Biochemical and Biophysical Research Communications 450 (2014) 1032–1037

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

c-Myb negatively regulates Ras signaling through induction of dual phosphatase MKP-3 in NIH3T3 cells

Young Jae Park [a], Jong Min Lee [b], Mi So Lee [b], Young Ho Kim [a,*], Soon Young Shin [b,*]

[a] School of Life Science and Biotechnology, College of Natural Sciences, Kyungpook National University, Daegu 702-701, Republic of Korea
[b] Department of Biological Sciences, College of Biological Science and Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea

ARTICLE INFO

Article history:
Received 14 June 2014
Available online 27 June 2014

Keywords:
c-Myb
ERK
MKP-3
H-Ras
NIH3T3 fibroblasts

ABSTRACT

Mitogen-activated protein kinase phosphatase-3 (MKP-3) negatively regulates ERK1/2 MAPK in a feedback loop. However, little is known about the molecular mechanism by which Ras signaling induces *MKP-3* expression. In the present study, we demonstrate that exogenous expression of constitutively active H-Ras increases the level of *MKP-3* mRNA. A transfection study using a series of *MKP-3* promoter deletion constructs revealed that the c-Myb binding site is required for Ras-induced transcriptional activation of the *MKP-3* gene promoter. Furthermore, we show that c-Myb directly binds to the *MKP-3* promoter, as revealed by electrophoretic mobility shift assay and chromatin immunoprecipitation. Knock-down of c-Myb expression using siRNA abrogated Ras-induced *MKP-3* promoter activity. These findings propose a novel mechanism through which Ras signaling activates c-Myb-dependent transcriptional activation of the *MKP-3* gene.

1. Introduction

Extracellular signal-regulated kinase 1 and 2 (ERK1/2) are members of the mitogen-activated protein kinase (MAPK) family, which is involved in the regulation of cell growth, differentiation, and survival. ERK1/2 are phosphorylated in response to many different signals on both threonine and tyrosine residues by upstream kinases and MAPK/ERK kinase (MEK). These signals include stimulation of receptor tyrosine kinases, G-protein coupled receptors and integrins. Once activated, ERK1/2 translocate to the nucleus, where they regulate many downstream targets such as ELK-1 and c-JUN. It has been demonstrated that retained phosphorylation of ERK1/2 by nerve growth factor (NGF) leads to neuronal differentiation, whereas transient phosphorylation of ERK1/2 by epidermal growth factor (EGF) results in cell proliferation in cultured rat PC12 cells [1]. Therefore, it seems likely that the strength and duration of ERK1/2 activity determine the biological responses to any given mitogenic or stress stimuli for each cell type [2,3], which reflects a balance between kinases and phosphatases in the cell.

ERK1/2 activity is inhibited by dephosphorylation of either threonine or tyrosine residues. This can be achieved using the serine/threonine-specific phosphatase PP2A, tyrosine-specific phosphatases or by dual-specificity (threonine/tyrosine) phosphatases [4]. Mitogen-activated protein kinase phosphatase-3 (MKP-3; also known as dual-specificity phosphatase 6 (DUSP6), Pyst1, or rVH6) selectively dephosphorylates both threonine and tyrosine residues within a Thr-X-Tyr motif of ERK1/2 in the cytoplasm. The dephosphorylation prevents nuclear translocation of ERK1/2 [5], while showing little effect on c-Jun NH2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38 MAPK [6]. MKP-3 expression is upregulated by ERK1/2 via the Ets response element within the *MKP-3* promoter upon stimulation of both the fibroblast growth factor 2 (FGF2) in NIH3T3 cells [7] and the epidermal growth factor (EGF) in human lung cancer cells [8]. These findings suggest that growth factor-induced ERK1/2 activity is controlled by a negative feedback loop involving the upregulation of MKP-3 expression. Other studies have demonstrated that MKP-3 is overexpressed in breast epithelial H-Ras MCF10A cells that stably express activated H-Ras [9] as well as in human melanoma cell lines harboring activating mutations in B-RAF and N-Ras [10]. However, the molecular mechanism through which constitutively active Ras induces MKP-3 expression remains largely unknown. In this study, we examined the molecular mechanism by which exogenous expression of constitutively active H-Ras regulates *MKP-3* transcription in NIH3T3 cells and found that c-Myb plays an important role in H-Ras-induced *MKP-3* transcription.

