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Non-visual arrestins regulate the focal adhesion formation via small GTPases RhoA and Rac1 independently of GPCRs

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ABSTRACT

Arrestins recruit a variety of signaling proteins to active phosphorylated G protein-coupled receptors in the plasma membrane and to the cytoskeleton. Loss of arrestins leads to decreased cell migration, altered cell shape, and an increase in focal adhesions. Small GTPases of the Rho family are molecular switches that regulate actin cytoskeleton and affect a variety of dynamic cellular functions including cell migration and cell morphology. Here we show that non-visual arrestins differentially regulate RhoA and Rac1 activity to promote cell spreading via actin reorganization, and focal adhesion formation via two distinct mechanisms. Arrestins regulate these small GTPases independently of G-protein-coupled receptor activation.

1. Introduction

Cell migration and chemotaxis are essential processes in embryonic development, the inflammatory response, and play a key role in metastatic cancers [1–3]. The signaling mechanisms cells use to sense chemical gradients in their environment are complex and include multiple functional steps involving activation of chemokine G protein-coupled receptors (GPCRs), as well as other GPCRs [4,5] and a network of actin regulatory signaling pathways. To ensure correct navigation of different cells to distinct destinations, the availability of the guiding cues and the cell's responsiveness to them must be tightly controlled. Thus, as the cell migrates, signaling must be quenched at the trailing edge. Arrestins, together with their partners in the GPCR desensitization process, G protein-coupled receptor kinases, are known to play the key role in regulating the sensitivity to chemokines and the signaling of other GPCRs involved in migration [6,7]. Migration requires the coordinated activation of hundreds of proteins in distinct compartments of the cell [8]. Because arrestins are multi-functional regulators capable of orchestrating signaling and localizing proteins to distinct subcellular compartments [9,10], they are also likely to affect the activity of various signaling proteins involved in generating the forces that promote movement. Indeed, over the last few years, arrestins have emerged as important regulators of the actin cytoskeleton [11–13].

Rho family GTPases are small G proteins that act as molecular switches that regulate the signal transduction pathways connecting plasma membrane receptors to the cytoskeleton [14,15]. GTPases of the Rho family, which includes 20 proteins from three distinct types, Rho, Rac and Cdc42, control separate signal transduction pathways regulating the remodeling of actin cytoskeleton [15]. Rac activation induces the formation of protrusions known as lamellipodia that drive the cell migration. Cdc42 activity produces filopodia, a different type of cell protrusions involving actin polymerization [16]. Cdc42 activity may be involved in the control of the movement direction in response to external cues [17]. Rho proteins also regulate the actin-myosin contractility required to propel the cell forward [15,18]. The functional information about other members of the Rho family is limited.

There is growing evidence for a role of the non-visual arrestins in facilitating small GTPase-mediated events. First, in was shown that arrestin-2[2] activates the small GTPase RhoA coordinately with Gαq following the activation of the angitotensin II 1A receptor ($ATII_{1A}R$) [11]. Arrestin-2 also regulates RhoA activity by binding and inhibiting ARHGAP21, a RhoA GTPase activating protein, in response to $ATII_{1A}R$

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[2] Different names of arrestin proteins are used in the field. We use the systematic names of arrestins, where the number after the dash indicates the order of cloning: arrestin-1 (historic names S-antigen, 48-kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin).

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stimulation [19]. Arrestin-3 interacts with the actin treadmilling protein cofilin upon activation of another GPCR, PAR2 [13], and both arrestins inhibit PAR-2-stimulated Cdk2 activity [20]. In contrast, the transforming growth factor beta (TGF-beta) superfamily co-receptor, the type III TGF receptor, activates Cdk2 via direct interaction with arrestin-3, which leads to inhibition of directed cell migration [21]. Both arrestin-2 and -3 regulate small GTPase guanyl nucleotide dissociation stimulator ralGDS upon activation of the fMLP receptor [22], and activates the ELMO-ARF cascade upon stimulation of the calcium-sensing receptor [12]. Furthermore, arrestins interact with tumor suppressor PTEN, and this interaction is enhanced by stimulation of the G_{12}-coupled lysophosphatidic acid receptor and subsequent activation of RhoA [23]. In the context of 3-D culture, PTEN regulates the arrestin-2 interaction with ARHGAP21/Cdk2 and the activity of Cdk2, which is essential for the multicellular morphogenesis [24]. Thus, collectively the data suggests that arrestins could act both upstream as RhoA regulators as well as downstream as RhoA effectors.

We were interested in determining whether ubiquitous non-visual arrestins [10] regulate the activity of these GTPases. Arrestins have been shown to regulate a variety of proteins independently of G-protein coupled receptor (GPCR) activation [25–30], but the effect of arrestins on the small GTPases under basal conditions has not been explored. Recently we found that arrestins promote focal adhesion disassembly, likely by recruiting clathrin to microtubules targeting focal adhesions to facilitate intergrin internalization [31]. Here we show that arrestins regulate the actin cytoskeleton to limit cell spreading by affecting the activity of the small GTPases RhoA and Rac1 in a receptor-independent manner. We also show that, in addition to microtubule dependent FA disassembly, arrestin-mediated regulation of the small GTPase RhoA likely contributes to the FA phenotype in arrestin null cells.

