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Involvement of PI3K-AKT-mTOR pathway in protein kinase CKII inhibition-mediated senescence in human colon cancer cells

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ABSTRACT

Cellular senescence is a tumor suppression mechanism. We previously reported that CKII downregulation induces senescence in human lung fibroblast IMR-90 and colon cancer HCT116 cells. In this study, potential longevity drugs, including rapamycin, vitamin C, and vitamin E, blocked CKII downregulation-mediated senescence through reduction of reactive oxygen species (ROS) production in HCT116 cells. Since rapamycin is a mammalian target of rapamycin (mTOR) inhibitor, we examined the roles of mTOR and its upstream regulators phosphatidylinositol 3-kinase (PI3K) and AKT in CKII inhibition-mediated senescence. CKIIα knock-down or CKII inhibitor treatment strikingly increased phosphorylation of mTOR, p70S6K, an mTOR substrate, and AKT, whereas CKIIα overexpression reduced this phosphorylation event. This result indicated that CKII inhibition activated the PI3K-AKT-mTOR pathway. Further, pharmacological inhibition of PI3K and AKT attenuated ROS production and senescence in CKII-downregulated cells. Taken together, these results demonstrate, for the first time, that the PI3K-AKT-mTOR-ROS pathway is necessary for CKII inhibition-mediated cellular senescence.

1. Introduction

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine protein kinase that plays an integral role in coordinating cell growth and division in response to growth factors, nutrients, and cellular energy status [1,2]. mTOR elicits its pleiotropic functions within the context of two functionally distinct signaling complexes, termed mTOR complex 1 (mTORC1) and complex 2 (mTORC2). In mammalian cells, mTORC1 is composed of four subunits: Raptor, PRAS40, mLST8, and mTOR. The most well characterized mTORC1 substrates are ribosomal p70 S6 kinase (p70S6K1) and eukaryotic initiation factor 4E-binding protein 1, through which mTORC1 controls protein synthesis. On the other hand, mTORC2 shares mTOR and mLST8 with mTORC1, but possesses three unique components, Rictor, PRR5, and mSin1. Signaling by mTORC1 is nutrient-sensitive, acutely inhibited by the anti-immunosuppressive drug rapamycin, and controls cell growth, angiogenesis, and metabolism. In contrast, mTORC2 signaling is not sensitive to nutrients, nor inhibited by acute rapamycin treatment.

Growth factors and hormones positively regulate mTORC1 through activation of the phosphatidylinositol 3-kinase (PI3K)-AKT pathway [3,4]. Activation of PI3K results in the addition of a phosphate group to its lipid substrate, phosphatidylinositol 3,4-bisphosphate (PIP2), resulting in the formation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) at the cell membrane. PIP3 then initiates a signaling cascade by interacting with the pleckstrin homology domain of the serine kinase AKT, which inactivates two negative regulators of mTOR, including TSC2 and PRAS40. On the other hand, the phosphatase PTEN acts as a negative regulator of the PI3K-AKT signaling pathway by dephosphorylating PIP3 into PIP2.

Normal primary cells withdraw from the cell division cycle after a finite number of divisions and enter an irreversible proliferation arrest designated replicative senescence or, more generally, cellular senescence [5]. Cellular senescence can also be induced by oxidative stress, DNA damage, or oncogenic activation [6,7]. Consequently, cellular senescence is thought to be an important tumor suppression process *in vivo*. Senescence is characterized by several molecular and cytological markers, including a large flat cell morphology and senescence-associated β-galactosidase activity (SA-β-Gal) [8,9]. We have recently shown that CKII activity is

Abbreviations: CKII, protein kinase CKII (also known as casein kinase II); DMEM, Dulbecco's modified Eagle's medium; DHE, dihydroethidium; DRB, 5,6-dichloro-1-γ-D-ribofuranosylbenzimidazole; ETH, ethidium; FBS, fetal bovine serum; HA, hemagglutinin; mTOR, mammalian target of rapamycin; p70S6K, ribosomal p70 S6 kinase; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 3,4-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; SA-β-gal, senescent-associated β-galactosidase; SDS, sodium dodecyl sulfate; siRNA, small-interfering RNA.