Abbreviations: ERK1/2, extracellular signal-regulated kinase 1 and 2; MAPK, the mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MKP-3, mitogen-activated protein kinase phosphatase-3; ChIP, chromatin immunoprecipitation.

* Corresponding authors.
E-mail addresses: ykim@knu.ac.kr (Y.H. Kim), shinsy@konkuk.ac.kr (S.Y. Shin).

http://dx.doi.org/10.1016/j.bbrc.2014.06.103
0006-291X/

2. Materials and methods

2.1. Cells and reagents

Tetracycline-inducible NIH3T3tet-on/H-RasG12R cells were generated as described elsewhere [11]. The expression plasmid for constitutively active Ras (pSG5/V12Ras) was donated by Julian Downward (Cancer Research UK London Research Institute, London, UK).

2.2. Western blot analysis

Cells were lysed in a buffer containing 20 mM HEPES (pH 7.2), 1% Triton X-100, 10% glycerol, 400 mM NaCl, 10 μg/mL leupeptin, and 1 mM PMSF. Western blot analysis was performed according to standard procedures using antibodies against H-Ras (1:500; Oncogene), phospho-ERK1/2 (Thr202/Tyr204; 1:1000; Cell Signaling), c-Myb (1:2000; Santa Cruz Biotechnology), MKP-3 (1:5000; Cell Signaling), and GAPDH (1:2000; Santa Cruz Biotechnology).

2.3. Northern blot analysis

Total RNA samples (10 μg) were separated by electrophoresis on a formaldehyde/agarose gel and transferred to a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech). Northern blotting was performed with [γ-^{32}P]dCTP-labeled MKP-3 cDNA probes using a High Prime DNA Labeling Kit (Roche), followed by hybridization with a *glyceraldehydes-3-phosphate dehydrogenase* (*Gapdh*) cDNA probe.

2.4. Electrophoretic mobility shift assay (EMSA)

Synthetic deoxyoligonucleotides (4 pmol) corresponding to the Myb binding sequence (5′-acggcaacagccccttc-3′) within the *MKP-3* promoter were end-labeled with [γ-^{32}P]dATP (Amersham Biosciences) through incubation with 10 units of T4 polynucleotide kinase (Promega) and 5 μL of T4 polynucleotide kinase buffer for 30 min at 37 °C, followed by inactivation at 65 °C for 10 min. For EMSA, 10 μg of nuclear extract was mixed with the binding buffer (50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 2.5 mM EDTA, 250 mM NaCl, 20% glycerol), and 1 μg of poly(dI-dC) (Amersham Biosciences) added as a non-specific competitor, as described previously [12].

2.5. Chromatin immunoprecipitation (ChIP) assay

NIH3T3tet-on/H-RasG12R cells cultured in the absence or presence of doxycycline (2 μg/mL) for 48 h were treated with 1% formaldehyde to cross-link the DNA. The cells were lysed and chromatin immunoprecipitated using a rabbit anti-c-Myb antibody or normal rabbit IgG. The cross-linking of protein to DNA and chromatin immunoprecipitation were performed as described previously [13]. The following promoter-specific primers were used to amplify the *MKP-3* gene promoter sequences by polymerase chain reaction (PCR): 5′-tgcactggggcttatccg-3′ (target region forward primer, −176/−158), 5′-gatacattctctcggtcagc-3′, (target region reverse primer, −42/−23), 5′-acaatagaaccgagcgcg-3′ (off-target region forward primer, −1475/−1458), 5′-agagacctggagcggaaaa-3′ (off-target region reverse primer, −1298/−1280).

