p53 deacetylation by SIRT1 decreases during protein kinase CKII downregulation-mediated cellular senescence

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Abstract

Cellular senescence is thought to be an important tumor suppression process in vivo. We have previously shown that p53 activation is necessary for CKII inhibition-mediated cellular senescence. Here, CKII inhibition induced acetylation of p53 at K382 in HCT116 and HEK293 cells. This acetylation event was suppressed by SIRT1 activation. CKIIα and CKIIβ were co-immunoprecipitated with SIRT1 in a p53-independent manner. Maltose binding protein pull-down and yeast two-hybrid indicated that SIRT1 bound to CKIIβ, but not to CKIIα. CKII inhibition reduced SIRT1 activity in cells. CKII phosphorylated and activated human SIRT1 in vitro. Finally, SIRT1 overexpression antagonized CKII inhibition-mediated cellular senescence. These results reveal that CKII downregulation induces p53 stabilization by negatively regulating SIRT1 deacetylase activity during senescence.

1. Introduction

Normal primary cells withdraw from the cell division cycle after a finite number of divisions and enter irreversible growth arrest designated as replicative senescence, or more generally, cellular senescence [1]. Cellular senescence can also be induced by oxidative stress, DNA damage, and oncogenic activation [2–6]. Senescence is thought to be an important tumor suppression process in vivo. In a state of senescence, cells become enlarged, acquire a flattened shape, and express several senescence-associated markers such as senescent-associated β-galactosidase (SA-β-gal), cyclin-dependent kinase inhibitor p21Cip1/WAF1, and tumor suppressor protein p53 [7–10]. p53 is a transcription factor that plays a major role in cell cycle regulation. It is activated in response to a variety of cellular stress signals, where upon it triggers cell cycle arrest or apoptosis depending on the circumstances. p53 activity is modulated by protein stability and post-translational modifications, including phosphorylation and acetylation [11].

The silent information regulator 2 (Sir2) is an NAD-dependent protein deacetylase that controls longevity in lower eukaryotes, such as budding yeast and nematode [12,13]. An increase in Sir2 activity extends the lifespan of these organisms. Mammals possess seven paralogs of the Sir2 gene, with SIRT1 being the most similar to Sir2. SIRT1 deacetylates not only histones (H1, H3, and H4), but also many non-histone proteins such as p53 [14]. It has been shown that SIRT1 is also important for various cellular functions, including apoptosis, differentiation, proliferation, and metabolism.

Protein kinase CKII (formerly known as casein kinase II) (CKII), a ubiquitous serine/threonine kinase, plays a significant role in the control of cell proliferation and transformation [15–17]. The holoenzyme of CKII is a heterotetramer composed of two catalytic (α and/or α′) and two regulatory (β) subunits. We previously demonstrated that CKII activity is downregulated at the transcriptional level in both senescent human lung fibroblast...
IMR-90 cells and aged rat tissues. CKII inhibition by CKII inhibitors or CKIIα small-interfering RNA (siRNA) induces premature senescence in IMR-90 cells [18]. In addition, silencing of the CKIIα and CKIIβ genes during cellular senescence is mediated by DNA methylation [19]. Studies designed to examine the mechanism through which CKII inhibition contributes to the development of senescence have indicated that p53 is involved in the development of senescence induced by CKII inhibition in HCT116 human colon cancer cells [20]. Superoxide anion generation by NADPH oxidase (NOX) activation is the upstream mediator of p53 stabilization in cells made senescent by CKII inhibition [21]. However, we cannot rule out the possibility that CKII downregulation also mediates p53 stabilization by another mechanism. Here, we provide evidence that CKII downregulation can stabilize p53 via inhibition of SIRT1 deacetylase activity in cells and that, conversely, exogenous SIRT1 delays CKII inhibition-mediated senescence.

2. Materials and methods

2.1. Cell culture and transfection

Human colon cancer HCT116 and human embryonic kidney (HEK) 293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum at 37°C (Danvers, MA). K382 antibody was obtained from Cell Signaling Technology (Danvers, MA) as described by the manufacturer.

2.2. Preparation of cell extracts

For Western blotting, cells in 100-mm dishes were washed with ice-cold phosphate-buffered saline (PBS), collected by scraping with a rubber policeman, and lysed in 100 μl of ice-cold RIPA buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, and 1 μg/ml of pepstatin]. For measurement of deacetylase activity, cells were lysed by sonication in lysis buffer [50 mM Tris–HCl (pH 8.0), 20 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 1% Nonidet P-40, 0.5 mM PMSF, 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, and 4 mM p-nitrophenyl phosphate]. The particulate debris was then removed by centrifugation at 12000 × g. The samples were then separated on a 10% SDS–polyacrylamide gel by the addition of purified CKII and incubated for 15 min at 30°C.