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downregulated at the level of transcription level in both senescent human lung fibroblast IMR-90 cells and aged rat tissues. CKII inhibition induces premature senescence in IMR-90 cells, whereas the p53-$p21^{Cip1/WAF1}$ pathway is required for the development of senescence induced by CKII inhibition in HCT116 human colon cancer cells [10,11]. Both superoxide anion generation due to NADPH oxidase activation and p53 acetylation due to SIRT1 downregulation are upstream triggers of p53 stabilization in cells made senescent by CKII inhibition [12,13]. DNA methylation and microRNAs are involved in silencing of CKIIα gene expression during senescence [14,15].

Although CKII inhibition has been found to induce senescence by stimulating ROS production, the molecular mechanisms by which CKII modulates ROS generation and senescence remain obscure. In this paper, for the first time, we demonstrate that rapamycin, vitamin C, and vitamin E attenuate senescence by inhibiting ROS generation. Most importantly, CKII inhibition activates the PI3K-AKT-mTOR pathway during cellular senescence.

2. Materials and methods

2.1. Cell culture and SA-β-gal activity assay

HCT116 human colon cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) under a humidified atmosphere of 5% (v/v) CO_2 at 37 °C. SA-β-gal activity was measured as described previously [8] with minor modifications. Cells in sub-confluent cultures were washed with ice-cold phosphate-buffered saline (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 $MgCl_2$ for 24 h at 37 °C. The number of blue-stained cells was counted in at least 10 fields at 20× magnification and expressed as the percentage of positive cells.

2.2. RNA interference and DNA transfection

siRNAs and pcDNA-HA-CKIIα were transfected into cells by using Lipofectamine (Invitrogen, Carlsbad, CA) as described by the manufacturer. siRNA for CKIIα was 5′-UCAAGAUGACUACCAGCUGdTdT. siRNA for the negative control was 5′-GCUCAGAUCAAUACGGAGAdTdT. At 48 h after transfection, the cells were harvested or stained for SA-β-gal activity.

2.3. Preparation of cell extracts

For preparation of cell extract, cells were lysed by sonication in lysis buffer [50 mM Tris–HCl (pH 8.0), 20 mM NaCl, 1 mM $MgCl_2$, 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]. Particulate debris was removed by centrifugation at 12,000×*g*. Volumes of the supernatants were adjusted to ensure equal protein concentration.

2.4. Western blotting

Proteins were separated on 12% polyacrylamide gels in the presence of SDS, and then transferred by electrophoresis to nitrocellulose membranes. The membranes were 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 membranes were washed three times in TBST and then treated with an 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 reprobed with anti-β-actin antibody as a control for protein loading. Antibodies specific for CKIIα, p53, $p21^{Cip1/WAF1}$, and β-actin were from Santa Cruz Biotechnology Inc. Antibodies specific for phopho-mTOR (S2448), phospho-AKT (S473), and phospho-p70S6K were from Cell Signaling Technology (Beverly, MA).

2.5. CKII activity assay

Standard assay for phosphotransferase activity of CKII was conducted in a reaction mixture containing 20 mM Tris–HCl (pH 7.5), 120 mM KCl, 10 mM $MgCl_2$, and 100 μM [γ-^{32}P]ATP in the presence of 1 mM synthetic peptide substrate (RRREEETEEE) in a total volume of 30 μl at 30 °C. Cell lysates were added to initiate the reactions and were incubated for 15 min. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 10%. The mixture was then centrifuged, after which 10 μl of supernatant was applied to P-81 paper. The paper was washed in 100 mM phosphoric acid, and radioactivity was measured by scintillation counting.

