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RLIP76 regulates Arf6-dependent cell spreading and migration by linking ARNO with activated R-Ras at recycling endosomes

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R-Ras small GTPase enhances cell spreading and motility via RalBP1/RLIP76, an R-Ras effector that links GTP-R-Ras to activation of Arf6 and Rac1 GTPases. Here, we report that RLIP76 performs these functions by binding cytohesin-2/ARNO, an Arf GTPase guanine exchange factor, and connecting it to R-Ras at recycling endosomes. RLIP76 formed a complex with R-Ras and ARNO by binding ARNO via its N-terminus (residues 1-180) and R-Ras via residues 180-192. This complex was present in Rab11-positive recycling endosomes and the presence of ARNO in recycling endosomes required RLIP76, and was not supported by RLIP76(Δ1-180) or RLIP76(Δ180-192). Spreading and migration required RLIP76(1-180), and RLIP76(Δ1-180) blocked ARNO recruitment to recycling endosomes, and spreading. Arf6 activation with an ArfGAP inhibitor overcame the spreading defects in RLIP76-depleted cells or cells expressing RLIP76(Δ1-180). Similarly, RLIP76(Δ1-180) or RLIP76(Δ180-192) suppressed Arf6 activation. Together these results demonstrate that RLIP76 acts as a scaffold at recycling endosomes by binding activated R-Ras, recruiting ARNO to activate Arf6, thereby contributing to cell spreading and migration.

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1. Introduction

Migrating cells rely on the concerted actions of small GTPases to regulate cytoskeletal dynamics, to establish and maintain polarity, and to modulate the formation and release of ECM attachments [1,2]. Formation of new integrin attachments at the cell front stimulates activation of Arf6 and Rac1 GTPases, which cause localized actin polymerization to create lamellipodia [3–6]. Similarly, integrin-mediated adhesion of detached cells to ECM substrates leads to rapid un-polarized Arf6 and Rac1 activation, resulting in lamellipodial extension around the cell perimeter in a process known as cell spreading [7]. Spreading, lamellipodial extension, and cell migration are thus closely related Arf6-and Rac1-mediated events. We have shown that the Ras family GTPase R-Ras participates in Arf6-and Rac1-dependent cell spreading and motility [8].

R-Ras has unique functions distinct from other Ras family GTPases, such as stimulating integrins and formation of attachment structures [9–13], and adhesion-mediated Rac1 activation and cell migration [14,15]. R-Ras has an almost identical effector-binding region to Ras [16,17], and couples to common Ras effectors including Raf-1 (albeit at much lower affinity), RalGDS, RapL/NORE1 and PI3-K [12]. However, until recently no unique R-Ras effectors had been described to account for its functions distinct from Ras and Rap1. We identified RLIP76 (Ral-interacting protein of 76 kDa, also Ral-binding protein 1 or RalBP1) as a requisite R-Ras-specific effector in R-Ras-dependent cell spreading and migration [8,18].

We previously showed that the role of RLIP76 in R-Ras-dependent spreading and migration is to regulate activation of Rac1 and

Abbreviations: Arf, ADP ribosylation factor; ArfGAP, Arf GTPase activating protein; ArfGEF, Arf guanine nucleotide exchange factor; ECM, extracellular matrix; GTP, guanosine triphosphate; GTPase, GTP hydrolyzing enzyme; RE, recycling endosome(s).

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Arf6. The N-terminal domain of RLIP76 interacts with ARNO (cytohesin 2), a Sec7-domain-containing ArfGEF which activates both Arf1 and Arf6 and promotes Arf6-dependent activation of Rac1 [19–21]. Furthermore, ARNO over-expression rescued the spreading defect in RLIP76-depleted cells [8]. The sub-cellular localization of GTPases and of the GEFs and GAPs which control their activities plays an important role in spatial segregation of signaling by small G proteins such as Arf6 [22,23]. We hypothesized that RLIP76 interaction with ARNO may contribute to its effects on R-Ras-dependent Arf6 activation leading to cell spreading and migration. In this study we describe an Arf6 activation complex consisting of activated R-Ras, RLIP76 and ARNO, localized to recycling endosomes. We present a model for a molecular mechanism of R-Ras stimulation of Arf6 leading to cell spreading and motility.

2. Materials and methods

2.1. Antibodies and reagents

Monoclonal anti-Rac1 antibody (23A8) was obtained from Millipore. α−human RalBP1 (RLIP76), α−R-Ras, α-Arf6, α-GFP, α-FLAG, α-myc and α-ARNO antibodies were from Santa Cruz Biotechnology, Inc. Anti-HA antibody was from Covance. Restriction endonucleases were from New England Biolabs.

2.2. Cell lines and transfections

RLIP76 knockout mice have been described previously(Awasthi et al., 2005). C57Bl/6 mice were from Jackson Laboratories (Bar Harbor, ME). Embryonic fibroblasts were derived from 8-week-old C57Bl/6 (wild type) and RLIP76-null female mice. At day E13, uteri containing embryos were removed and placed in ice-cold PBS. The embryos were separated from the uteri and then dissected, removing the head, organs, and appendages from the fetuses. The carcasses were placed in trypsin for 1 h, then centrifuged to remove larger particles. The cell suspensions were transferred to culture plates for propagation. Cell culture and transfections were as described [24]. All animal experimental procedures were approved by the Temple University Institutional Animal Care and Use Committee.

