Biochimica et Biophysica Acta 1863 (2016) 2189–2200

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Symmetry breaking in spreading RAT2 fibroblasts requires the MAPK/ERK pathway scaffold RACK1 that integrates FAK, p190A-RhoGAP and ERK2 signaling

Zuzana Klímová [a], Vojtěch Bráborec [a], Miloslava Maninová [a], Josef Čáslavský [a], Michael J. Weber [b], Tomáš Vomastek [a,*]

[a] Institute of Microbiology AS CR, Prague, Czech Republic
[b] Department of Microbiology, Immunology, and Cancer Biology, University of Virginia, Charlottesville, VA, USA

ARTICLE INFO

Article history:
Received 18 January 2016
Received in revised form 22 April 2016
Accepted 17 May 2016
Available online 19 May 2016

Keywords:
RACK1
ERK
FAK
p190A-RhoGAP
Polarity
Feed-forward loop

ABSTRACT

The spreading of adhering cells is a morphogenetic process during which cells break spherical or radial symmetry and adopt migratory polarity with spatially segregated protruding cell front and non-protruding cell rear. The organization and regulation of these symmetry-breaking events, which are both complex and stochastic, are not fully understood. Here we show that in radially spreading cells, symmetry breaking commences with the development of discrete non-protruding regions characterized by large but sparse focal adhesions and long peripheral actin bundles. Establishment of this non-protruding static region specifies the distally oriented protruding cell front and thus determines the polarity axis and the direction of cell migration. The development of non-protruding regions requires ERK2 and the ERK pathway scaffold protein RACK1. RACK1 promotes adhesion-mediated activation of ERK2 that in turn inhibits p190A-RhoGAP signaling by reducing the peripheral localization of p190A-RhoGAP. We propose that sustained ERK signaling at the prospective cell rear induces p190A-RhoGAP depletion from the cell periphery resulting in peripheral actin bundles and cell rear formation. Since cell adhesion activates both ERK and p190A-RhoGAP signaling this constitutes a spatially confined incoherent feed-forward signaling circuit.

1. Introduction

The establishment of cell polarity plays an essential role in many physiological and pathophysiological processes including cell motility, embryonic development, morphogenesis, immune response as well as the migration and dissemination of cancer cells. Polarized cells display asymmetrical distributions of protein complexes, signaling components and cytoskeletal networks that allow the spatial segregation and regulation of distinct intracellular processes [1–3]. Cell polarity also plays a critical role in motile adherent cells where it promotes directional migration. In migrating cells the polarity axis develops between a functionally and morphologically distinct protruding cell front and a retracting cell rear. The presence of a branched F-actin filament network and abundant dynamic adhesions are typical for the cell front. Dendritic actin polymerization and cellular protrusivity are limited at the cell sides and the cell rear where actin predominantly forms long actomyosin bundles that terminate in less abundant but large stable adhesions [4,5]. The spatial differences in the organization of the actin cytoskeleton and focal adhesions at the cell front and rear are reflected in asymmetrical cell shapes that range from conical or crescent to more irregularly shaped cells [6].

The establishment of a front–rear axis and the direction of migration are generally thought to be determined by external directional signals such as chemotactic gradients or mechanical stimuli and physical constraints. These stimuli induce the formation of protruding lamellipodium at cell front and the establishment of front–rear polarity axis. Once front–rear asymmetry is made, the actin cytoskeleton, microtubules and other cellular organelles are arranged along the front–rear axis, and this organization promotes directional cell migration [7–11]. The establishment of cell polarity and directional migration can occur spontaneously in the absence of external cues by a self-organizing process. Spontaneous polarization is initiated by the by the breaking of cell's radial symmetry and adoption of asymmetrical shape with segregated rear and front. Symmetry breaking seems to be prerequisite for the establishment of cell polarity and directional cell migration. Depending on the cell type, symmetry breaking can be induced either by the establishment of a single cell front with a localized increase in protrusion, or by the retraction of the cell rear [12].

Abbreviations: ERK, extracellular signal-regulated kinase; p190, p190A-RhoGAP; RACK1, receptor for activated C kinase 1; FAK, focal adhesion kinase.