2.6. Measurement of intracellular ROS

The oxidation-sensitive fluorescent probes CM-H_2DCFDA and dihydroethidium (DHE) were used to monitor production of hydrogen peroxide and cytosolic superoxide anions, respectively. Probes were purchased from Invitrogen. HCT116 cells were treated with CKII inhibitors for 4 days and then incubated with 5 μM CM-H_2DCFDA or DHE for 20 min at 37 °C in the dark. Cells were then rinsed twice with PBS, and digital images were obtained with a Leica DM IRB inverted microscope (Leica Microsystems, Germany) equipped with a coolsnap HQ camera (Roper Scientific, NJ) operated by Metamorph Image Software (Universal Imaging Corporation, PA). For flow cytometry analysis, cells in culture dishes were detached by trypsinization and washed with PBS. Fluorescence intensity was determined with a Colter Elite ESP Cell Sorter (Beckman Colter Inc., Brea, CA). The forward and side scatter gates were set to exclude any dead cells from the analysis; at least 10,000 events within this gate were acquired per sample.

2.7. Statistical analysis

The statistical significance of β-gal staining data was analyzed by one-way ANOVA with the SPSS package program. The results were considered significant if the value of P was less than 0.05. Duncan's multiple-range test was performed if the differences between the groups were identified as $\alpha = 0.05$.

3. Results

3.1. Rapamycin, vitamin C, and vitamin E suppress CKII inhibition-mediated senescence in HCT116 cells

We previously reported that CKII downregulation induces senescence in HCT116 cells [11]. To investigate whether or not rapamycin, vitamin C, and vitamin E suppress senescence induced by CKII inhibition, these reagents were added to HCT116 cells in the presence of the CKII inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). There was a significant increase in SA-β-gal activity in response to CKII inhibition induced by DRB treatment.

However, co-treatment of cells with rapamycin, vitamin C, or vitamin E apparently reduced the rate of SA-β-gal staining (Fig. 1A). To confirm reduction of SA-β-gal staining by rapamycin, vitamin C, and vitamin E in cells with downregulated CKII, we knocked-down CKIIα in HCT116 cells by gene silencing with siRNA duplexes. Transfection of CKIIα siRNA markedly increased SA-β-gal staining, whereas rapamycin, vitamin C, and vitamin E treatment suppressed SA-β-gal staining (Fig. 1B). These results indicate that CKII downregulation-mediated senescence can be blocked by rapamycin, vitamin C, and vitamin E.

We have previously demonstrated that CKII inhibition induces senescence through the p53-p21$^{Cip1/WAF1}$-dependent pathway [11]. Thus, we determined whether or not treatment with rapamycin, vitamin C, or vitamin E decreases the protein expression level of p53 in cells with downregulated CKII. Immunoblotting showed that the protein level of p53 was upregulated in cells treated with DRB or CKIIα siRNA compared with control cells. However, co-treatment with rapamycin, vitamin C, or vitamin E apparently reduced expression of p53 protein in cells (Fig. 1C). These results suggest that rapamycin, vitamin C, and vitamin E inactivate p53 in cells made senescent by CKII inhibition.

3.2. Rapamycin, vitamin C, and vitamin E antagonize CKII inhibition-mediated ROS production in HCT116 cells

Previously, we have shown that ROS generation is an upstream trigger of p53 stabilization in cells made senescent by CKII inhibition [12]. To examine whether or not rapamycin, vitamin C, and vitamin E reduce ROS production in cells made senescent by CKII inhibition, HCT116 cells were incubated with CM-H_2DCFDA or DHE. Within cells, CM-H_2DCFDA is hydrolyzed to DCFH, which is then oxidized by hydrogen peroxide via intracellular peroxidases to yield fluorescent DCF [16]. DHE is relatively specific for superoxide anion and reacts only minimally with hydrogen peroxide [17]. Hydrogen peroxide is produced from superoxide anion ($O_2^{\cdot-}$), which is a key radical in ROS generation, in a reaction catalyzed by superoxide dismutase. Fig. 1D shows representative images of DCF and DHE fluorescence in untreated cells and cells treated with DRB. DRB significantly increased hydrogen peroxide and superoxide anion levels relative to untreated control cells, as indicated by green and red fluorescence. However, incubation with rapamycin, vitamin C, and vitamin E apparently prevented both fluorescent signals in DRB-treated cells. Vitamin C and vitamin E are well known ROS scavengers, whereas rapamycin is an mTOR inhibitor. Thus, these findings suggest that CKII inhibition likely stimulates ROS production through mTOR activation.

