Nanjing, Jiangsu 211166, China
[e] Department of Gastroenterology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China
[f] Department of Physiology, School of Medicine, Jiangsu University, Zhenjiang, Jiangsu 212013, China

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ABSTRACT

Non-small cell lung cancer (NSCLC) remains one of the most metastasizing tumors, and directional cell migration is critical for targeting tumor metastasis. GIT2 has been known to bind to Paxillin to control cell polarization and directional migration. However, the molecular mechanisms underlying roles of GIT2 in controlling cell polarization and directional migration remain elusive. Here we demonstrated GIT2 control cell polarization and direction dependent on the regulation of Golgi through RUSC2. RUSC2 interacts with SHD of GIT2 in various lung cancer cells, and stabilizes GIT2 (Mazaki et al., 2006; Yu et al., 2009) by decreasing degradation and increasing its phosphorylation. Silencing of RUSC2 showed reduced stability of GIT2, defective Golgi reorientation toward the wound edge and decreased directional migration. Moreover, short-term EGF stimulation can increase the interaction between RUSC2 and GIT2, prolonged stimulation leads to a decrease of their interaction through activating Rab35. Silencing of Rab35 also reduced stability and phosphorylation of GIT2 and decreased cell migration. Taken together, our study indicated that RUSC2 participates in EGFR signaling and regulates lung cancer progression, and may be a new therapeutic target against lung cancer metastasis.

Introduction

Directional cell migration is important for various physiological processes such as embryonic development, angiogenesis, tumor invasion and metastasis [1]. It involves the coordination of multiple subcellular processes such as reorganization of cell adhesion, actin assembly and membrane remodeling. In order to migrate effectively, polarization signals and morphology must be stabilized to form a dominant leading-edge protrusion [2–4]. For this, various signaling molecules are essential during directional migration. In particular, the small GTPases and their guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) play key roles in regulating the reorganization of the cytoskeleton and intracellular trafficking [5–9].

Among the signaling molecules that are related to small GTPases, G protein-coupled receptor kinase interacting ArfGAP 2 (GIT2) is involved in diverse cellular processes such as cytoskeletal dynamics, membrane trafficking and focal adhesion turnover [10–14]. As one of the GAPs of ARF6, GIT2 is essential for down-regulating ARF6 activity to maintain osteoclast polarity [15]. And its GAP activity for ARF6 might be in coordination with the phosphatidylinositol 3-kinase (PI3K) signaling pathway [16]. In addition to GAP activity, GIT2 also regulates cell polarity and orientation through other activities. Research has shown that interaction between paxillin and GIT2 was essential for normal cell spreading and generation of lamellipodia [17]. In GIT2-deficient mouse neutrophils, loss of GIT2 resulted in a partial loss of directionality and defective chemotaxis toward various chemoattractants, such as N-formyl-MetLeu-Phe (fMLP), C5a and IL-8 [18]. Similar to this result, the GIT–PIX complex was involved in the regulation of chemotaxis in the fMLP-stimulated rat

Abbreviations: SHD, Spa2 homology domain; GIT1/2, G protein-coupled receptor kinase interacting ArfGAP 1and 2; RUSC2, RUN and SH3 domain containing 2; EGF, epidermal growth factor; ALP, alkaline phosphatase; CHX, cycloheximide; NSCLC, non-small cell lung cancer.

* Corresponding author. Tel.: +86 02586862016; fax: +86 02586862016.
E-mail address: lgu@njmu.edu.cn (L. Gu).

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basophilic leukemia RBL-2H3 cell line [19]. Knockdown of GIT2 in HeLa cells also caused aberrant cell spreading through dysregulation of Rac1 [20]. Importantly, GIT2 is also phosphorylated by FAK/Src and its tyrosine phosphorylation is necessary for cell spreading and directional migration [21]. In fibroblasts, knockdown or expression of phosphorylation mutants of GIT2 led to dysregulation of Rac1 and PAK activities, reduction of Cdc42 and Erk signaling, as well as perturbation of front–rear cell polarity and directional cell migration upon PDGF stimulation [22]. Recent studies indicated that GIT2/Vav2 and GIT2/β-PIX signaling played a coordinate role in directional persistence and polarization in HT1080 cells in response to adhesion and EGF stimulation [23]. These studies suggest that GIT2 is an integral component of directional migration, cell polarization and growth factor signaling. However, the mechanism of GIT2 regulating cell polarization and directional migration remains to be clarified. Here, we identified GIT2 interacts with RUSC2, and involves a possible regulation through affection of Golgi to control directional cell migration.

