Cellular Signalling 25 (2013) 630–638

Contents lists available at SciVerse ScienceDirect

Cellular Signalling

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

RhoGEF17, a Rho-specific guanine nucleotide exchange factor activated by phosphorylation via cyclic GMP-dependent kinase Iα

Susanne Lutz [a,b,1], Marion Mohl [a,1], Julia Rauch [a], Pamina Weber [a], Thomas Wieland [a,*]

[a] Institute of Experimental and Clinical Pharmacology and Toxicology, Mannheim Medical Faculty, University of Heidelberg, Maybachstrasse 14, 68169 Mannheim, Germany
[b] Dept. of Pharmacology, University of Göttingen, Robert-Koch-Strasse 40, D-37075,Göttingen, Germany

ARTICLE INFO

Article history:
Received 11 May 2012
Received in revised form 29 October 2012
Accepted 12 November 2012
Available online 27 November 2012

Keywords:
Cyclic GMP-dependent kinase
Guanine nucleotide exchange factor
RhoA
Rho dependent kinase
Serum response factor

ABSTRACT

RhoGEF17, the product of the *ARHGEF17* gene, is a Rho-specific guanine nucleotide exchange factor (GEF) with an unusual structure and so far unknown function. In order to get insights in its regulation, we studied a variety of signaling pathways for activation of recombinantly expressed RhoGEF17. We found that in the presence of stable cGMP analogs RhoGEF17 associates with and is phosphorylated by co-expressed cGKIα at distinct phosphorylation sites leading to a cooperative activation of RhoA, the Rho dependent kinases (ROCK) and serum response factor-induced gene transcription. Activation of protein kinase A did not induce phosphorylation of RhoGEF17 nor altered its activity. Furthermore, we obtained evidence for a ROCK-driven positive feedback mechanism involving serine/threonine protein phosphatases, which further enhanced cGMP/cGKIα-induced RhoGEF17 activation. By using mutants of RhoA which are phosphorylation resistant to cGK or mimic phosphorylation at serine 188, we could show that RhoGEF17 is able to activate RhoA independently of its phosphorylation state. Together with the ROCK-enforced activation of RhoGEF17 by cGMP/cGKIα, this might explain why expression of RhoGEF17 switches the inhibitory effect of cGMP/cGKIα on serum-induced RhoA activation into a stimulatory one. We conclude that RhoGEF17, depending on its expression profile and level, might drastically alter the effect of cGMP/cGK involving signaling pathways on RhoA-activated downstream effectors.

1. Introduction

RhoGTPases play a major role in many physiological processes, like cell migration, proliferation and cellular contraction [1]. The regulation of these processes is based most importantly on their ability to stimulate gene transcription through several transcription factors, e.g. the serum response factor (SRF) or NFκB [2], and on their capacity to control the dynamics of the actin cytoskeleton [3]. The family of RhoGTPases comprises at least 20 different members, whereas only few of them are currently matter of intense research. The most prominent and best described members RhoA, Rac1 and Cdc42 are shown to induce stress fibers, lamellipodia and filopodia formation in different cell types, respectively, and to stimulate gene transcription through SRF. The activity of RhoA, Rac1 and Cdc42 is complexly regulated by a subset of accessory proteins, which are under a strict control of upstream signal mediators. In brief, like all G proteins, RhoGTPases cycle between an inactive GDP bound and an active GTP bound state. Their low intrinsic GTPase activity is accelerated by GTPase activating proteins (GAPs), the GDP-bound inactive state is stabilized by guanine dissociation inhibitors (GDIs), which bind to the isoprenoid moiety of the GTPases and inhibit the translocation to the membrane. For the activation of RhoGTPases, the presence of a guanine nucleotide exchange factor (GEF) is necessary to catalyze the GDP/GTP exchange. Nowadays at least 70 distinct partial or full-length genes encoding putative GEFs for RhoGTPases (RhoGEF) [4,5] are described. They all share a common protein domain, the Dbl homology (DH) domain, which holds the catalytic activity. In most RhoGEFs the DH domain is followed by a pleckstrin homology domain (PH) with an invariant gap of about 10 amino acids. The function of the PH domain is not fully understood and may vary from RhoGEF to RhoGEF. It apparently confers to membrane attachment by binding phospholipids but can exert also autoinhibitory constraint on the DH domain [6,7]. Besides this DH/PH tandem motif, many RhoGEFs possess additional signal integration domains, allowing their interaction with different signal mediators. Although the detection of distinct protein domains by using bioinformatical programs has become more reliable in the last years, not much is known about the regulation of most of the more than 70 potential RhoGEFs.

