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Shoc2-tranduced ERK1/2 motility signals — Novel insights from functional genomics

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ABSTRACT

The extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway plays a central role in defining various cellular fates. Scaffold proteins modulating ERK1/2 activity control growth factor signals transduced by the pathway. Here, we analyzed signals transduced by Shoc2, a critical positive modulator of ERK1/2 activity. We found that loss of Shoc2 results in impaired cell motility and delays cell attachment. As ERKs control cellular fates by stimulating transcriptional response, we hypothesized that the mechanisms underlying changes in cell adhesion could be revealed by assessing the changes in transcription of Shoc2-depleted cells. Using quantitative RNA-seq analysis, we identified 853 differentially expressed transcripts. Characterization of the differentially expressed genes showed that Shoc2 regulates the pathway at several levels, including expression of genes controlling cell motility, adhesion, crosstalk with the transforming growth factor beta (TGFβ) pathway, and expression of transcription factors. To understand the mechanisms underlying delayed attachment of cells depleted of Shoc2, changes in expression of the protein of extracellular matrix (lectin galactoside-binding soluble 3-binding protein; LGALS3BP) were functionally analyzed. We demonstrated that delayed adhesion of the Shoc2-depleted cells is a result of attenuated expression and secretion of LGALS3BP. Together our results suggest that Shoc2 regulates cell motility by modulating ERK1/2 signals to cell adhesion.

1. Introduction

The mechanisms leading to activation of RAF, MEK and ERK kinases in the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway have been studied extensively [1,2]. However, what determines the activity of the pathway in the context of a specific set of downstream targets and how ERK signals result in distinct biological outcomes are not yet clear. Several studies suggest that divergent cell fates induced by the ERK pathway are the result of a tight spatio-temporal control of ERK1/2 targeting, sequestration and activation of the kinases and phosphatases, as well as modulation of the strength and duration of the ERK signaling [3,4]. Scaffold proteins have been proposed to fulfill some of these requirements. Scaffolds have been implicated in controlling the spatial organization of signaling enzymes, insulation of active modules and prevention of a spurious cross-talk of signaling networks [5,6]. Yet, detailed mechanisms allowing scaffolds to elicit specific cellular responses at the molecular level remain to be elucidated.

Scaffold proteins of the ERK1/2 pathway represent a diverse group of proteins [2]. A well-studied scaffold kinase suppressor of Ras 1 (KSR1) [7–9] is the multifunctional protein that binds to and accelerates activity of MEK and RAF kinases thereby stimulating expression of genes that drive cell proliferation and differentiation [10,11]. Other ERK1/2 scaffolds, mitogen-activated protein 1 (MP1) and p14 (also called the LAMTOR2/3 complex) are believed to regulate cytoskeletal dynamics [12–15], and the MP1/p14 complex is involved in remodeling of focal adhesion and actin structures during cell spreading [16].

Ras–RAF-1–ERK1/2 signaling is accelerated by the scaffolding protein Shoc2 [17,18]. This evolutionarily well-conserved protein is essential for normal development [19–21]. Loss of Shoc2 in mammalian cultured cells and *Caenorhabditis elegans* leads to a dramatic decrease in ERK1/2 activity [17,22,23]. As a scaffold protein, Shoc2 provides a molecular platform for multi-protein assemblies that modulate ERK1/2 activity [24,25]. In addition to its signaling partners Ras and RAF-1, Shoc2 tethers the catalytic subunit of protein phosphatase 1c (PP1c) as well as

Abbreviations: EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated kinase 1 and 2; KSR1, kinase suppressor of Ras (KSR1); MP1, mitogen-activated protein 1; PNGase F, Peptide-*N*-Glycosidase F; Endo H, Endoglycosidase H; PP1c, protein phosphatase 1c; qPCR, real-time quantitative polymerase chain reaction; RPKM, reads per kilobase of exon per million reads mapped; TGFβ, transforming growth factor beta.

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proteins of the ubiquitin machinery HUWE1 and PSMC5 [23,26,27]. The ability of this non-catalytic scaffold to mediate ERK1/2 signaling is controlled through allosteric ubiquitination [24]. Alterations in the mechanisms controlling ubiquitination of the scaffold affect Shoc2-mediated ERK1/2 signals and cell motility [27].

Activation of the ERK1/2 pathway in response to epidermal growth factor (EGF) stimulation of the EGF receptor falls into three major regulatory loops: immediate, delayed, and late (secondary) [28–30]. The immediate regulatory loop induces phosphorylation of transcription factors such as FOS, Jun and EGR1 and does not require new protein synthesis for their transcription [30]. Expression of the genes of the immediate response induces transcription of delayed genes, such as the RNA-binding protein ZFP36 or dual specific phosphatases, which dephosphorylate ERK1/2 kinases that terminate the activity of the immediate loop [30]. Late (secondary) transcriptional response leads to expression of genes such as actin-binding proteins or genes encoding proteins that are involved in cell metabolism and biogenesis of membranes and appear to define cellular outcomes [31].

In the current study, we aimed to determine the specific ERK1/2 response elicited through the Shoc2 scaffolding module. Results of this study provide evidence that Shoc2-mediated ERK1/2 activity contributes to maintenance of the ERK1/2 feedback loop that regulates expression of genes of the TGFβ pathway. We also found that Shoc2–ERK1/2 signals control cell motility and adhesion, in part, through mechanisms that monitor expression of the protein of extracellular matrix — lectin galactoside-binding soluble 3-binding protein or LGALS3BP (also called Mac-2 binding protein) [32]. Deficient expression and secretion of this heavily glycosylated protein led to attenuated attachment of Shoc2-depleted cells. These results indicate that Shoc2 transduces signals to unique cellular responses and identifies novel molecular targets of the Shoc2–ERK1/2 signaling axis.