2. Material and methods

2.1. Materials

Restriction endonucleases and other DNA modifying enzymes were from New England Biolabs (Ipswich, MA). Cell culture reagents and media were from Mediatech-Corning (Manassas, VA) or Life-technologies (Carlsbad, CA). DNA purification kits were from Zymo Research (Irvine, CA). All other reagents were from Amresco (Solon, OH) or Sigma-Aldrich (St Louis, MO).

2.1.1. Antibodies

Rhodamine-phalloidin (for actin staining) was from Invitrogen (Carlsbad, CA); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-HA, monoclonal anti-Cdc42 antibodies, anti-GFP monoclonal antibody, and monoclonal paxillin antibodies were from BD Biosciences (Palo Alto, CA); monoclonal rat from Roche Molecular Biochemicals (Indianapolis, IN) or monoclonal rabbit (from Cell Signaling Technology) anti-HA antibodies were used for cell staining; antibodies against mouse FAK were from Abcam (Cambridge, MA). Pan-arrestin mouse monoclonal F4C1 antibody [32] was a gift of Dr. L. A. Donoso. Monoclonal RhoA antibody was a part of the Rho activation pulldown kit (Cytoskeleton, Denver, CO). Anti-Rac1 mouse monoclonal antibody was from Cytoskeleton (Denver, CO).

2.2. Cell culture, transfection, and retroviral infection of cells

Arrestin DKO and WT MEF cell lines [33,34] (a generous gift from Dr. R.J. Lefkowitz, Duke University) were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin (P/S) at 37 °C and 5% CO_2. HA-tagged RhoN19, RhoV14, RacN17, RacV12 genes were cloned into PFB murine retrovirus vector (Stratagene). Cells were retrovirally infected after transfecting the retroviral vector using Lipofectamine 2000 (Sigma, St. Louis, MO) into Phoenix cell line. Fugene HD (Promega, Fitchburg, WI) (1:3 DNA:lipid) or Lipofectamine2000 (1:2.5 DNA:lipid) was used to transfect cells in some cases. Cells were kept in serum-free DMEM with 1% (P/S) for approximately 24 h prior to every experiment.

2.3. Protein preparation and Western-blotting

Cells were lysed in Lysis solution (Ambion) or 1% SDS lysis buffer and heated for 5 min at 95 °C. Protein concentration was measured with Bradford reagent (BioRad). The protein was precipitated with nine volumes of methanol, pelleted by centrifugation (10,000 × *g*, 10 min at RT), washed with 90% methanol, dried and dissolved in SDS sample buffer at 0.5 mg/ml. Equal amounts of protein were analyzed by reducing SDS-PAGE and Western blotting onto Immobilon-P (Millipore, Bedford, MA). The membrane was blocked with 5% non-fat dry milk in TBS with 0.1% Tween 20 (TBST) and incubated with appropriate primary and then secondary antibodies coupled with horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA) in TBST with 1% BSA. Bands were visualized with SuperSignal enhanced chemiluminescence reagent (Pierce, Rockford, IL) and detected by exposure to X-ray film. The bands were quantified using VersaDoc and QuantityOne software (BioRad).

2.4. GTPase pulldown assays

The levels of GTP-liganded Rho were analyzed using the Rho activation pulldown kit according to manufacturer's instructions (Cytoskeleton, Denver, CO). Equal volumes of cell lysates were used to measure total Rho. PAK1-PBD-conjugated glutathione-Sepharose beads were prepared as described [35]. Cells were serum-starved for 24 h and lysed. Equal volumes of lysates were added to 30 µl of PAK1-PBD–conjugated glutathione-Sepharose beads and incubated at 4 °C for 1 h with gentle rocking. After four washes with 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% Igepal CA-630, 50 mM MgCl2, 5 mM EDTA and 10% glycerol, the beads were resuspended in 15 µl of 2× Laemmli SDS sample buffer, boiled for 5 min, and resolved on 15% SDS-PAGE. Equal volumes of total lysates were run for comparison. The proteins were transferred to a nitrocellulose membrane and incubated with anti-Rac1 or anti-Cdc42 antibodies in 2% BSA at 4 °C overnight.

2.5. RhoA inhibition

WT cells were infected with retrovirus to express either dominant-negative RhoN19-HA or GFP and plated on fibronectin or poly-D-lysine for 2 h. Cells were stained with Rhodamine-phalloidin and anti-HA or anti-paxillin and anti-HA. Alternatively, cells were allowed to spread for 2 h, and then incubated with 0.5 µg/ml C3 Transferase (Cytokeleton, Denver, CO), a permeable Rho inhibitor, or with 1 µM Y-27632, a selective inhibitor for Rho-associated kinases (ROCK) (EMD Chemicals, Inc., Darmstadt, Germany) for 4 h. Cells were fixed and stained with rhodamine-phalloidin and anti-paxillin antibody.

2.6. Image and statistical analysis

Cell size. FA size, and FA number analysis was from 10 to 15 randomly selected fields per experiment, cell area was measured using Image J or Nikon NIS software. Cells with moderate expression of HA-GTPases were selected for cell size and focal adhesion number rescue measurements. Cells were imaged either on Nikon TE2000-E automated inverted microscope with 40x oil objective with additional 1.5 optic magnification or on LSM 510 Meta Confocal with 40X oil objective. Focal adhesion size and number were measured from confocal images using ImageJ with qualifications for focal adhesion area: 0.5–100 µm^2. Using threshold tool, global background staining was removed from the images, leaving only punctate focal adhesions. Using Analyze Particles feature of ImageJ, Focal adhesion size and number were binned with qualifications for focal adhesion area: 0.5–100 µm^2. When three or