3.3. CKII inhibition induces mTOR activation in senescent HCT116 cells

To address whether or not CKII inhibition activates mTOR, HCT116 cells were pretreated with rapamycin for 1 h and then transfected with CKIIα siRNA. mTOR activation was measured by monitoring phosphorylation of mTOR as well as its downstream target, p70S6K. Probing of HCT116 cells by Western blot analysis

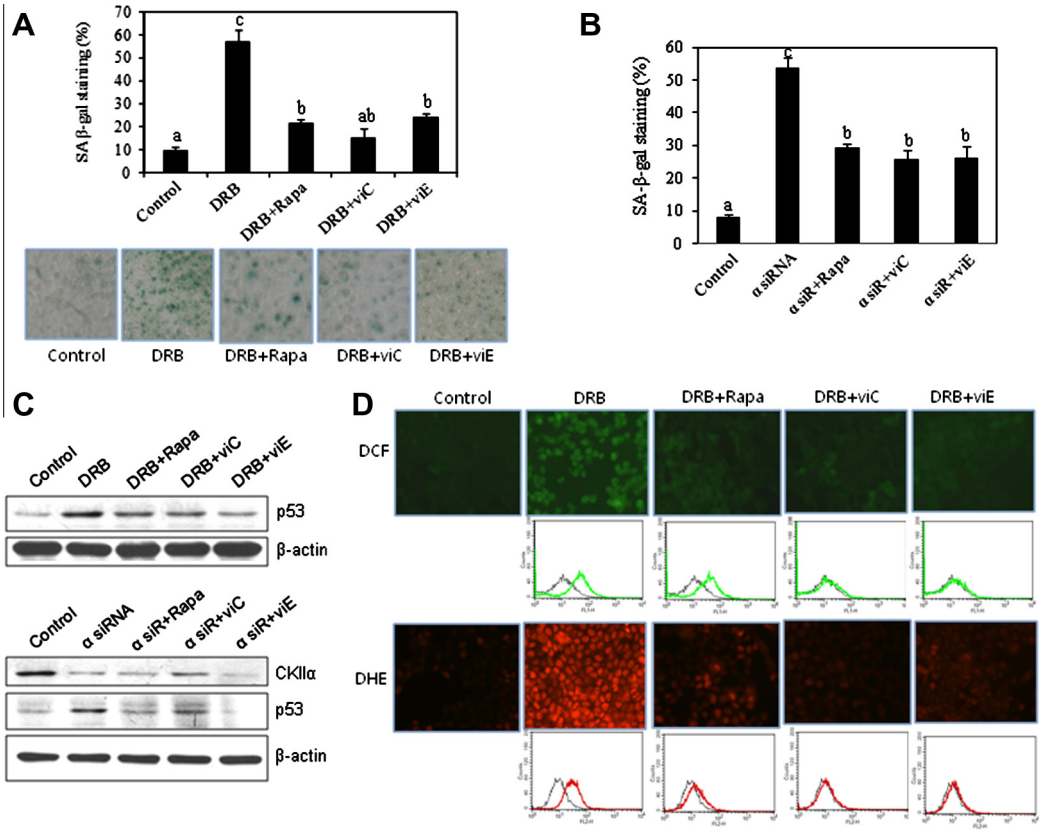

Fig. 1. Rapamycin, vitamin C, and vitamin E suppress CKII inhibition-mediated senescence and ROS production. HCT116 cells were treated with the CKII inhibitor DRB (20 μM) or transfected with CKIIα siRNA (α siR) in the presence or absence of 0.1 μM rapamycin (Rapa), 100 μM vitamin C (viC), and 100 μM vitamin E (viE) for 2 days. (A and B) Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside, and the percentage of positively stained cells was measured. Data are shown as the means ± SEM. Bars that do not share a common letter (a, b, c) are significantly different among the groups at $P < 0.05$. Representative images were obtained at 20× magnification. (C) Cells were lysed, electrophoresed on a 10% (w/v) SDS–polyacrylamide gel, and visualized by immunoblotting with anti-p53 antibody. (D) Cells were incubated with CM-H_2DCFDA or DHE as described in Section 2. Representative images illustrate the green fluorescence of DCF produced by ROS (upper panel) and the red fluorescence of ETH produced by superoxide anion (bottom panel). Fluorescence intensity was determined by flow cytometry analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