2.3. Immuno-blotting

Proteins were separated by polyacrylamide gel electrophoresis in the presence of SDS and then transferred by electrophoresis to a nitrocellulose membrane. The membrane was blocked with 5% (w/v) non-fat, dry skim milk in TBST [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20] for 2 h and then incubated with specific antibodies in 1% (w/v) non-fat, dry skim milk for 1 h. The membrane was washed three times in TBST and then analyzed with the ECL system (Amersham Pharmacia Biotech, Korea). Some membranes were stripped in stripping buffer [2% SDS, 100 mM β-mercaptoethanol, and 50 mM Tris–HCl (pH 7.0)] at 50°C for 1 h with gentle shaking and then reprobed with anti-β-actin antibody as a control for protein loading. Anti-p53, -CKIIα, -SIRT1, and -β-actin antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-acetyl p53 K382 antibody was obtained from Cell Signaling Technology (Danvers, MA).

2.4. Maltose binding protein (MBP) pull-down assay and immunoprecipitation

An MBP pull-down assay was performed by incubating amylase agarose beads with MBP-CKII and cell lysates in 200 μl of binding buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF]. The reaction was allowed to proceed for 1 h while rocking at 4°C. For immunoprecipitation, cell lysates were pre-cleared with normal mouse or rabbit IgG along with protein A Sepharose (Amer sham Biosciences, Piscataway, NJ) for 1 h at 4°C. The supernatant was then incubated with anti-CKII or anti-SIRT1 antibody along with protein A Sepharose with mixing for 12 h at 4°C. The beads were collected by centrifugation and washed three times with PBS.

2.5. Yeast two-hybrid assay

The reporter strain Saccharomyces cerevisiae HF7c (MATa ura3-52 his3-200 ade2-101 lys2-801 trpl-901 leu2-3112 gal4-542 gal80-538 LYS2::GAL1::GAL1TATA-HIS3 URA3::GAL417merFEN(x3)-CyC1TA-TA-lacZ) was co-transformed with various combinations of hybrid plasmids containing a DNA-binding domain or a transcriptional activation domain. Transformants were plated on synthetic medium lacking tryptophan and leucine. After 4 days of growth, the transformants were cultured on selective medium lacking tryptophan, leucine, and histidine but including 1 mM 3-amino-triazole, after which they were incubated for 4 days at 30°C. Interactions between the hybrid proteins were monitored by growth on selective media.

2.6. RNA interference

The siRNA for CKIIα was 5'-UCAGUGACGUACACGUGTdT. The siRNA for the negative control was 5'-GCUCAGAUCAUUAG-GAGAdTdT. Both siRNAs were transfected into cells using Lipofectamine (Invitrogen) as described by the manufacturer. Five hours after transfection, the medium was changed, and the cells were grown for another 3 days before being harvested or stained for SA-β-gal.

2.7. Purification of CKII and SIRT1 phosphorylation by CKII

CKII holoenzyme and CKII subunits tagged with MBP were expressed in Escherichia coli and purified as described previously [22,23]. Phosphorylation of human SIRT1 (BIOMOL International, Plymouth Meeting, PA) by CKII was carried out in a reaction mixture containing 20 mM Tris–HCl (pH 7.5), 100 mM KCl, 10 mM MgCl₂, 1 mM DTT, 1 mM EGTA, 100 μM [γ-32P]ATP, and 1 μg of human SIRT1 in a total volume of 30 μl. The reactions were initiated by the addition of purified CKII and incubated for 15 min at 30°C. The samples were then separated on a 10% SDS–polyacrylamide gel. The gel was stained with Coomassie blue, dried, and subjected to autoradiography.