2.3. Complementary DNAs

pEGFP-C1 was from CLONTECH Laboratories, Inc. (Mountain View, CA). pEGFP-C1-Rac1 WT was a gift from Miguel del Pozo (Fundación Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain). pEGFP-R-Ras(G38V) was as described [24]. pEGFP-Rab11 (wt) was a gift from Eugene Tkatchenko (University of California, San Diego, La Jolla, CA). FLAG-ARNO was a gift from Lorraine Santy (Penn State University, State College, PA). Human RLIP76 cDNA containing a 5′ influenza HA tag and shRNA-insensitive (mismatch) mutations, RLIP76 truncation plasmids, and R-Ras(43N) were as described [8,19]. HA-RLIP76(Δ180-192) was generated by PCR from the same template using the following primer set: 5′-GGAGCCAGAGGTGCCTCTGGTTGCTGCTCAAAGTCA-CAATCGCGTTTTGGAAACTCCTTTGGCTGATGC-3′ and 5′-CATCAGC-CAAAGGAGTTTCCAAAACGCGATTGTGACTTTGAGCAGCAACCAGAGG CACCTCTGGCTCC-3'. For shRNA-mediated RLIP76 knockdown, complementary single-strand oligonucleotides with overhangs (5′-GATCCCCGTAGAGAGGACCATGATGTTTCAAGAGAACATCATGGTCCT CTCTAC TTTTTA-3′ and 5′-AGCTTAAAAAGTAGAGAGGACCATGAT GTTCTCTTGAAACATCATGGTCCTCTCTACGGG-3′) targeting human and mouse RLIP76 (IDT Technologies) were dissolved in TE buffer (10 mM Tris-Cl, pH 7.5, 0.1 mM EDTA). Sequence-scrambled complementary oligonucleotides were also obtained. Double-stranded oligonucleotides were generated by annealing in annealing buffer (100 mM KOAc, 30 mM HEPES-KOH, pH 7.4, and 2 mM MgOAc), and duplexes were ligated directly into HinDIII/BglII-digested pSUPER.retro.puro vector (OligoEngine).

2.4. Cell spreading and migration assays and microscopy

Cell spreading and confocal microscopy were performed as described previously [8,24].

2.5. Arf6 activation assays and immunoprecipitations

Adhesion-induced Arf6 activation and co-immunoprecipitation assays were performed as described previously [8,21].

2.6. Statistical analysis

One-way ANOVA followed by Fisher PLSD analysis was used for all statistical data analysis, using StatView (SAS). A 5% probability was considered significant. All experiments in this study were performed at least in triplicate, except where indicated otherwise, and for microscopy results representative images are shown.

3. Results

We recently identified the RLIP76 N-terminus (aa 1-180) as the ARNO interaction domain [19]. We sought to map the R-Ras interaction site in RLIP76 and determine whether R-Ras and ARNO can interact in a tri-molecular complex with RLIP76. RLIP76(Δ180), which does not interact with ARNO [19], retained R-Ras binding (Fig. 1A); however, a larger RLIP76 N-terminal fragment (1-192) also bound activated R-Ras; suggesting that aa 180-192 are important for R-Ras interaction. We mutated this region to a randomized sequence in the full-length protein (RLIP76(Δ180-192), which disrupted RLIP76 interaction with R-Ras(Fig. 1B), demonstrating that the 180-192 region of RLIP76 is responsible for R-Ras interaction. RLIP76(Δ180) and RLIP76(Δ180-192) also disrupted ARNO/R-Ras interaction (Fig. 1C). Thus, whereas ARNO interacts with RLIP76 in a region between aa 1-180, activated R-Ras binding to RLIP76 requires the region between aa 180-192. Together, these findings demonstrate that activated R-Ras, RLIP76 and ARNO can form a tri-molecular complex in cells, RLIP76 interacts with R-Ras and ARNO through distinct sites, and ARNO association with R-Ras requires RLIP76. Thus, RLIP76 acts as an adapter protein to link activated R-Ras to ARNO in cells.

3.1. R-Ras, ARNO and RLIP76 co-localize to recycling endosomes

R-Ras has been localized to Rab11-positive vesicles, presumed to be recycling endosomes (RE) [25,26]. To investigate a correlation of this property of R-Ras with RLIP76 localization, we assessed the cellular distribution of R-Ras and RLIP76 by confocal microscopy. WT R-Ras localized to Rab11-positive structures, likely RE, and RLIP76 was also partially localized to the Rab11-positive structures, overlapping with R-Ras (Fig. 1D). In addition, both R-Ras and RLIP76 were observed in other unidentified sub-cellular compartments and at the plasma membrane; these observations are also consistent with previous reports for R-Ras [25–27]. RLIP76 staining did not overlap with the distribution of GFP-tagged markers for early endosomes (Rab5) [28], cis-Golgi (GM130) [29], or cis-Golgi-to-ER transport vesicles (p23) [30], (Supplemental Figure 1). Thus, RLIP76 partially co-localizes with R-Ras in recycling endosomes.

We next investigated whether the R-Ras/RLIP76 complex associates with ARNO at RE. Both ARNO and R-Ras were present in Rab11-positive endosomes (Fig. 2A and B). ARNO was also observed in diffuse patterns throughout the cytosol and at the plasma

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