* Corresponding author at: Institute of Microbiology AS CR, Videnska 1083, 142 20 Prague, Czech Republic.
E-mail address: vomy@biomed.cas.cz (T. Vomastek).

http://dx.doi.org/10.1016/j.bbamcr.2016.05.013
0167-4889/

Polarizing neutrophils break the symmetry by localized actin polymerization and protrusion formation that define the leading edge and precede the formation of cell rear [13,14]. On the contrary, in spontaneously polarizing fibroblasts and fish keratocytes the symmetry breaking is initiated by localized cell edge retraction that results in the establishment of cell rear [15,16].

Spontaneous symmetry breaking and cell polarization generally is believed to be initiated by stochastic fluctuations in the cellular environment that are amplified and stabilized by positive feedback signaling [17–20]. It is presumed that symmetry breaking could also be elaborated by the competition of locally activated signaling with globally exerted inhibition. Such an antagonistic signaling circuit composed of a short range activator–long range inhibitor system is sufficient for the generation of a signaling gradient from spatially homogenous conditions [3,19,21–23].

An important question in understanding the symmetry breaking is, what is the signaling mechanism that governs spatially restricted intracellular signaling from uniform external cues. Scaffold/adaptor proteins, because they are capable of promoting the activation of a signaling pathway in a specific subcellular compartment, represent one mechanism by which external or stochastically generated signals can be directed to specific locations [24]. We have previously shown that the scaffold protein RACK1 specifically controls activation of the extracellular signal-regulated kinase (ERK) MAP kinase and its localization to focal adhesions during integrin dependent adhesion [25]. Here, we show that the extracellular signal-regulated kinase (ERK) pathway scaffold RACK1 regulates symmetry breaking in radially spreading RAT2 fibroblasts by enabling cells to form a non-protruding cell rear. RACK1 promotes adhesion-mediated activation of ERK that in turn locally suppresses p190A-RhoGAP (hereafter p190) signaling by depletion of p190 from the cell periphery. We show that knockdown of RACK1 or ERK2 enables a uniform p190 cellular distribution and a uniform, non-polar spreading without symmetry breaking. Since both ERK and p190 are activated by cell adhesion we hypothesize that adhesion induces a signaling circuit termed "incoherent feed-forward loop" [22,26]. In this signaling circuit spatially restricted and sustained ERK signaling opposes globally activated p190 to induce cell rear formation.

2. Material and methods

2.1. Antibodies and materials

Following antibodies were used for immunofluorescence staining: *anti*-p190A-RhoGAP (mouse, BD Transduction Laboratories), *anti*-paxillin (clone 5H11, mouse, Upstate Biotechnology), *anti*-phospho ERK (rabbit, Cell Signaling) and fluorescent secondary goat anti mouse IgG labeled with Alexa Fluor 488 (Invitrogen). Avidin–Biotin blocking kit, biotinylated goat anti rabbit IgG and Texas Red–avidin were from Vector Laboratories. Actin was stained with rhodamine–phalloidin (Invitrogen) and nucleus was visualized by DAPI. Immunoblotting was performed with following antibodies: *anti*-RACK1 (clone B3, mouse, Santa Cruz), *anti*-p190A-RhoGAP (mouse, BD Transduction Laboratories), *anti*-FAK (clone 4.47; mouse, Upstate Biotechnology), *anti*-FAK (pY397) (rabbit, Invitrogen), *anti*-phosphotyrosine (clone 4G10, mouse, Millipore), anti-ERK1/2 (clone 3 A7, mouse, Cell Signaling), anti-p120RasGAP (mouse, ECM Biosciences). *Anti*-ERK2 (mouse) and *p*-ERK (rabbit) antibodies were described elsewhere [25]. Secondary HRP conjugated goat *anti*-mouse IgG and goat *anti*-rabbit antibodies were from Sigma-Aldrich. Glass-bottom dishes for life cell imaging were obtained from In Vitro Scientific.

2.2. Cell culture, plasmid and siRNA transfection

RAT2 cell lines were maintained in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). RAT2 cell line stably expressing mCherry–LifeAct was established by transfection of RAT2 cells with mCherry–LifeAct plasmid (kindly provided by Dr. D. Rosel) and by puromycin selection (3.5 μg/ml). RAT2-mCherry–LifeAct cells were maintained in DMEM with 10% FBS and puromycin (1 μg/ml). pcDNA5-mRFP-Actin [27] was transiently transfected using Lipofectamine 2000.

