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HCV-induced PKR activation is stimulated by the mitogen- and stress-activated protein kinase MSK2

Ju-Il Kang, Byung-Yoon Ahn *

School of Life Sciences & Biotechnology, Korea University, Republic of Korea

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ABSTRACT

The replication of viral nucleic acids triggers cellular antiviral responses. The double-stranded RNA (dsRNA)-activated protein kinase (PKR) plays a key role in this antiviral response. We have recently reported that JFH-1 HCV replication in Huh-7 cells triggers PKR activation. Here we show that the HCV-induced PKR activation is further stimulated by the mitogen- and stress-activated protein kinase 2 (MSK2), a member of the 90 kDa ribosomal S6 kinase (RSK) family that has emerged as an important downstream effector of ERK and p38 MAPK signaling pathways. We show that MSK2 binds PKR and stimulates PKR phosphorylation, whereas the closely related MSK1 and RSK2 have no effect. Our data further indicate that MSK2 functions as an adaptor in mediating PKR activation, apparently independent of its catalytic activity. These results suggest that, in addition to viral dsRNA, stress signaling contributes to the regulation of cellular antiviral response.

1. Introduction

The dsRNA-activated protein kinase (PKR) is a key mediator of the cellular antiviral action of type I interferon (IFN). PKR is expressed constitutively in many tissues at a low level, before it is transcriptionally induced upon viral infection or following IFN treatment. Binding of dsRNA at the N-terminal domain of PKR further triggers the activation of its catalytic function, which resides in the C-terminal kinase domain. Besides auto-phosphorylation of PKR itself, the alpha-subunit of the heterotrimeric eukaryotic translation initiation factor 2 (eIF2α) is the best characterized substrate of PKR. Phosphorylation of eIF2α leads to the inhibition of viral and cellular protein synthesis and the subsequent restriction of viral spread. PKR is also implicated in the response to stress signals, cell proliferation and apoptosis [1].

Hepatitis C virus (HCV) is an enveloped virus with a single-stranded, positive-sense, RNA genome. Worldwide, more than 170 million people are chronically infected with the virus, and many of them are at the risk of developing chronic liver diseases and liver cancer [2]. At present, there are limited therapies for HCV infection and many patients do not adequately respond to IFN treatment. The antiviral role of PKR against HCV has been demonstrated with the recently developed HCV replicon systems [3,4]. We have shown that HCV replication triggers phosphorylation of PKR and eIF2α, and thereby inhibits viral protein synthesis and replication [5]. Alternatively, others have shown that HCV-mediated PKR activation can lead to the attenuated translation of cellular proteins as well, including the IFN-induced antiviral proteins [6] and IFN itself [7]. In the latter cases, PKR function was considered to restrict or delay cellular antiviral responses, which potentially contributes to the establishment of persistent infection.

Although dsRNA is the typical activator of PKR, several proteins can also activate PKR. Protein activator of the IFN-inducible protein kinase (PACT) interacts with PKR and activates it in response to cellular stresses that are induced by cytokines, ceramides and other chemicals [8]. The adaptor proteins of the TNF-receptor associated factor (TRAF) family were also shown to activate PKR in LPS-mediated TLR signaling pathways [9]. The mitogen- and stress-activated protein kinase (MSK) is a serine–threonine kinase related to the 90-kDa ribosomal S6 kinase (RSK or $p90^{rsk}$) family that mediates signal transduction downstream of the MAP kinase pathways [10]. Two MSKs (MSK1 and MSK2) and four RSKs (RSK1–RSK4) have been identified in mammals. While RSKs are activated by ERK, MSKs are activated by both ERK and p38 in response to mitogens and cellular stress. It was shown that RSK2 is involved in PKR activation following UVA-induced stress signaling [11], and in the antiviral response against influenza virus [12].

In this study, we show that MSK2 specifically binds PKR and stimulates PKR phosphorylation in HCV-replicating cells. These findings provide a pathway that connects cellular stress signaling to the regulation of PKR.

* Corresponding author. Address: School of Life Sciences & Biotechnology, Korea University, 5-1 Anamdong, Seoul 136-701, Republic of Korea. Fax: +82 2 923 9923.
E-mail address: ahnbyung@korea.ac.kr (B.-Y. Ahn).

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2. Materials and methods

2.1. Cells and virus

Human hepatoma Huh-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM HEPES, 50 μg/ml of gentamicin and 5% fetal bovine serum (Invitrogen) at 37 °C in 5% CO_2. JFH-1 HCV (genotype 2a) RNA was synthesized in vitro and electroporated with Gene Pulser II RF module (Bio-Rad) as described [5]. Transient suppression of RSKs was achieved by electroporation of Huh-7 cells ($\sim 4 \times 10^6$) with 1 nmol of siRNA duplexes (Sigma) of the following target sequences followed by two [dT] residues at 3′-ends of both strands: MSK1 (5′-CUGCU AAUUU GACAG GACA), MSK2 (5′-CCUUC UGUGG CACCA UCGA) and RSK2 (5′-GCAAC AUUCU UUAUG UGGA). RNA of unrelated sequence (5′-GAUCA UACGU GCGAU CAGA) was used as a negative control. Huh-7 cells stably expressing a siRNA against PKR (PKR-si cell) have been described [5]. Stable MSK2-si cells were selected with 1 μg/ml of puromycin (Sigma) after transfection of pSiMSK2. Ro 31-8220, GF109203X and H-89 were from Sigma–Aldrich. PD-98059 and SB-203580 were from Assay Designs.