2.8. Measurement of histone deacetylase (HDAC) and SIRT1 activity

HDAC activity was assessed with the HDAC Colorimetric Assay/Drug Discovery Kit (BIOMOL International) according to the manufacturer’s instructions. Briefly, cell extracts and Color de Lys™ substrate were added to the wells of a microtiter plate and incubated at 37°C for 15 min. HDAC inhibitor trichostatin A and Color de Lys™ developer were added to stop the HDAC reaction. The mixture was incubated at 37°C for 10 min and read on a microtiter-plate reader (PerkinElmer, Waltham, MA) at 405 nm. The enzyme activity of SIRT1 was measured with the SIRT1 Fluorimetric Drug Discovery Kit (BIOMOL International) based on Fluor de Lys–SIRT1.
substrate peptide. The reaction was carried out at 37 °C for 30 min. The deacetylase activity was detected as a fluorescent emission at 460 nm with an excitation wavelength of 355 nm. The fluorescence intensity of the compounds at 460 nm was subtracted from the values measured in the assay. The resulting value was treated as the baseline value in the absence of compound. The experiments were performed at least three times.

2.9. SA-β-gal activity assay

SA-β-gal activity was measured as described previously [8] with minor modifications. Cells in sub-confluent cultures were washed with PBS, fixed in 3% (v/v) formaldehyde in PBS for 10 min at room temperature, and then incubated with a stain solution containing 1 mg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactoside, 40 mM citric acid-sodium phosphate (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM MgCl₂ for 24 h at 37 °C. The number of blue-stained cells was counted in at least 10 fields at 400× magnification and expressed as percentage of positive cells.

2.10. Growth curves

Cells were seeded in six-well dishes at a starting density of 5000 cells/well with duplicate wells for each sample. Every 24 h, cells were trypsinized and counted in triplicate using a hemocytometer. Trypan blue was used to distinguish viable cells from non-viable cells.

2.11. Statistical analysis

Results are presented as the means ± standard errors. Statistical analysis was performed with SPSS version 11.0 (SPSS Inc., Chicago, IL). Data were analyzed by one-way ANOVA, and Duncan’s multiple-range test was performed if differences were identified among the groups at \( P < 0.05 \). Analysis between two groups was determined by using unpaired Student’s t-test.

3. Results and discussion

3.1. SIRT1-dependent p53 K382 deacetylation decreases during CKII inhibition-mediated senescence

We previously demonstrated that CKII inhibition induces cellular senescence through the p53-dependent pathway in HCT116 cells [20]. SIRT1 has been shown to be involved in cellular senescence [12,13] and regulate p53 K382 acetylation [14]. To examine whether or not p53 K382 acetylation contributes to CKII inhibition-induced p53 stabilization in senescent cells, we treated HCT116 cells with CKII inhibitors, apigenin and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), and then determined the protein level and acetylation status of p53 by immunoblotting. As shown in Fig. 1A, expression of p53 was upregulated in apigenin- or DRB-treated HCT116 cells. In addition, acetylation of p53 K382 was induced by treatment with CKII inhibitors. To determine if SIRT1 activity blocks CKII inhibition-induced p53 K382 acetylation, we added the SIRT1 activator resveratrol to HCT116 cells in the presence of CKII inhibitor. Apigenin-induced acetylation of p53 K382 was inhibited by resveratrol, indicating that SIRT1 reduced p53 acetylation during CKII inhibition-induced senescence (Fig. 1B). The apigenin-induced acetylation of p53 K382 was also inhibited by resveratrol in HEK293 cells (Fig. 1C). However, the protein levels of SIRT1 were unchanged in these cells, indicating that SIRT1 deacetylase activity was regulated by post-translational modification (Fig. 1A and C). We performed time-course studies and found that treatment of cells with resveratrol (2 μM) decreased p53 K382 acetylation levels within 2 days (Fig. 1D). To confirm the role of resveratrol in p53 K382 acetylation induced...
Fig. 2. CKIIβ subunit interacts with SIRT1. (A) Cell lysates from HCT116 cells were subjected to immunoprecipitation (IP) with anti-SIRT1, anti-CKIIα, and anti-CKIIβ antibodies, or normal rabbit IgG. The precipitated proteins were separated by 10% (w/v) SDS–polyacrylamide gel electrophoresis and visualized by Western blotting with appropriate antibodies. (B) Purified human SIRT1 was incubated with MBP-CKII subunit-coated amylose resin. Precipitation with MBP-coated amylosel resin was used as a negative control. The immobilized complexes were recovered with 20 mM maltose and analyzed by Western blotting. (C) The reporter strain was co-transformed with pGADGH-SIRT1 and pGBT9-CKIIα or pGBT9-CKIIβ. Double transformants grew on non-selective medium lacking tryptophan and leucine (−TL) and on selective medium lacking tryptophan, leucine, and histidine (−TLH) but including 1 mM 3-amino-triazole. Interactions between the hybrid proteins were tested to assess the ability to support growth on selective medium. (D) SIRT1 was precipitated from lysates of wild-type (WT) and p53−/− HCT116 cells with MBP-CKII subunit-coated amylose resin. The immobilized complexes were recovered with 20 mM maltose and analyzed by Western blotting.