The siRNAs oligonucletides targeting FAK (GCTAGTGACGTATGGA TGT), p190A-RhoGAP (GGTGGTGACGATCTGGGCT), RACK1#1 (AAGG TGTGGAATCTGGCTAAC) and RACK1#2 (GCTAAAGACCAACCACATTTT) were described previously [25,28,29]. Additional siRNAs targeting RACK1 RACK1 #4 (CTGTCCAGGATGAGAGTCA), RACK1#5 (TCTGGCTA ACTGCAAGCTA) as well as Non-Specific control (AGGTAGTGTAATCG CCTTG) were used. Where indicated, RACK1 oligonucleotides were used as a pool of RACK1#1, RACK1#2 and RACK1#4 siRNAs. The sequence for the siRNA oligonucleotides targeting ERK1 and ERK2 are as follows: ERK1 #1 (GACCGGATGTTAACCTTTA), ERK1#2 (GAAACTACCT ACAGTCTCT), ERK2 #1 (AGTTCGAGTTGCTATCAAG), ERK2#2 (GGTGCC ATGGAACAGGTTG). To silence ERK1 and ERK2 expression RAT2 cells were transfected by siRNA pools for ERK1 (ERK1#1and ERK1#2) and ERK2 (ERK2#1 and ERK2#2). All siRNA oligonucleotides were synthesized with 3′TT overhangs by Eurofins MWG Operon. Specific siRNAs were transfected into RAT2 cells using the calcium phosphate protocol as described previously [25,28] and analyzed 48 h post-transfection.

2.3. Replating assay

To determine roundness index and cell area, cells were plated 2 days before in DMEM with 10% FBS to reach 60–80% confluency on the day of replating experiment. Cells were detached by trypsin, treated with trypsin inhibitor (1 μg/ml; Sigma-Aldrich), washed with serum free DMEM and kept in suspension at 37 °C for 60 min. Cells were then plated on fibronectin (10 μg/ml) coated dishes for indicated times. For quantification of cell shape phase contrast images were acquired from cells fixed with 2% paraformaldehyde.

2.4. Live cell microscopy and immunocytochemistry

For live cell fluorescence microscopy cells were replated on fibronectin (10 μg/ml) coated glass bottom dishes and cell live microscopy was performed at 37 °C using Olympus CellR imaging station (Olympus IX81 inverted microscope, MT20 illumination system and Olympus FV2T CCD camera). Time lapse phase contrast or epifluorescence images were captured with CellR software.

For phase contrast microscopy and immunostaining cells were kept in suspension for 60 min in medium with 2% FBS and replated on glass coverslips coated with fibronectin (10 μg/ml) for 60 min. Cells were fixed with 2% paraformaldehyde in PBS for 25 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min. Coverslips were blocked with 20% normal goat serum in PBS, stained with indicated antibodies and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories). Active ERK was visualized following modified Avidin–Biotin amplification protocol [25,30]. Briefly, cells were fixed in 1% paraformaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 4 mM $MgSO_4$, pH 6.9) supplemented with 0.2 mM vanadate and 50 mM β-glycerophosphate for 20 min and extracted with 1% CHAPS in PHEM buffer for 5 min. Cells were blocked in 20% normal goat serum in MBST (50 mM MOPS, 150 mM NaCl, 0.05% Tween 20, pH 7.4) for 1 h and for additional 30 min with 20% normal goat serum supplemented with Avidin. Cells were then incubated for 1 h with rabbit polyclonal *anti*-pERK antibody in 5% normal goat serum supplemented with Biotin followed by the incubation with biotinylated secondary goat *anti*-rabbit antibody conjugated to biotin (Vector laboratories) for 1 h, and by incubation with Texas Red–avidin for 30 min. Paxillin was detected with mouse monoclonal *anti*-Paxillin antibody and visualized with goat anti mouse IgG antibody labeled with Alexa Fluor 488. Fluorescent images were acquired by epifluorescence microscope Olympus IX81, MT20 illumination system and Olympus FV2T CCD camera (Fig. 6) or