2.6. Construction and mutagenesis of the MKP-3 promoter-reporter construct

A fragment of the mouse *MKP-3* gene spanning nucleotides −1597 to −10 (transcription start site numbered as +1) was amplified from mouse genomic DNA (Promega) by PCR using the primers 5′-agctccttttccctgggacc-3′ (forward; −1597/−10) and 5′-agagaatgtatccattgagacgc-3′ (reverse; −34/−10). The amplified PCR products were ligated into a T&A vector (RBC Bioscience), digested with *Hind*III, and then subcloned into the luciferase reporter plasmid pGL3-basic (Promega), yielding pMkp3-Luc(−1597/−10). A series of deletion constructs was generated using pMkp3-Luc(−1597/−10) as a template. Forward primer sequences were 5′-ctaacttaagattgtaagcgtcg-3′ (−386/−10), 5′-gcagcttgtttg-cactggggc -3′ (−186/−10), 5′-tgaatgacaaactcattaacaa-3′ (−133/−10), and 5′-cagcgcgctcattggctgacc-3′ (−56/−10). One reverse primer (−34/−10) was used for all deletion constructs. The amplified PCR products were ligated into the *Kpn*I/*Bgl*II sites of the pGL3-basic vector, yielding pMkp3-Luc(−386/−10), pMkp3-Luc(−186/−10), pMkp3-Luc(−133/−10), and pMkp3-Luc(−56/−10). The pMkp3-Luc(−1129/−10) and pMkp3-Luc(−712/−10) were generated by digestion of pMkp3-Luc(−1597/−10) with *Nhe*I/*Hind*II (−1129/−10) or *Sma*I/*Hind*III (−712/−10). Site-specific mutation of the Myb binding site was performed with a QuickChange site-directed mutagenesis system (Stratagene) using pMkp3-Luc(−186/−10) as a template. All mutations were verified by DNA sequencing.

2.7. Transient transfection and luciferase reporter assay

For the promoter reporter assay, cells were seeded into 12-well plates and transfected with 0.2 μg of the *MKP-3* promoter constructs using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. For Myb-dependent transcriptional activity, NIH3T3 cells cultured in 12-well plates were transfected with 0.1 μg of the pMyb-Luc plasmid (RIKEN BioResource Center, Ibaraki, Japan), along with 50 ng of the pRL-null plasmid encoding *Renilla* luciferase. To assess transfection efficiency, 50 ng of pRL-null plasmid encoding Renilla luciferase was included in all samples. At 24 h post-transfection, the levels of firefly and Renilla luciferase activities were measured using a Dual-Glo™ Luciferase assay system (Promega). Luminescence was also measured using a dual luminometer (Centro LB960; Berthold Tech). The firefly luciferase activity was normalized to the Renilla activity, and the relative amount of luciferase activity in the control cells was set to "1".

2.8. siRNA-mediated c-Myb silencing

A mixture of double-stranded RNA nucleotides targeting four different sequences of mouse Myb mRNA (ON-TARGETplus SMARTpool L-044112-00-0005) and a non-targeting negative control siRNA were obtained from Dharmacon (Thermo Fisher Scientific., Lafayette, CO). NIH3T3 cells were transfected with siRNA oligonucleotide pools using DharmaFECT reagent according to the manufacturer's protocol (Dharmacon).

2.9. Statistical analysis

Each experiment was performed at least three times. The results are plotted as means with SD. Statistical comparisons were made by a one-way ANOVA followed by the Turkey–Kramer Multiple Comparisons Test using GraphPad InStat v3.0 software. A p value of <0.05 was considered statistically significant.

3. Results

3.1. MKP-3 is induced by activation of Ras signaling pathway

We previously described a NIH3T3 cell line (NIH3T3tet-on/H-RasG12R), in which the expression of constitutively active Ras (H-RasG12R) can be induced by addition of doxycycline [11]. In