RUSC2, as a poorly researched multidomain protein, has been identified as a putative binding partner of GIT2 through yeast two-hybrid screen [24]. RUSC2 contains a SH3 domain, two polyproline stretches and a RUN domain, which is linked particularly to the functions of Rap and Rab GTPase families and might interact with motor proteins such as kinesin or myosin [25–27]. As an ubiquitously expressed protein, RUSC2 can trap GTP-Rab35 specifically, and overexpression of RBD35 (Rab35-binding site of RUSC2) strongly inhibits nerve growth factor-induced neurite outgrowth of PC12 cells [28]. RUSC2 also has been predicted to be involved in vesicle-mediated transport and secretory pathway, and even regulate cell polarization and directional migration [29]. In addition, Rab35, as a member of Rab GTPase families, has been implicated in diverse processes that include T-cell receptor recycling, neurite outgrowth, cytokinesis and actin dynamics [30–33]. Our previous studies have shown that Rab35 acted upstream of Rac1 to regulate Wnt5a-induced MCF-7 cell migration [34]. However, whether the interaction between Rab35 and RUSC2 mediates directional cell migration has not been determined.

In this study, we hypothesize that Rab35 may regulate localization of RUSC2 through binding to RUSC2 to promote phosphorylation and stabilization of GIT2 during directional cell migration. Here, we show RUSC2 plays a crucial role in EGF-induced directional cell migration through forming a complex with GIT2. We moreover show that activation of Rab35 by EGF is crucial to promote the intracellular association between RUSC2 and GIT2 contributing successful phosphorylation and stabilization of GIT2 and directional cell migration.

Materials and methods

Cell lines and cell culture

Human cell lines (H1299, A549, H446, SPC-A-1, PC9, HEK-293T) were obtained from the Cell Biology Institute of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose) (Gibco, Thermo Scientific, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) and antibiotics (100 U/ml streptomycin and 100 μg/mL penicillin) (Invitrogen, USA) in a humidified incubator at 37 °C with 5% CO_2. Cells were grown on coverslips for fluorescence staining and on plastic dishes for protein extraction. Cells were starved in serum-free medium for 12 h, and then treated with EGF (R&D Systems, Minneapolis, MN, USA) for various time periods as indicated before harvest.

Plasmids and siRNAs

The pSV-HA-RUSC2 plasmid, pBK(Δ)-Flag-GIT2-long plasmid, and pCMV-Flag-GM130 and pEGFP-Rab35 (WT, S22N and Q67L) plasmids were kindly provided by Dr. Angelika Barnekow (Department of Experimental Tumorbiology, University of Muenster, Badestr. 9, D-48149 Muenster, Germany), Dr. Richard T. Premont (Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, NC 27710, USA), Dr. Shilai Bao (Key Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China) and Dr. Matthew P. Scott (Department of Medicine, Stanford University, Stanford, California, USA), respectively. Full-length RUSC2 cDNA was amplified from pSV-HA-RUSC2 plasmid using the following primer set, sense: 5′-CCGGAATTCCTTATGGATAGTCCCCCAAAGC-3′ and antisense: 5′-CCGCTCGAGATTCAGTTTTGGCTGCTTCCAG-3′ or sense: 5′-CCGGAATTCAGATGGATAGTCCCCCAAAG-3′ and antisense: 5′-CCGCTCGAGTCAGTTTTGGCTGCTTCCAG-3′. In these primers, EcoR I and Xho I restriction site sequences have been underlined. The polymerase chain reaction (PCR) products were cloned into the pCMV-N-Flag (Beyotime, Nantong, China) or pEBG vector. Full-length GIT2 cDNA was amplified from pBK(Δ)-Flag-GIT2-long using the following primer set, sense: 5′-TTGCGGCCGCATGTCGAAACGGCTCC-3′ and antisense: 5′-CCGCTCGAGGTTGTTGTTCTCTTTGG-3′ or sense: 5′-ACGCGTCGACGATGTCGAAACG GCTCCG-3′ and antisense: 5′-CCGCTCGAGTCAGTTGTTGTTCTCTTTGG-3′. Sal I, Not I and Xho I restriction site sequences have been underlined. The PCR products were cloned into the pCMV-C-HA (Beyotime, Nantong, China) or pEBG vector. RUSC2 cDNA fragments corresponding to the RUSC2-N1 (aa 1–1429), RUSC2-N2 (aa 1–1239), RUSC2-C1 (aa 984–1516), and RUSC2-C2 (aa 1256–1516) were amplified by PCR and ligated into pEBG. GIT2 cDNA fragments corresponding to the GIT2-N1 (aa 1–642), GIT2-N2 (aa 1–265), GIT-C1 (aa 125–759), and GIT-C2 (aa 297–759) were amplified by PCR and ligated into pEBG. The cells were seeded in 6-well plates, cultured to 80–90% confluence, and then transiently transfected with those plasmids by using ExFect™ Transfection Reagent (Vazyme Biotech, Piscataway, NJ, China) according to the reverse transfection method provided by the manufacturer.

