Experimental Cell Research 352 (2017) 255–264

Contents lists available at ScienceDirect

Experimental Cell Research

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

The atypical Rho GTPase RhoD is a regulator of actin cytoskeleton dynamics and directed cell migration

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ARTICLE INFO

Keywords:
RhoD
Rho GTPase
Actin
Stress fiber
Cell migration

ABSTRACT

RhoD belongs to the Rho GTPases, a protein family responsible for the regulation and organization of the actin cytoskeleton, and, consequently, many cellular processes like cell migration, cell division and vesicle trafficking. Here, we demonstrate that the actin cytoskeleton is dynamically regulated by increased or decreased protein levels of RhoD. Ectopic expression of RhoD has previously been shown to give an intertwined weave of actin filaments. We show that this RhoD-dependent effect is detected in several cell types and results in a less dynamic actin filament system. In contrast, RhoD depletion leads to increased actin filament-containing structures, such as cortical actin, stress fibers and edge ruffles. Moreover, vital cellular functions such as cell migration and proliferation are defective when RhoD is silenced. Taken together, we present data suggesting that RhoD is an important component in the control of actin dynamics and directed cell migration.

1. Introduction

The actin cytoskeleton machinery is involved in many cellular processes, such as cell migration, morphogenesis, vesicle transport and cell division. A diverse range of proteins have been found to regulate actin filament reorganization, which is required for its highly dynamic properties. The Rho GTPases is a branch of the Ras protein superfamily and are known regulators of the actin cytoskeleton. Out of its 20 members, a limited number have been well studied, in particular RhoA, Cdc42 and Rac1, while the functions of the remaining members are poorly understood. One of the least studied proteins in this family, RhoD, was discovered more than 20 years ago as a protein regulating endosome dynamics and trafficking [1,2]. Many Rho GTPases are well conserved through evolution. RhoD, however, is a recent family member which most likely appeared by a gene duplication event in therians and is the only Rho GTPase to be expressed exclusively in mammals [3]. The Rho GTPases are small GTPases, which act as molecular switches, and bind to their effector proteins when in an active conformation, i.e. in a GTP-bound state. When GTP is hydrolyzed, a conformational change prevents interaction with effector proteins. The ability to bind and hydrolyze nucleotides is in turn regulated by three groups of proteins; the GEFs (Guanine nucleotide Exchange Factors), which exchange GDP for GTP, the GAPs (GTPase Activating Protein) which catalyze the GTP hydrolysis and GDIs (Guanosine nucleotide Dissociation Inhibitors), which sequester and inhibit the protein in an inactive GDP-bound state. However, such regulators have not been found for all Rho GTPases, including RhoD. Instead, regulation of those Rho GTPases has been proposed to occur at the transcriptional level and by protein degradation [4,5]. The Rho GTPases can be categorized into two groups, classical and atypical proteins [6]. An atypical Rho GTPase has a slower GTP hydrolysis than the classical ones (such as RhoA, Cdc42 and Rac1), or no hydrolysis at all, rendering the protein constitutively active. One example of an atypical Rho GTPase is RhoBTB2 (also called DBC2), which has an aberrant domain structure and might not bind GTP [7,8]. The Rnd1, 2 and 3 are examples of Rho GTPases with low hydrolysis activity, leading to a constitutive active conformation of the proteins [9,10]. Wrch1 is another example of an atypical Rho GTPase, which is constitutively active due to a high intrinsic nucleotide exchange activity. Since GTP exceeds the level of GDP by the factor of ten in the cell, the protein will quickly bind new GTP after hydrolysis, leaving the protein in an active state [11]. RhoD was first categorized as a classical Rho GTPase, but was later found to possess a high intrinsic nucleotide exchange activity and is therefore now considered an atypical Rho GTPase, which is also true for the RhoD-like GTPase Rif [12].

Most of the 20 Rho GTPase members have been found to control various aspects of actin cytoskeletal dynamics, which can be clearly

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http://dx.doi.org/10.1016/j.yexcr.2017.02.013
Received 22 November 2016; Received in revised form 7 February 2017; Accepted 10 February 2017
Available online 11 February 2017

seen when ectopically expressed in cells [13]. In addition to the original finding of RhoD as a regulator of endosome dynamics, the protein has been discovered to play roles in filopodia formation, ER-to-Golgi transport, Golgi homeostasis and receptor trafficking [13–16]. Processes like cell cycle regulation, centrosome duplication and cell migration have also been proven to be influenced by RhoD [17–19]. In this study, we widen the perspective of RhoD and show that increase or decrease of RhoD protein levels induces different actin cytoskeletal phenotypes in different cell types, and that fundamental cellular functions such as cell migration, chemotaxis and proliferation are to various degrees regulated by RhoD.

2. Materials and methods

2.1. Cell culture, transfection and immunofluorescence

BJ hTERT SV40T, HeLa, U2OS and U251MG were cultured in Dulbecco's modified Eagle's medium supplemented with 10% Fetal Bovine Serum (FBS) at 37 °C and 5% CO_2. When starved, cells were cultured overnight in DMEM media without serum. For plasmid transfections, JetPEI transfection reagent (PolyPlus Transfection) was used according to manufacturer's protocol. 24 h post transfection, cells were fixed in 3% paraformaldehyde for 20 min at 37 °C, followed by permeabilization in 0.2% Triton-X100/PBS and blocking in 5% FBS/PBS for 1 h. Samples were incubated 1 h each with primary and secondary antibodies, which were diluted in blocking solution. Myc-tagged murine RhoD wild-type, RhoD G26V, RhoD T31N and RhoD-SAAX (with cysteines at positions 206 and 207 replaced with serines) have been described before [16]. The murine RhoD T31N-SAAX mutant was subcloned into the pRK5-Myc vector and the murine RhoD G26V into the pRK5-mCherry vector. GFP-LifeAct was a generous gift from Roger Karlsson, Stockholm University, Stockholm, Sweden.