The cyclic nucleotides cAMP and cGMP are important second messengers in many signaling pathways. Both mediate most of their downstream signaling by activation of the cAMP- and cGMP-dependent kinases protein kinase A (PKA) and cGMP-dependent kinase (cGK) [8], respectively, or direct interaction with effector proteins, e.g. cyclic nucleotide gated channels [9] or GEFs for the monomeric Rap GTPases

* Corresponding author. Tel.: +49 621 383 9610; fax: +49 621 383 9611.
E-mail address: thomas.wieland@medma.uni-heidelberg.de (T. Wieland).
[1] These authors contributed equally.

0898-6568/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cellsig.2012.11.016

[10]. With regard to RhoGEFs, it has been demonstrated that by fusion of the PDZ domain of syntrophin and an interacting motif which is sensitive to PKA phosphorylation to known DH/PH domains, cAMP-PKA regulated RhoGEFs can be created in which an intramolecular autoinhibitory constraint is released upon phosphorylation [11]. In the present study we describe for the first time a similar mechanism of activation for a natural occurring exchange factor, i.e. RhoGEF17, the full length variant of p164-RhoGEF [12]. It is directly interacting with and phosphorylated by the cGK variant Iα (cGKIα) in a cGMP-dependent manner. We further demonstrate that the extent of phosphorylation and thus activation of RhoGEF17 are controlled by the downstream effector Rho-dependent kinase (ROCK) and serine/threonine protein phosphatases, providing evidence for a positive feedback loop in the regulation of RhoGEF17 activity.

2. Material and methods

2.1. Expression plasmid construction

For cloning of full length RhoGEF17-cDNA a RT-PCR with 2 μg total RNA from human embryonic kidney cells was performed with the following oligonucleotides: p164for 5′-AAAGGTACCGAATTCTGGCGG ACGGGGCACCC-3′, p164rev5′-CCTGTGGTACCCAGGCCGCAA-3′. The amplificate was digested with EcoRI/KpnI and cloned in EcoRI/KpnI partially digested pCMV-Tag3C-p164-FL [10]. For pCMV-Tag3C-RhoGEF17ΔDH, aXhoI fragment (3022–6579 bp) of pCMV-Tag3C-p164ΔDH was subcloned in XhoI digested pCMV-Tag3C-RhoGEF17. pcMV-Tag3A-RhoGEF17-Δ55 and pCMV-Tag3B-RhoGEF17-Δ109 were created by subcloning of fragments obtained by digest with convenient restriction enzymes. The cDNA encoding cGKIα, a kind gift of Dr. P. Ruth, Tübingen, Germany, was subcloned into the EcoRI site of pcDNA3.1 (Invitrogen, Karlsruhe, Germany). The expression vectors encoding cGKII (pcDNA3-cGKII) and constitutive active PKA (pcDNA3-PKA-cat-YFP) were kind gifts from Dr. Stepan Gambaryan, Würzburg, Germany and Dr. Enno Klussmann, Berlin, Germany, respectively.

2.2. Site directed mutagenesis

For site directed mutagenesis the QuikChange Site-Directed Mutagenesis Kit from Stratagene (Amsterdam, Netherlands) was used. In brief, each sample reaction was prepared as described by the manufacturer's recommendations with 50 ng of the indicated dsDNA template and 125 ng of each sense and anti-sense oligonucleotide (Sigma Genosys) in a final volume of 50 μl. The PCR was carried out with 1 μl PfuTurbo DNA polymerase (2.5 U/μl, Stratagene) and 16 amplification cycles. The PCR product was digested with DpnI and transformed into XL1-Blue electrocompetent cells. The mutation was verified by restriction digest and automated sequencing. The following sense oligonucleotides were used for the respective mutagenesis: pCMV-Tag3C-RhoGEF17-S1331A: 5′-TCCCTGCGGCGCATCCGCGATGAG CCTGTAC-3′, pCMV-Tag3C-RhoGEF17-S1331E: 5′-TCCCTGCGGCGCAGC GAGGATGAGCCTGTAC-3′, pcDNA3.1-RhoA S188A: 5′-CGTGGGAAGAA AAAAGCCGGTTGCCTTGTCTTG-3′, and pcDNA3.1 RhoA S188E: 5′-CGTG GGAAGAAAAAAGAGGGTTGCCTTGTCTTG-3′.