Duplex oligonucleotides were chemically synthesized and purified by China GenePharma Co. The small interfering RNA (siRNA) duplexes used were RUSC2, #1, 5′-CCCUGGAGUUCUG GUUUAAdTdT-3′, #2, 5′-GCACCCUCUACAACAAGAUdTdT-3′, and #3, 5′-GGGACAAGUA UACACGAAUdTdT-3′; and GIT2, #1, 5′-CCUCGUGGAAA UACAGUAUdTdT-3′, #2, 5′-CUG CCAAAGAUCUUAGCAAdTdT-3′, and #3, 5′-GACCCUGCGUCUAUUAUGAdTdT-3′; and Rab35, #1, 5′-GCAGCAACAACAGAACGAUdTdT-3′, #2, 5′-GCUCACGAAGAACAGUAAA dTdT-3′ and #3, 5′-GAUGAUGUGUGCCGA AUAUdTdT-3′. Cells were transfected with siRNA duplexes using Lipofectamine 2000 (Invitrogen) according to the reverse transfection method provided by the manufacturer.

Reagents and antibodies

EGF was purchased from R&D systems (Minneapolis, MN, USA). Cyclohexamide (CHX) was purchased from Sigma (USA). Phospho-ERK, AKT, phospho-GIT2 (Tyr392), GFP and HA rabbit mAb were purchased from Cell Signaling Technology (Danvers, MA, USA). GIT2 and GM130 rabbit mAb were purchased from Cell Signaling Technology for western blotting and immunofluorescence, and GIT2 goat pAb and phospho-AKT pAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) for immunoprecipitation. RUSC2 rabbit pAb was purchased from Abcam (USA). Rab35 rabbit pAb was purchased from Biogot Technology (Nanjing, Jiangsu, China). Flag mouse mAb was purchased from Sigma for immunofluorescence and immunoprecipitation, and ABclonal (USA) for western blotting. GST mouse mAb were obtained from cwbiotech (Beijing, China). GAPDH rabbit mAb and HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology.

Western blotting

Sample protein extraction and concentration determination of whole cells were performed as previously described [34]. Briefly, equal amounts of protein were run on SDS polyacrylamide gels and transferred to nitrocellulose membrane. The resulting blots were blocked with 5% non-fat dry milk and incubated with primary antibodies overnight at 4 °C. Then protein bands were detected by incubating with HRP-conjugated secondary antibodies (Santa Cruz, CA, USA) for 1-2 h at room-temperature and visualized with ECL reagent (Millipore, Billerica, MA, USA) by ChemiDoc XRS+ gel imaging system (Bio-Rad, USA). Densitometry analysis was performed using Quantity One software, and band intensities were normalized to those of GAPDH.

In vitro pull down assay and immunoprecipitation

All cells were lysed with cell lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3VO_4, 0.5 μg/ml leupeptin, 1 mM PMSF). For in vitro binding assays, GST fusion proteins were first purified on MagneGST glutathione particles (Promega, Madison, WI). 500 μg of HEK-293T cell lysates transfected with GST fusion proteins was then incubated with 500–800 μg of HEK-293T cell lysates transfected with target proteins. For coprecipitation assays, 500 μg of cell lysates was subjected to immunoprecipitation, and proteins precipitated were detected by western blot, as described previously [34]. Antibodies against GIT2 and HA were used at dilutions of 1:50, 1:100, and 1:100 for immunoprecipitation, respectively.

Measurement of Rab35 activity

The Rab35-binding domain (RBD35) of mRUSC2 (aa 739–862) was amplified by PCR and ligated into pGEX-2T using EcoRI and SalI sites. It was generated as previously reported. GST-RBD35 was expressed in bacteria and purified by MagneGST