Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 336 (2005) 1285–1291
BBRC
www.elsevier.com/locate/ybbrc

Src regulates the activity of the mammalian formin protein FHOD1

Sreenivas Koka [a,*,1], Gerald T. Minick [b], You Zhou [c], Jennifer J. Westendorf [d], Matthew B. Boehm [b]

[a] Department of Dental Specialties, Mayo Clinic, Rochester, Minnesota, USA
[b] Department of Oral Biology and Nebraska Center for Cell Signaling, UNMC College of Dentistry, USA
[c] The Center for Biotechnology, University of Nebraska-Lincoln, USA
[d] The Cancer Center and Department of Orthopaedic Surgery, University of Minnesota, USA

Received 26 August 2005
Available online 12 September 2005

Abstract

The mammalian formin homology domain containing protein FHOD1 influences a variety of cellular events including cell migration, cytoskeletal arrangement, signal transduction, and gene expression. In this paper, we show that Src regulates a variety of FHOD1-associated effects. FHOD1 distribution to lamellipodia was prevented by the absence of Src. However, stress fiber formation induced by a C-terminal truncated form of FHOD1 was unaffected. Gene expression from an SRE-dependent promoter and from the skeletal actin promoter was induced by two truncated forms of FHOD1 and in both instances, inhibition of Src tyrosine kinase activity abrogated induction of gene expression. Furthermore, Src activity was necessary to maintain mRNA levels of FHOD1 itself, and as such, this finding represents the first description of mechanisms involved in the regulation of formin gene expression in mammals. In summary, we have identified Src as a key regulator of FHOD1 biology.

Keywords: Src; Formin; Cytoskeleton; Gene expression; Serum response element; FHOD1

In mammalian cells, formin homology (FH)-domain containing proteins regulate cytoskeletal organization, embryonic patterning, cell survival, migration, and gene transcription [1–8]. One sub-family of FH proteins, termed Diaphanous-related formins (DRFs), contain FH1 and FH2 domains, the former responsible for self-interaction (homodimerization), interaction with profilin (an actin-binding protein) in many DRFs, as well as an N-terminal GTPase-binding domain (GBD) and a carboxy-terminal Diaphanous autoregulatory domain (DAD) that appears to permit intramolecular binding between the amino and carboxy termini. Truncated forms of mDia induce changes in cell shape, actin distribution, and cytoskeletal organization. Similarly, expression of a truncated form of formin-related gene in leukocytes (FRL) containing the Rac1-binding domain inhibits cell spreading and chemokine-induced migration of an FRL-expressing macrophage cell line by blocking cell adhesion to fibronectin [8]. Thus, FH proteins appear to regulate several aspects of signaling events related to actin polymerization.

FHOD1 (formin homology 2 domain-containing protein) is a 1165 amino acid FH protein that was previously described as FHOS [3,9]. Its designation was changed to meet the Guidelines for Human Gene Nomenclature. FHOD1 is a characteristic DRF family member in that it contains a GTPase-binding domain (GBD), FH1 and FH2 domains, a coiled-coil, and a Diaphanous-like autoregulatory domain (DAD) [10,11]. FHOD1 regulates cytoskeletal architecture, specifically F-actin distribution, in a Rho- and Rac-dependent manner and its actin-modulating effects are proposed to occur as a result of homodimerization or oligomerization [7,12,13]. Functional assays indicate that FHOD1 enhances cell migration [7], regulates gene expression [4,9], and

* Corresponding author. Fax: +1 507 284 8082.
E-mail address: koka.sreenivas@mayo.edu (S. Koka).
[1] Formerly at Department of Oral Biology and Nebraska Center for Cell Signaling, UNMC, USA.

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.257

modulates intracellular vesicular trafficking [14]. Self-interactions between the amino- and carboxy-termini prevent full-length FHOD1 from activating gene transcription from the serum response element (SRE); however, deletion of the amino- or carboxy-termini allows FHOD1 to induce SRE transcription [9]. Despite the important role for FHOD1 in key cell processes, the factors responsible for activating or regulating FHOD1 remain unidentified. Recently, FHOD1 has been shown to be a substrate for protein kinase G in smooth muscle cells, with serine 1131 being the target residue [15]. However, whether phosphorylation at this site contributes to activation of FHOD1 is unknown.