2.2. Plasmid constructs

The human PKR and MxA gene constructs were described [5]. MSK1 (Accession No. NM_004755) and N- and C-terminal kinase-inactivated constructs (NT-KI, $MSK1^{D195A}$; CT-KI, $MSK1^{D565A}$; NCT-KI, $MSK1^{D195A/D565A}$) were kindly provided by Dr. J.H. Yoon [13] and subcloned into p3XFLAG-CMV-10 (Sigma), with three copies of the Flag tag at the N-terminus. MSK2 (Accession No. NM_003942) sequence was amplified from HeLa cell cDNA library and cloned into the same vector. The N- and C-terminal kinase-inactivated MSK2 (NT-KI, $MSK2^{D179A}$; CT-KI, $MSK2^{D551A}$; NT-KI, $MSK2^{D179A/D551A}$) were constructed by PCR-based mutagenesis. For stable knockdown of MSK2, pSiMSK2 was constructed to contain 5′-gcg gcc gag atc atg tgc aat tca aga gat tgc aca tga tct cgg ccg ttt ttt gga a (underlined sequences correspond to ntd 1862–1880 of MSK2 protein coding sequence) in pSilencer 2.1-U6 puro (Ambion).

2.3. Immunoblotting

Cells were lysed in RIPA buffer (10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.1% SDS and 0.25% Sodium deoxycholate) supplemented with protease inhibitors (1 mM PMSF, 1 μg/ml Leupeptin and 4 μg/ml Aprotinin) and phosphatase inhibitor cocktails (P2850, Sigma). Proteins were separated by gel electrophoresis and transferred onto Hybond ECL membrane (GE healthcare). Blots were incubated with antibody in TBST buffer (20 mM Tris–HCl [pH 7.6], 137 mM NaCl and 0.1% Tween-20) containing 5% skim milk and visualized with SuperSignal® West Pico Chemiluminescent reagents (Thermo Scientific). Anti-PKR (sc-707), anti-MSK1 (sc-25417), anti-RSK2 (sc-9986) and anti-GAPDH (sc-47724) antibodies were purchased from Santa Cruz Biotechnology. Anti-Phospho Thr446-PKR (ab32036) and anti-MSK2 (ab42101) antibodies were from Abcam Inc. Anti-HA (H6908), anti-Flag (F3165), anti-actin (A2668) and anti-tubulin (T9026) antibodies were from Sigma.

2.4. Immunoprecipitation

Huh-7 cells transfected with plasmids by using Lipofectamine and Plus reagent (Invitrogen) were washed with ice-cold PBS and lysed for 30 min in buffer (20 mM Tris–HCl [pH 7.4], 135 mM NaCl

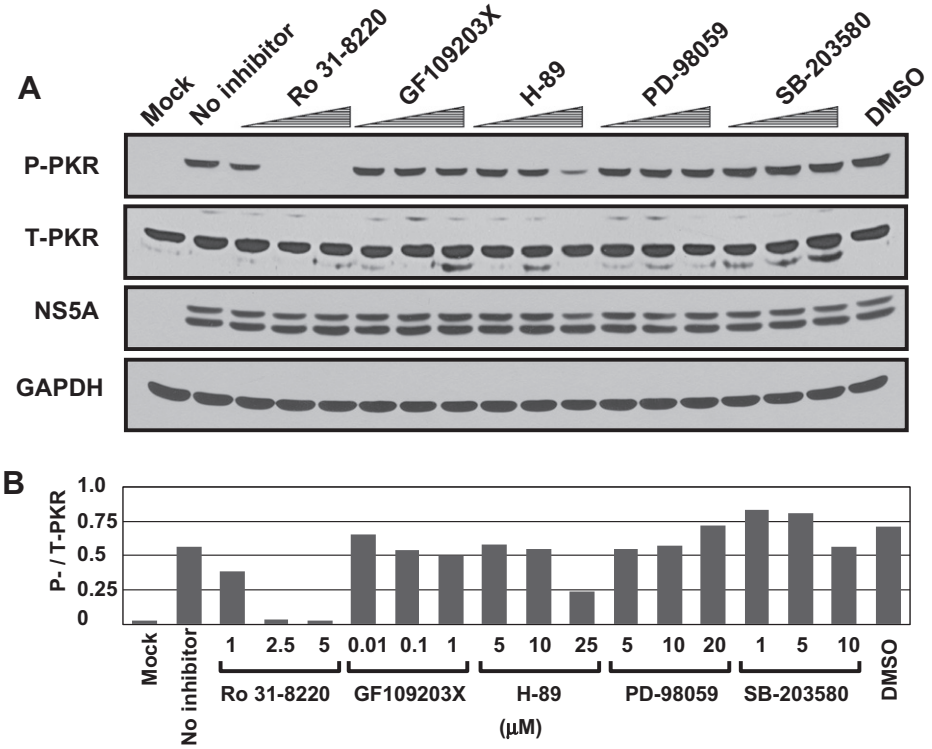

Fig. 1. PKR phosphorylation is inhibited by RSK inhibitors. Huh-7 cells carrying JFH-1 HCV RNA were treated with kinase inhibitors for 24 h: 1, 2.5, or 5 μM of Ro 31-8220; 0.5, 1, or 2 μM of GF109203X; 5, 10, or 25 μM of H89; 10, 20, or 40 μM of PD-98059; and 5, 10, or 25 μM of SB-203580. (A) Immunoblots were performed for the phosphorylated (T446) PKR, total PKR or HCV NS5A proteins. Cell without HCV RNA (first lane), and JFH-1 replicon cells, either untreated (second lane) or treated with 0.1% DMSO (last lane), respectively, were used as controls. GAPDH was used as a loading control. The blot shown here is one of more than three repeated experiments that gave similar results. (B) Intensity of phosphorylated and total PKR bands was quantitated with the ImageJ software (NIH, USA) and the ratio was shown in bar.