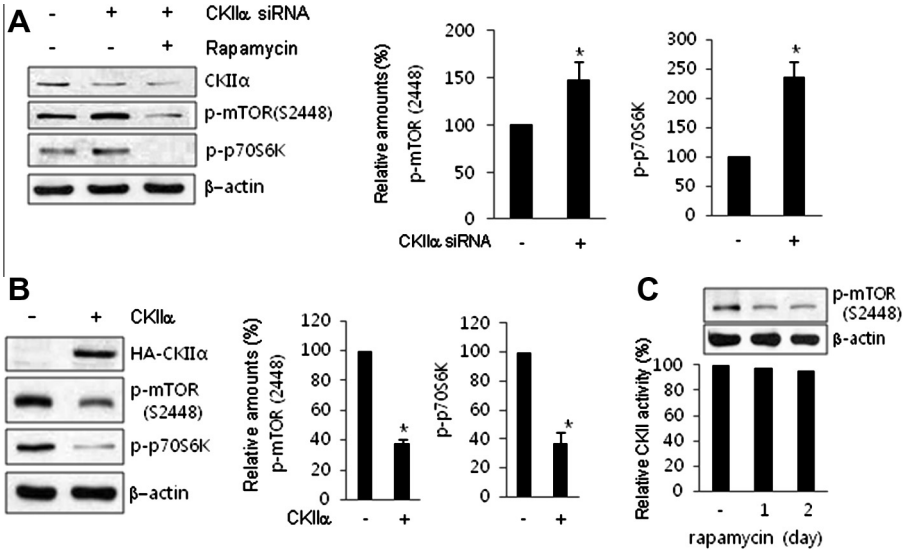

Fig. 2. Involvement of mTOR in CKII inhibition-induced senescence. (A) HCT116 cells were transfected with CKIIα siRNA in the presence or absence of 0.1 μM rapamycin. After 2 days, the level of each protein was determined by immunoblot analysis. β-Actin was used as a control. Quantitation of p-mTOR (2448) and p-p70S6K bands was performed by densitometry (right panel). Data are shown as the means ± SEM. $^{*}P < 0.05$. (B) HCT116 cells were transfected with empty vector or pcDNA-HA-CKIIα for 2 days, after which protein levels were determined by immunoblot analysis. β-actin was used as a control. Quantitation of p-mTOR (2448) and p-p70S6K bands was performed by densitometry (right panel). Data are shown as the means ± SEM. $^{*}P < 0.05$. (C) HCT116 cells were treated with 0.1 μM rapamycin. Lysates from rapamycin-treated cells were utilized in kinase assays using [γ-^{32}P]ATP and the specific CKII substrate peptide under standard assay conditions. ^{32}P incorporation into the substrate peptide was measured by scintillation counting. The level of p-mTOR (2448) was measured by immunoblot analysis (upper panel).