Src has a central role in tumor cell invasion and subsequent metastasis by regulating cell motility and gene expression [16,17]. Due to previous reports describing a function for FHOD1 in enhancing cell migration and gene expression, we investigated the relationship between Src and FHOD1. Here, we identify novel mechanisms by which Src regulates a variety of FHOD1-related effects including cellular distribution, F-actin distribution, and the expression of the FHOD1 gene itself.

Materials and methods

Plasmids. pCMV5-HA-FHOD1 expression plasmids have been described previously [7,9]. The plasmid constructs used in this study were generous gifts from Dr. Michael Schneider (skeletal actin, pSkA) [18], Dr. Richard Treisman (serum response element, pSRE), and Dr. Richard Cerione (dominant negative Rho, dnRho).

Cell culture. NIH3T3, SYF, Src+, and YF murine fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 25 U/ml penicillin, 25 μg/ml streptomycin, and 10% fetal bovine serum (unless otherwise indicated). SYF cells harbor functional null mutations in both alleles of the Src family protein tyrosine kinases, Src, Yes, and Fyn. Src+ cells harbor functional mutations in both alleles for Yes and Fyn and maintain endogenous levels of Src, while YF cells harbor mutations in both alleles for Src, Yes, and Fyn but with c-Src reintroduced [19]. All murine fibroblast cell lines were purchased from American Type Culture Collection (NIH3T3: CRL-1658; SYF: CRL-2459; Src+: CRL-2497; YF: CRL-2498).

In situ immunofluorescence assay. NIH3T3 cells were grown on uncoated and fibronectin-coated (Becton–Dickinson) coverslips, and SYF, YF, and Src+ cells were grown on fibronectin-coated coverslips in 35 mm culture wells and transfected with expression vectors as indicated using Geneporter 2 transfection reagent according to manufacturer's recommendations (Gene Therapy Systems, San Diego, CA). Briefly, for each well a total of 0.5–1.0 μg of plasmid was mixed with 8–10 μl of Geneporter in 1.2 ml of non-supplemented DMEM and allowed to stand at room temperature for 45 min. Cells were washed in non-supplemented DMEM once prior to addition of plasmid/Geneporter mixture and incubation for 5–8 h at 37 °C at which time 1 ml DMEM supplemented with 20% FBS was added to each well. In appropriate cultures, 24–36 h after transfection, cells were fixed and subjected to in situ immunofluorescence as previously described [7]. Briefly, cells were washed in 37 °C-cytoskeleton buffer (CB) (10 mM Mes, 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$, and 5 mM glucose, pH 6.1) and fixed in 3% paraformaldehyde in cytoskeleton buffer for 10 min at room temperature [7]. Cells were subsequently washed with CB, exposed to 0.1% Triton X-100 in CB for 2 min, prior to washing and blocking in 5% normal goat serum/2% BSA in TBS. Primary antibodies for HA (mouse: Covance; rabbit: Santa Cruz Biotech) were used at a concentration of 1:400. For assays involving F-actin, phalloidin rhodamine (0.2 U/coverslip) (Molecular Probes, Eugene, OR) was utilized, followed by a 1 h incubation with Cy2-conjugated and/or Cy5-conjugated anti-mouse or anti-rabbit secondary antibodies (1:400 Jackson Immunolabs, West Grove, PA) as appropriate. All antibodies were suspended in blocking buffer. Between and after antibody/phalloidin incubations, coverslips were washed in TBST and then mounted on glass slides in Gel Mount mounting medium (Sigma) and visualized at 600× or 1000× using an Olympus FV500 confocal microscope. All images were collected in sequential capture mode using specific laser-excitation lines for corresponding fluorescent conjugates.