2. Materials and methods

2.1. Reagents and antibodies

EGF was obtained from BD Bioscience. U0126 and PD98059 were obtained from LC Laboratories. Respective proteins were detected using specific primary antibodies, including: GAPDH, phospho-ERK1/2, ERK1/2, MEK1/2, COL1A1 and EGFR (Santa Cruz Biotechnology); His, Shoc2 and LGALS3BP (Proteintech); phospho-AKT, KSR1, and phospho-MEK1/2 (Cell Signaling).

2.2. Constructs

Shoc2-tRFP was described previously [25,33]. The plasmid carrying full-length His-tagged LGAL3SBP was obtained from Dr. Enza Picollo (Chieti, Italy). The plasmid carrying shRNA specifically recognizing KSR1 was kindly provided by Dr. Tianyan Gao (University of Kentucky) and was obtained from the Sigma Mission collection. The shRNA sequence used to target the KSR1 transcripts was as follows: #1 — 5′-CCGGCAACAAGGAGTGGAATGATTTCTCGAGAAATCATTCCACTCC TTGTTGTTTTTG-3′; and #2 — 5′-CCGGTCGTACACAAAGATCTCAAATC TCGAGATTTGAGATCTTTGTGTACGATTTTTG-3′. Efficiency of the shRNA knockdown was validated by western blotting. Plasmid DNAs were purified using Zymo Research. All constructs were verified by dideoxynucleotide sequencing.

2.3. Cell culture and DNA transfections

Cos1 (ATCC) and stable cell lines (NT, LV1, SR) (derivative of Cos1 cells) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with sodium pyruvate, MEM-NEAA, penicillin, streptomycin, and L-glutamate (Invitrogen). MCF7, T47D and stable cell lines (NT, LV1, SR) (derivative of T47D cells) were grown in RPMI 1640 Medium containing 10% FBS. MCF7 and stable cell lines (NT, LV1, SR) (derivative of MCF7 cells) were grown in MEM containing 10% FBS. The transfections of DNA constructs were performed using PEI (Neo Transduction Laboratories, Lexington, KY) reagent.

2.4. Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was isolated using PureZOL/Aurum Total RNA Isolation Kit (Bio-Rad) according to the manufacturer's instructions. Aliquots containing equal amounts of RNA were subjected to RT-PCR analysis. The RNA quality for RNA-seq was tested using Agilent Bioanalyzer 2100. Quantitative RT-PCR was performed using SoAdvanced™ SYBR® Green supermix and the Bio-Rad CFX detection system (Bio-Rad). Relative amounts of RNAs were calculated using the comparative C_T method [34]. HPRT1 gene expression was used as a reference. Sequence-specific primer sets are presented in Suppl. Table 3.

2.5. Western blot analysis

Cells were placed on ice and washed with phosphate-buffered saline (PBS); proteins were then solubilized in lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton, 1 mM Na_3OV_4, 10 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin and 10 μg/ml of aprotinin for 15 min at 4 °C. Lysates were centrifuged for 15 min to remove insoluble material. Cell lysates were denatured in the sample buffer at 95 °C, resolved by electrophoresis, and probed with various antibodies, followed by chemiluminescence detection. Quantification was performed using the densitometry analysis mode of Image Lab software (Bio-Rad, Inc.).

2.6. Wound healing assays

Cells were seeded at a density of 1×10^5/ml in each well of IBIDI™ culture-inserts placed on a glass-bottom 33 mm culture dish. To create 500 μm cell-free gap, culture-inserts were removed 18 h upon seeding. Cells were then treated with 10 nM EGF and observed with a 10× objective on a Zeiss Axiovert microscope. Images were acquired using Slidebook software (Intelligent Imaging Innovations, Denver, CO). Detection of GFP fluorescence was performed using a FITC filter channel every 12 h up to 48 h. The distance of closure was measured at three spots and the average from three images was shown as mean ± SD.

2.7. Migration assay

Cell migration assays were performed using 8.0 μm pore 24-well TC inserts (Greiner Bio-one, Monroe, NC, USA). Filters were coated with 15 μg/ml collagen at 37 °C for 30 min. Cells were trypsinized (with 0.05% Trypsin–EDTA; Invitrogen), collected with serum-free medium containing soybean trypsin inhibitor (1 mg/ml), centrifuged (500 ×*g* for 5 min), and then resuspended in serum-free medium. Cells (5×10^4) were then placed in the upper chamber and the lower chamber was filled with complete medium with 10% serum or 10 nM EGF. Cells were migrated at 37 °C for 4 h. After removing non-migrated cells, membranes were fixed in methanol and stained with 1% crystal violet. Migrated cells were counted in three random fields per membrane under the microscope at ×20. Each assay was repeated more than three times. To test migration of T47D cells, plates were incubated for 24 h at 37 °C.

2.8. Cell attachment assay

Cells were trypsinized (with 0.05% Trypsin–EDTA; Invitrogen) and collected with serum-free medium containing soybean trypsin inhibitor (1 mg/ml) as described previously [35,36]. Cells were centrifuged,