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SIRT1 negatively regulates the protein stability of HIPK2

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ABSTRACT

In the present study, we investigated whether a histone deacetylase sirtuin 1 (SIRT1) can regulate the protein stability of homeodomain-interacting protein kinase 2 (HIPK2). We observed the evidence of molecular interaction between SIRT1 and HIPK2. Interestingly, overexpression or pharmacological activation of SIRT1 promoted ubiquitination and the proteasomal degradation of HIPK2 whereas inhibition of SIRT1 activity increased the protein level of HIPK2. Furthermore, a SIRT1 activator decreased the level of HIPK2 acetylation whereas an inhibitor increased the acetylation level. These results suggest that SIRT1 may deacetylate and promote the ubiquitination and subsequent proteasomal degradation of HIPK2.

1. Introduction

Homeodomain-interacting protein kinase 2 (HIPK2) is a serine/threonine kinase that regulates DNA damage response and development [1]. HIPK2 functions as a tumor suppressor by activating p53-dependent proapoptotic pathway [2]. Proapoptotic function of HIPK2 was reported to be mediated also by phosphorylation and downregulation of transcriptional corepressor carboxyl-terminal binding protein [3]. Therefore, regulation of HIPK2 activation is of much importance in managing DNA damage response.

Under normal condition, the protein level of HIPK2 is tightly regulated by ubiquitin–proteasomal degradation system [1]. However, HIPK2 escapes from the degradation pathway upon DNA damage and accumulates to aid p53-mediated DNA damage response [4]. The accumulated HIPK2 phosphorylates p53 at serine 46 and this further stabilizes p53 so that p53 transcriptionally activates its downstream genes regulating cell cycle arrest or apoptosis [2]. Therefore, it is important to keep the protein level of HIPK2 low under unstressed conditions or recovering phase following DNA damage response. In unstressed cells, HIPK2 interacts with an E3 ubiquitin ligase, seven in absentia homolog 1 (Siah1). This interaction promotes polyubiquitination and proteasomal degradation of the HIPK2 [5]. The steady state level of HIPK2 protein is also regulated by another E3 ubiquitin ligase WD repeat and SOCS box-containing protein 1 [6]. Siah2 has been reported to promote the degradation of HIPK2 under hypoxic conditions [7].

SIRT1 is a member of mammalian sirtuin family and a class III histone deacetylase (HDAC) [8]. Sirtuins are the mammalian homologues of yeast silent information regulator 2 (Sir2) which has been reported to extend life span of yeast under calorie-restricted conditions [8]. Like Sir2, SIRT1 has drawn much attention as a possible 'longetivity' gene. It has been suggested that SIRT1 is a versatile cytoprotector against diverse cellular stresses. The cytoprotective function of SIRT1 is mediated by deacetylation of histones and nonhistone proteins. The nonhistone substrates of SIRT1 include many important regulators of cell survival, death, and metabolism such as p53, p73, p300, retinoblastoma, and forkhead box class O (FOXO) [9]. via NAD+-dependent deacetylation of these targets, SIRT1 regulates metabolism, aging and many aging-related diseases like cancer and neurodegenerative diseases [9].

Much of antiapoptotic function of SIRT1 is mediated by deacetylation of the tumor suppressor p53 [10]. Deacetylation of p53 by SIRT1 has been shown to decrease transcription-dependent apoptotic and senescence-inducing functions of p53 [10]. Since SIRT1 and HIPK2 play an opposite role in p53 stabilization and activation, we set out to test a possibility that SIRT1 directly antagonizes HIPK2. In the present study, we present evidence that SIRT1 deacetylates HIPK2 and promotes its proteasomal degradation.

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Our study proposes a novel mechanism of HIPK2 protein stability regulation mediated by SIRT1.

2. Materials and methods

2.1. Cell culture and transfection

Human Embryonic Kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle's medium (Welgene, Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotics plus antimycotics solution (Welgene, Daegu, Korea). Transfection of HEK293 cells with expression vectors encoding SIRT1 or HIPK2 was carried out using Mirus reagent according to the manufacturer's protocol (Mirus Bio, Madison, WI, USA).

2.2. Plasmids

Construction of the expression plasmids for full-length Myc-HIPK2, GFP–HIPK2 and various HIPK2 deletion mutants was described previously [11]. GFP–SIRT1, Flag-SIRT1, and Flag-SIRT1(HY) deacetylase-inactive mutant expression plasmids were kindly provided by Dr. S.J. Um (Sejong University, Korea).

2.3. Antibodies and immunocytochemistry

Antibodies used for the immunoblot and immunostaining were anti-SIRT1 (Millipore, Billerica, MA, USA), anti-HIPK2 (Abcam, Cambridge, UK), anti-α-tubulin (Sigma, St. Louis, MO, USA), anti-Myc, anti-Flag, anti-GFP, anti-HA (abm, Richmond, Canada), anti-ubiquitin (Millipore, Billerica, MA, USA), anti-acetylated lysine (Cell signaling, Danvers, MA, USA). Immunocytochemistry for HEK293 cells was performed as described previously [16].