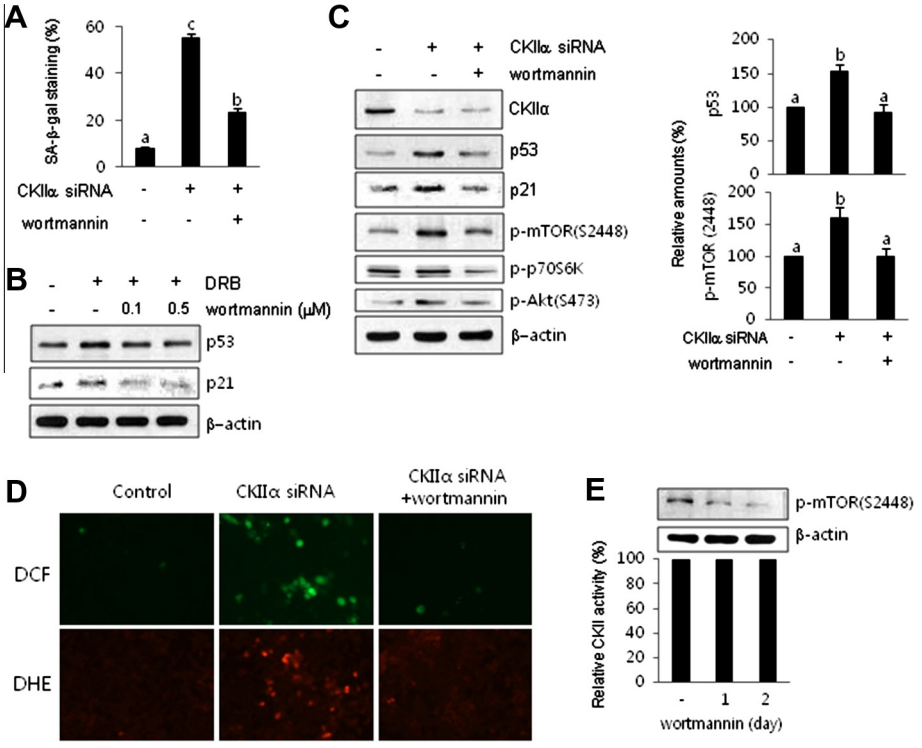

Fig. 3. Involvement of PI3K in CKII inhibition-induced senescence. (A) HCT116 cells were transfected with CKIIα siRNA in the presence or absence of the PI3K inhibitor wortmannin (0.1 μM) for 2 days. Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside, and the percentage of positively stained cells was measured. Data are shown as the means ± SEM. Bars that do not share a common letter (a, b, c) are significantly different among the groups at $P < 0.05$. (B) HCT116 cells were treated with 20 μM DRB in the presence or absence of wortmannin (0.1 μM) for 2 days. Cells were lysed, electrophoresed on a 12% (w/v) SDS–polyacrylamide gel, and visualized by immunoblotting with anti-p53 and anti-p21$^{Cip1/WAF1}$ antibodies. (C) HCT116 cells were transfected with CKIIα siRNA in the presence or absence of wortmannin (0.1 μM) for 2 days, after which protein levels were determined by immunoblot analysis. β-Actin was used as a control. Quantitation of p53 and p-mTOR (2448) protein bands was performed by densitometry (right panel). Data are shown as the means ± SEM. $^{*}P < 0.05$. (D) HCT116 cells were transfected with CKIIα siRNA in the presence or absence of 0.1 μM wortmannin. After 2 days, cells were incubated with CM-H_2DCFDA or DHE as described in Section 2. Representative images illustrate the green fluorescence of DCF produced by ROS (upper panel) and the red fluorescence of ETH produced by superoxide anion (bottom panel). (E) HCT116 cells were treated with 0.1 μM wortmannin. Lysates from wortmannin-treated cells were utilized in kinase assays using [γ-^{32}P]ATP and the specific CKII substrate peptide under standard assay conditions. ^{32}P incorporation in the substrate peptide was measured by scintillation counting. The level of p-mTOR (2448) was measured by immunoblot analysis (upper panel). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

revealed that CKII inhibition strikingly increased phosphorylation of mTOR (S2448) and p70S6K, which was completely blocked by rapamycin treatment (Fig. 2A). Quantitation by densitometry revealed that transfection with CKIIα siRNA increased phosphorylation of mTOR (S2448) and p70S6K by 50% and 140%, respectively, compared with levels in control cells. Next, we examined whether or not CKIIα overexpression inactivates mTOR. Western blot analysis revealed that overexpression of CKIIα reduced phosphorylation of both mTOR (2448) and p70S6K by 60% compared to control cells (Fig. 2B). Collectively, these data suggest that CKII downregulates mTOR activity. Consistent with this, rapamycin had no effect on the catalytic activity of CKII in cells, indicating that CKII is an upstream regulator of mTOR (Fig. 2C).