For siRNA transfections, SilentFect reagent (BioRad) was used according to manufacturer's protocol. 20 nM of RhoD siRNA; GAAGUGAAUCAUUUCUGCAtt or control siRNA (Ambion-Applied Biosystems) was used per transfection. siRNA-silenced cells were analyzed 48 h after transfection if not stated otherwise.

The following antibodies were used: mouse anti-phospho-tyrosine (PY99) 1:200 and rabbit anti-Myc 1:50 (Santa Cruz Biotechnology), mouse anti-Myc 1:500 (Nordic BioSite), aminomethylcoumarin (AMCA)-conjugated goat anti-mouse 1:50 (Jackson ImmunoResearch Laboratories), Alexa Fluor 488-conjugated donkey anti-rabbit 1:1000 (Invitrogen Molecular Probes). F-actin was stained either with tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin 1:250 (Sigma) or Alexa Fluor 488-conjugated phalloidin 1:500 (Invitrogen Molecular Probes).

Pictures were acquired with a Zeiss AxioVert 40 CFL microscope equipped with a Zeiss AxioCAM MRm digital camera and the AxioVision software. Quantifications were performed in blind when possible.

2.2. Live cell imaging

2.2.1. Actin dynamics

BJ hTERT SV40T and U251MG cells were transfected with GFP-LifeAct together with an mCherry-vector, or mCherry-tagged RhoD G26V. GFP-LifeAct was also transfected into cells treated with control or RhoD siRNA. Cells were kept at 37 °C and 5% CO_2 during acquisition.

2.2.2. Mitosis and cell migration

To follow random cell migration and to see if mitosis was hampered in RhoD silenced BJ hTERT SV40T, cells were seeded at low confluence. 48 h after siRNA transfection, the cells were filmed during 24 h. Cells were kept at 37 °C and 5% CO_2 during acquisition. The migrating distances of the cells were analyzed using the Gradientech Tracking Tool software (Gradientech AB, Uppsala, Sweden).

2.3. MCC – Microfluid Chemotaxis Chamber

The procedure essentially followed the published protocol [20]. BJ hTERT SV40T were transfected with control or RhoD siRNA 48 h prior experimental onset as described above. Cells were seeded in serum-free media overnight on dishes that had been pre-coated with 0,2% gelatin for 15 min. A hill-shaped flow-dependent gradient of 0–20 ng/ml PDGF-BB in DMEM was applied to the starved cells. Phase-contrast pictures were acquired with a 10 x objective every 5 min for 50 cycles (approximately 4 h) using a Zeiss Axiovert 200 microscope. The migrating distances of the cells were analyzed using the Gradientech Tracking Tool software (Gradientech AB, Uppsala, Sweden). The acquired pictures were divided in three equal sections, where only cells in the first and third sections were quantified, as these two regions hold approximately linear gradients (in contrast to the middle section where the level of PDGF-BB is relatively even) (see Fig. 6A). Hence, the migration of cells towards increasing concentration of PDGF-BB occurs in two directions. In order to avoid obtaining a misleading total migrating distance after the four hours of acquisition, cells migrating out or into the field of view were excluded. In addition, cells dying or dividing during the acquired time were excluded. To quantify the fraction of cells migrating towards the gradient or perpendicular to the gradient, cells migrating in and out of the field of view were included. Data was analyzed using a two-way unpaired Student's *t*-test with equal variance.

2.4. Scratch-wound assay

The IncuCyte Zoom Scratch Wound assay (Essen Bioscience) was used to follow the migration of BJ hTERT SV40T cells, during wound closure. The scratches were introduced using a wound maker, which creates wounds of equal width. This was done 48 h post siRNA transfection and pictures were acquired every two hours with a 10 x phase-contrast objective. The IncuCyte scratch-wound analysis software allowed quantification of the increasing cell confluence inside the wound. The percentage of the closing wound at each time point was analyzed using paired two-way Student's *t*-test with equal variance.

2.5. Cell proliferation assay

The IncuCyte Zoom Cell Proliferation assay was used to monitor the cell growth rate of control and RhoD siRNA-treated BJ hTERT SV40T cells. Since the assay continued for longer than 48 h, the cells had been pre-transfected with siRNA for only 24 h at the experimental onset to make sure the knock down effect would last throughout the experiment. The cells, seeded in a multi-well plate, were scanned every second hour with a 10 x phase contrast objective to follow the growth rate. The IncuCyte proliferation software application was used to quantify the time required for cells to grow from 20% to 80% of confluence. The initial values were 15.3–25.8% for control-silenced samples and 14.3–24.6% for RhoD-silenced samples. Three samples of the control were excluded since their starting confluence values were below 10%, leading to a total of 9 and 12 quantified fields of view for control and RhoD siRNA treated cells respectively.

2.6. qPCR

Total cellular RNA of HeLa, U2OS and U251MG cells was isolated with Quick-RNA MiniPrep Kit (Zymo Research) and reverse transcribed with SuperScript VILO cDNA synthesis kit (Invitrogen). The following primers were used: human RhoD (forward 5′-TGGTCAACCTGCAAGTGAA-3′ and reverse 5′- GGAGGCGGTCATAGT CATC-3′), and human GAPDH (forward 5′-GGA AGG TGA AGG TCG