2.4. Immunoprecipitation and immunoblotting

Immunoprecipitation was performed using 2×10^7 cells in a lysis buffer (20 mM Hepes, pH 7.5, 0.1 M KCl, 0.4 mM EDTA, 0.2% Nonidet P-40, 10 mM β-Mercaptoethanol, 1 μg/ml sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 mM PMSF). After incubation at 4 °C on a rotating wheel for 15 min and centrifugation at 13,000 rpm in a benchtop microcentrifuge for 10 min at 4 °C, equal volumes of protein were diluted with lysis buffer lacking NaCl and KCl, then incubated overnight with antibodies. Then protein A Sepharose beads were added (Sigma, St. Louis, MO, USA). After incubation at 4 °C on a rotating wheel for 1 h, the beads were washed three times with lysis buffer. Immunoblotting was performed by conventional methods.

2.5. Reverse transcription polymerase chain reaction

Total RNA was isolated using RNeasy minikit (Qiagen, Valenia, CA). cDNA was synthesized using the Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol. Reverse transcription (RT) reaction product was used as a template for PCR using the following primer pairs: mouse HIPK2 (forward 5′-GTC ACC ATG ACA CAC CTG CT-3′, reverse 5′-AGG GGG ACA CAC GAT GAG AG-3′), human HIPK2 (forward 5′-CCA CAG CAC ACA CGT CAA ATC-3′, reverse 5′-TTT GCT CTG GTT CAC CGT GTC-3′), β-Actin (forward 5′-CTG GGA CGA CAT GGA GAA-3′, reverse 5′-AAG GAA GGC TGG AAG AGT-3′). Annealing temperature was 58 °C. Reaction products were analysed on 2% agarose gels.

3. Results

3.1. HIPK2 interacted with SIRT1

Previous studies independently reported the presence of SIRT1 and HIPK2 in the promyelocytic leukemia nuclear bodies [12,13]. However, it has not been addressed whether these two enzymes colocalize in the nucleus or interact with each other. To find out if HIPK2 and SIRT1 interact, we first examined their cellular localization by immunocytochemistry. HEK293 cells were transfected with GFP–HIPK2 expression vector and then immunostaining was performed for the endogenous SIRT1. As shown in Fig. 1A, many of the HIPK2-positive nuclear speckles were also positive for the SIRT1, suggesting HIPK2 and SIRT1 colocalized in the nuclear speckles.

We then examined whether HIPK2 and SIRT1 interact *in vivo* by performing coimmunoprecipitation assay. HEK293 cells were transfected with Myc-HIPK2 and Flag-SIRT1 and then the whole cell lysates were immunoprecipitated with anti-Myc, anti-Flag or control IgG. As shown in Fig. 1B, the anti-Myc precipitates contained Flag-SIRT1 but those of control IgG did not, suggesting Myc-HIPK2 interacted with the Flag-SIRT1. The overexpressed Flag-SIRT1 was coimmunoprecipitated with the Myc-HIPK2 (Fig. 1C). In addition, endogenous SIRT1 was also coimmunoprecipitated with the overexpressed Myc-HIPK2 (Fig. 1D).

To examine which domain(s) of the HIPK2 interacted with SIRT1, various deletion mutants of HIPK2 was tested for the coimmunoprecipitation with SIRT1. As shown in Fig. 1E, the interaction domain of HIPK2 (residues 503–860) was coimmunoprecipitated with SIRT1. A deletion mutant spanning the speckle retention signal domain (residues 860–1049) also interacted with SIRT1 to a lesser degree. However, neither N-terminal half containing the kinase domain (residues 1–629) nor the autoinhibitory domain (residues 1049–1189) interacted with SIRT1. These results suggest that HIPK2 interact with SIRT1 mainly via its interaction domain.

3.2. Overexpressed SIRT1 decreased the protein level of HIPK2

SIRT1 is known as a prosurvival molecule against various cellular stressors while HIPK2 is proapoptotic in general [1,9]. Therefore, the interaction of SIRT1 and HIPK2 as we observed in Fig. 1 is likely to cause an antagonistic regulation between these molecules. Since HIPK2 is under a tight regulation of proteasomal degradation [1], we first examined if SIRT1 affects the protein level of HIPK2. To test this possibility, HIPK2 was cotransfected with SIRT1 and the protein level of HIPK2 was examined by immunoblot assay. As shown in Fig. 2A, an increasing amount of SIRT1 resulted in the decrease of HIPK2 protein. In addition, the accumulation of overexpressed HIPK2 over time was suppressed when SIRT1 was cotransfected (Fig. 2B). To find out at which level SIRT1 downregulated the expression of HIPK2, mRNA levels of the exogenous and endogenous HIPK2 were examined by RT-PCR analysis in the presence or absence of SIRT1 overexpression. Unlike protein level, the mRNA levels of both exogenous and endogenous HIPK2 were not altered by SIRT1 overexpression (Fig. 2C). This result suggests that SIRT1 may regulate the expression of HIPK2 at a post-transcriptional level.

3.3. SIRT1 activity was required for the downregulation of HIPK2 protein

To examine if deacetylase activity of SIRT1 is required for the downregulation of HIPK2 protein, the HIPK2-transfected cells were incubated in the presence of a SIRT1 activator, resveratrol. As shown in Fig. 3A, an increasing amount of resveratrol decreased

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