3.4. PI3K is involved in CKII inhibition-mediated cellular senescence in HCT116 cells

PI3K and AKT are well known upstream regulators of mTOR [1–4]. To establish the role of PI3K in CKII inhibition-induced senescence, HCT116 cells were pretreated with wortmannin, a PI3K inhibitor, for 1 h and then transfected with CKIIα siRNA. There was a significant increase in SA-β-gal activity in response to CKII downregulation. However, co-treatment of cells with wortmannin dramatically reduced the rate of SA-β-gal staining (Fig. 3A). We next examined whether or not treatment with wortmannin decreases p53 and $p21^{Cip1/WAF1}$ protein levels in cells with downregulated CKII. Whereas p53 and $p21^{Cip1/WAF1}$ expression levels were elevated in DRB-treated cells compared with to control cells, wortmannin apparently downregulated the expression of these proteins (Fig. 3B). Consistent with this, wortmannin almost completely blocked activation of p53 and $p21^{Cip1/WAF1}$ in cells transfected with CKIIα siRNA. Furthermore, wortmannin attenuated phosphorylation of mTOR (S2448), p70S6K, and AKT (S473), suggesting that PI3K acted as a positive regulator of mTOR in cells made senescent by CKII inhibition (Fig. 3C). CKIIα knockdown significantly increased hydrogen peroxide and superoxide anion levels relative to control cells, as indicated by green and red fluorescence. However, incubation with wortmannin apparently prevented green and red fluorescence in CKIIα-downregulated cells (Fig. 3D). Finally, wortmannin had no effect on the catalytic activity of CKII in cells, indicating that