2.3. Cell culture and transfection

Human embryonic kidney cells (HEK) were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated bovine calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine at 37 °C in an atmosphere of 5% CO_2. DNA transfections were performed with Polyfect (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

2.4. RhoA activity assay

The cellular levels of GTP-loaded RhoA were determined using GST fusion proteins containing the RhoGTPase-binding domain of Rhotekin (GST-RBD) as described before [11]. In brief, GST fusion proteins were expressed in and purified from *Escherichia coli*. HEK cells (6-wells) were transfected with the indicated amounts of plasmid DNA or the corresponding empty vectors and cultured for 48 h in serum-reduced (0.5%) DMEM. Prior to the assay the cells were incubated with or without 8-Br-cAMP (Biolog, Bremen, Germany), 8-Br-cGMP (Biolog), 8-pCPT-cGMP (Biolog), Y27632 (Calbiochem, Darmstadt, Germany), H-1152P (Calbiochem), fetal calf serum, okadaic acid (Santa Cruz, Heidelberg, Germany) or calyculin A (Santa Cruz). The respective used concentrations and the incubation periods are given in the figure legends. Thereafter, the cells were lysed in a buffer containing 1% (v/v) Nonidet P-40, and the particular fraction was pelleted by centrifugation. The GTPase-containing supernatant was then incubated for 1 h at 4 °C with GST-RBD bound to glutathione Sepharose beads. After two washing steps the bound proteins were eluted with sample buffer and separated by SDS-PAGE. Rho GTPases were then detected by immunoblotting with commercially available specific anti-RhoA (26C4, Santa Cruz, 1 μg/ml) antibodies.

2.5. Co-immunoprecipitation

HEK cells were seeded in 10 cm dishes and transfected at a confluence of 80% with 8 μg of the indicated cDNA constructs. Prior to the assay the cells were incubated with or without 8-Br-cAMP, 8-pCPT-cGMP or KT-5823 (Calbiochem). The respective used concentrations and the incubation periods are given in the figure legends. 48 h after transfection, the cells were solubilized in 600 μl IP buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 μM PefablocSC, 0.1% Triton X-100). After incubation on ice for 20 min and centrifugation (26,000 ×*g* for 10 min), 8 μg of anti-c-myc antibody were added to the cleared supernatants (2 mg of protein) and incubated for 1 h at 4 °C. After addition of 40 μl 1:1 slurry of protein-A sepharose (GE Healthcare), the mixture was gently shaken for 4 h at 4 °C. Then the beads were washed four times with IP buffer and bound proteins were eluted with 25 μl sample buffer for 5 min at 95 °C.

2.6. Immunoblot analysis

10 to 40 μg protein was separated by discontinuous SDS-PAGE (6–15% acrylamide) and subsequently transferred onto a nitrocellulose membrane. For detection of specific proteins the following antibodies were used: anti-c-myc antibody (clone 9E10, 0.2 μg/ml) for detection of N-terminally myc-tagged RhoGEF17, anti-RhoGEF17 antibody (custom made by Biogenesis, Berlin Germany, 1 μg/ml), anti-RhoA antibody (26C4, Santa Cruz, 1 μg/ml), anti-cGKI antibody (a kind gift from Dr. J. Schlossmann, Regensburg, Germany, 1:1000), anti-cGKII antibody (a kind gift by Dr. Stepan Gambaryan, Würzburg, Germany, 1:2000), anti-GFP antibody (sc-8334, Santa Cruz, 1:1000) and anti-Phospho-PKA Substrate (RRXS/T) antibody(100 G7, New England Biolabs, Frankfurt, Germany, 1:5000).

2.7. SRF activation assay

Luciferase reporter gene assays were performed with the Dual Luciferase Reporter Assay System (Promega) according to manufactures protocol. In brief, HEK cells were seeded into 48-well plates and co-transfected with the indicated plasmids together with pSRE.L encoding firefly luciferase reporter plasmid (kindly provided from Dr J. Mao and Dr. D. Wu, Rochester, NY) and pRL-TK (Promega, Mannheim, Germany) encoding renilla luciferase control vector. The transfected cells were maintained in DMEM with 0.5% FCS for 24 h and stimulated for further 24 h either with 8-Br-cAMP, 8-Br-cGMP