Fig. 3. CKII phosphorylates and activates SIRT1. HCT116 cells were treated with CKII inhibitors for 6 days (A, B) or transfected with CKIIα siRNA for 4 days (C) in the presence or absence of resveratrol. SIRT1 activity was measured in cell lysates as described in Section 2. Data are shown as the means ± S.E.M. *P < 0.05. Bars that do not share a common letter (a, b, c) are significantly different among the groups at P < 0.05. (D) Human SIRT1 was incubated without (lane 1) or with (lane 2) CKII holoenzyme in the presence of [γ-32P]ATP and analyzed by SDS–polyacrylamide gel electrophoresis, with Coomassie Blue staining (CBB, left panel), autoradiography (middle panel), and Western blotting with anti-SIRT1 antibody (right panel). (E) The purified SIRT1 was incubated without or with CKII holoenzyme in the presence of ATP. Then, SIRT1 activity was assayed as described in Section 2. Data are shown as the means ± S.E.M. *P < 0.05.
by CKII downregulation, we knocked down CKIIα in HCT116 cells by gene silencing using siRNA. Treatment of cells with CKIIα siRNA stimulated acetylation of p53 at K382, and resveratrol suppressed this acetylation event (Fig. 1E). To examine whether or not overexpression of SIRT1 affects CKII inhibition-induced p53 K382 acetylation, we transfected HCT116 cells with pECE-Flag-SIRT1. As shown in Fig. 1F, exogenous expression of wild-type SIRT1, but not the catalytically inactive mutant of SIRT1 (H363Y), suppressed CKII inhibition-induced p53 K382 acetylation. Taken together, these results demonstrate that CKII inhibition can promote acetylation of p53 K382 through downregulation of SIRT1 deacetylase activity in cells.

3.2. SIRT1 interacts with CKII through direct binding to CKIIβ subunit

To determine whether or not SIRT1 is associated with CKII in HCT116 cells, we immunoprecipitated SIRT1 and CKII from HCT116 cell extracts. Normal IgG immunoprecipitation was used as a control. As shown in Fig. 2A, both CKIIα and CKIIβ were co-precipitated with SIRT1. SIRT1 was also co-precipitated with CKIIβ.

**Fig. 4.** SIRT1 overexpression suppresses CKII inhibition-mediated senescence. HCT116 (A) and HEK293 (B) cells were treated with CKIIα siRNA and pECE-Flag-SIRT1 for 2 days. Cells were lysed and subjected to electrophoresis on a 10% (w/v) SDS–polyacrylamide gel. Protein bands were visualized by immunoblotting with anti-CKIIα, anti-SIRT1, and anti-β-actin antibodies (upper panels). Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside, and the percentage of positively stained cells was measured. Representative images were obtained at 20× magnification (bottom panels). Data are shown as the means ± S.E.M. Bars that do not share a common letter (a, b, c) are significantly different among the groups at \( P < 0.05 \). (C) HCT116 cells that were transfected with CKIIα siRNA and pECE-Flag-SIRT1 were seeded in six-well dishes. Every 24 h, cells were trypsinized and counted. These experiments were performed twice with similar results. (D) Possible model illustrating CKII downregulation-mediated cellular senescence. CKII downregulation generates ROS by activating NADPH oxidase [21] and also inhibits SIRT1 deacetylase, leading to acetylation of p53. This process results in activation of the p53-p21WAF1/CIP1-Rb-dependent cellular senescence pathway [20]. ROS, reactive oxygen species; CDK, cyclin-dependent kinase.
and CKIIβ, indicating that SIRT1 interacts with CKII holoenzyme in HCT116 cells. Next, to examine which subunit of CKII bound directly to SIRT1, we purified MBP-CKIIα and MBP-CKIIβ and tested their ability to form a complex with SIRT1. When MBP-CKII sub-units were mixed with purified human SIRT1, SIRT1 bound only to MBP-CKIIβ and not to CKIIα (Fig. 2B). The yeast two-hybrid system confirmed this finding (Fig. 2C). p53 has already been shown to interact with SIRT1 [14] and CKIIβ [24]. To examine whether or not interaction of SIRT1 with CKII is p53-dependent, we used p53−/− HCT116 cells in which the p53 gene was inactivated by homozogous knockout. When MBP pull-down assay was carried out with p53−/− cell extracts, SIRT1 was able to bind to CKIIβ regardless of p53 protein, indicating that the interaction between CKII and SIRT1 was p53-independent (Fig. 2D). Taken together, these results suggest that SIRT1 binds to CKIIβ independently of p53.