Serum response element reporter assays. The pSRE luciferase (Luc) reporter plasmid (from the Treisman laboratory) contains three tandem copies of the promoter element fused to the herpes simplex virus-thymidine kinase TATA-like promoter. The serum response element (SRE) sequence is identical to the SRE in the c-*fos* promoter. Luciferase reporter plasmid was mixed with pCMV5-secreted alkaline phosphatase (SEAP) and the indicated pCMV5-HA-FHOS expression vectors. COS-7 cells were transfected and incubated for 26–40 h in DMEM containing 0.1% FBS. Some cultures were exposed to the STK inhibitor PP2 (10 μM) (Calbiochem/EMD Biosciences, San Diego, CA). Luciferase activity was measured with the Luciferase Assay Systems (Promega, Madison, WI) as instructed by the manufacturer. SEAP activity was measured. Luciferase activity was normalized for transfection efficiency using SEAP values for each sample. Fold activation was determined relative to samples transfected with pCMV5. Values represent means of three independent transfections ± SD of the mean.

Skeletal actin promoter–reporter assays. COS-7 cells were seeded at a density of 2×10^4 cells/well in 24-well plates and allowed to adhere and grow for 24 h. Two forms of promoter were used, the wild type form (Fig. 3A) and a promoter mutated in the SRF-binding site. Luciferase reporter plasmid (0.5 μg) was mixed with 100 ng of *Renilla* luciferase plasmid in order to determine transfection efficiency and pCMV5-HA-FHOS expression vectors and/or dnRho (0.5 μg) as indicated. Cells were transfected using Gene Porter 2 Transfection Reagent (Gene Therapy Systems) as instructed by the manufacturer. Briefly, for each well a total of 0.5–1.0 μg of plasmid was mixed with 8–10 μl of Geneporter in 1.2 ml of non-supplemented DMEM and allowed to stand at room temperature for 45 min. Cells were washed in non-supplemented DMEM once prior to addition of plasmid/Geneporter mixture and incubation for 5–8 h at 37 °C at which time 1 ml DMEM supplemented with 20% FBS was added to each well for 8–12 h. Following on, cells were given DMEM containing 0.1% FBS for 24 h. Indicated cultures received media supplemented with the STK inhibitor PP2 (10 μM) (Calbiochem/EMD Biosciences, San Diego, CA). Luciferase activity was measured with the Dual-Reporter Luciferase Assay System (Promega, Madison, WI) as instructed by the manufacturer. Luciferase activity was normalized for transfection efficiency using *Renilla* luciferase values for each sample. Fold activation was determined relative to samples transfected with pCMV5. Values represent means of three independent transfections ± SD of the mean.

RNA isolation and cDNA preparation. NIH3T3, SYF, Src+, and YF cells were grown in 35 mm wells. When cells were 70–80% confluent, total RNA was isolated using the RNAEasy system (Qiagen, Valencia, CA). Final sample concentration and sample purity were determined with a spectrophotometer. Total RNA (1 μg) was treated with DNAse I (Invitrogen, Carlsbad, CA), reverse-transcribed using Superscript II (Invitrogen), and exposed to RNaseH (Invitrogen) as per manufacturer's recommendations.

Real-time quantitative polymerase chain reaction. cDNA quantitation for FHOD1 and an internal reference standard (18S rRNA) was performed using a fluorescence-based real-time detection method (ABI PRISM 7000) Sequence Detection System (TaqMan); Applied Biosystems, Foster City, CA. The PCR mixture consisted of 900 nM each of the primers; 250 nM probe; 0.3 U/μl AmpliTaq Gold Polymerase; 200 nM each of dATP, dCTP dGTP, and dTTP; 3.5 mM $MgCl_2$; and 1× TaqMan Buffer A, which contains a reference dye, to a final volume of 25 μL (all reagents were from Applied Biosystems). Cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Forward primer: 5′-CTACATACCGTGAGCGCAA CAA-3′; reverse primer: 5′-GCCACTGGGACAGACAGGTTATT-3′; probe 5′-ACCGAGACAGAGAAGT-3′. In addition, parallel reactions