3.3. CKII stimulates SIRT1 deacetylase activity by phosphorylation

The finding that CKII inhibition-mediated acetylation of p53 K382 was inhibited by a SIRT1 activator suggested that CKII down-regulation might inhibit the activity of SIRT1. To investigate this possibility, we examined the effects of CKII inhibition on SIRT1 activity. Treatment with CKII inhibitors or CKII subunit is known to determine substate specificity [15–17], we examined whether or not CKII catalyzes the phosphorylation of SIRT1. Purified human SIRT1 became phosphorylated when incubated with CKII holoenzyme in the presence of [γ-32P]ATP. Phosphorylation of SIRT1 was not observed when CKII was omitted from the reaction mixture, indicating that SIRT1 was not autophosphorylated (Fig. 3D). To examine whether or not CKII modulates human SIRT1 deacetylase activity in vitro, we assessed the activity of SIRT1 after preincubation with or without CKII in the presence of ATP. As shown in Fig. 3E, preincubation with CKII stimulated human SIRT1 activity, indicating that CKII phosphorylation was involved in the upregulation of SIRT1 activity. Consistent with this finding, it was recently reported that CKII phosphorylates mouse SIRT1 and regulates its deacetylase activity [25].

HDAC1 is also known to catalyze the deacetylation of p53 K382 [26]. To examine whether or not CKII downregulation inhibits the activity of HDAC1, we determined the effect of CKII inhibition on HDAC1 activity in HCT116 cells. CKIIα siRNA treatment actually resulted in stimulation of HDAC activity, suggesting that HDAC1 did not affect the acetylation of p53 K382 in cells made senescent by CKII inhibition (data not shown). Taken together, these results suggest that CKII phosphorylates and activates human SIRT1 and that CKII downregulation leads to a reduction in SIRT1 deacetylase activity in cells.

3.4. SIRT1 overexpression suppresses CKII inhibition-mediated senescence

We next examined whether or not SIRT1 antagonizes the cellular senescence induced by CKII inhibition. To detect senescence, HCT116 cells were transfected with CKIIα siRNA and then stained for SA-β-gal activity. There was a significant increase in SA-β-gal activity in response to CKIIα knock-down. However, co-transfection of these cells with wild-type SIRT1 resulted in a decrease in the rate of SA-β-gal staining. In contrast, overexpression of the catallytically inactive mutant of SIRT1 (H363Y) did not affect SA-β-gal activity in the cells (Fig. 4A). Similar results were obtained with HEK293 cells (Fig. 4B). To confirm this finding, we generated growth curves with HCT116 cells. As expected, CKIIα siRNA treatment decreased HCT116 cell proliferation. This reduction in proliferation was reversed by expression of wild-type SIRT1 but not by mutant SIRT1 H363Y (Fig. 4C).

We previously showed that the activated p53−/p21[1]WAF1 pathway acts as a mediator of CKII inhibition-induced cellular senescence [20]. Stress signals, such as reactive oxygen species (ROS), ionizing radiation, and DNA damage, converge on p53 and activate CBP/p300 acetyltransferase, which acetylates p53 in the C-terminal domain where K382 is located [11,27]. Recently, we revealed that NOX-dependent superoxide anion production is an upstream contributor to the CKII downregulation-mediated senescence signaling cascade [21]. Here, we presented an additional mechanism for p53 stabilization during CKII downregulation-mediated senescence. The present results indicate that CKII inhibition stimulates p53 stability by negatively regulating SIRT1 deacetylase activity and that CKII inhibition-mediated senescence can be antagonized by SIRT1 overexpression. Therefore, based on previous findings and the current study, we can conclude that through NOX activation, CKII inhibition generates ROS that activate CBP/p300 acetyltransferases, which, in turn, acetylate p53 K382. Furthermore, through downregulation of SIRT1 deacetylase activity, CKII inhibition maintained the p53 acetylation state, which is required for p53 activation during cellular senescence (Fig. 4D). Cellular senescence is thought to be an important tumor suppression process in vivo. Our results illustrate the potential of targeting pathways involved in the regulation of p53 for therapeutic use in various tumors.

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