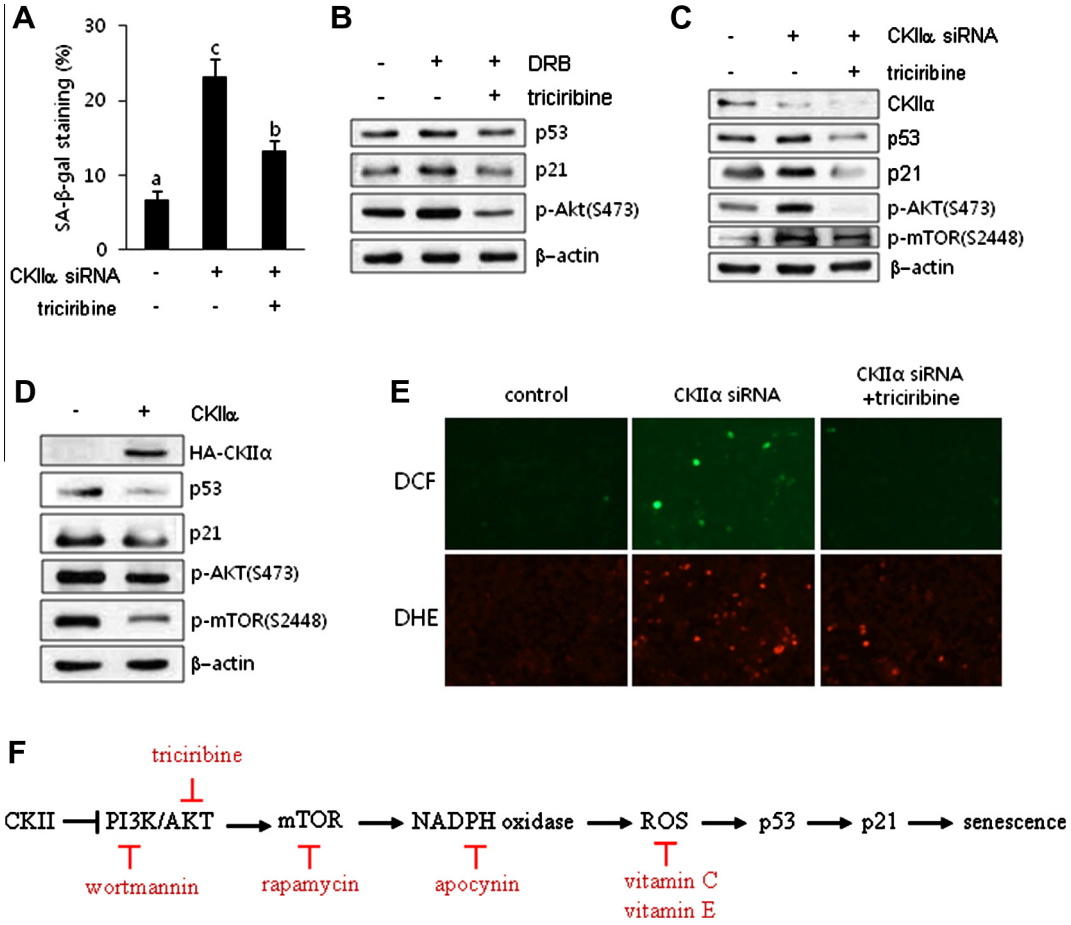

Fig. 4. Involvement of AKT in CKII inhibition-induced senescence. (A) HCT116 cells were transfected with CKIIα siRNA in the presence or absence of the AKT inhibitor triciribine (10 μM) for 2 days. Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside, and the percentage of positively stained cells was measured. Data are shown as the means ± SEM. Bars that do not share a common letter (a, b, c) are significantly different among the groups at $P < 0.05$. (B and C) HCT116 cells were treated with 20 μM DRB (B) or CKIIα siRNA (C) in the presence or absence of triciribine (10 μM) for 2 days, after which protein levels were determined by immunoblot analysis. β-Actin was used as a control. (D) HCT116 cells were transfected with empty vector or pcDNA-HA-CKIIα for 2 days, after which protein levels were determined by immunoblot analysis. β-Actin was used as a control. (E) HCT116 cells were transfected with CKIIα siRNA in the presence or absence of triciribine (10 μM). After 2 days, cells were incubated with CM-H_2DCFDA or DHE as described in Section 2. Representative images illustrate the green fluorescence of DCF produced by ROS (upper panel) and the red fluorescence of ETH produced by superoxide anion (bottom panel). (F) Possible model illustrating CKII inhibition-mediated cellular senescence. In this study, CKII inhibition induces cellular senescence by ROS generation by activating the PI3K-AKT-mTOR pathway. This process results in activation of the p53-$p21^{Cip1/WAF1}$-Rb-dependent senescence pathway [11]. The NADPH oxidase inhibitor apocynin [12], PI3K inhibitor wortmannin, AKT inhibitor triciribine, mTOR inhibitor rapamycin, and ROS scavengers vitamin C and E can suppress this senescence signaling pathway. ROS, reactive oxygen species. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CKII is an upstream regulator of PI3K (Fig. 3E). Taken together, these results indicate that PI3K is involved in CKII inhibition-mediated cellular senescence.

3.5. AKT is involved in CKII inhibition-mediated cellular senescence in HCT116 cells

To determine the relationship between AKT and CKII inhibition-induced senescence, we treated HCT116 cells with triciribine, an AKT inhibitor, for 1 h before transfection with CKIIα siRNA. We found that triciribine reduced the rate of CKII inhibition-induced senescence (Fig. 4A). Consistent with this, we observed that triciribine completely abolished CKII downregulation-induced overexpression of p53 and $p21^{Cip1/WAF1}$ (Fig. 4B and C). In addition, triciribine reduced phosphorylation of mTOR (S2448) and AKT (S473), suggesting that AKT acts as a positive regulator of mTOR in cells made senescent by CKII inhibition. Furthermore, overexpression of CKIIα clearly suppressed phosphorylation of AKT (473) as well as mTOR (2448) (Fig. 4D). Finally, incubation with triciribine reduced production of hydrogen peroxide and superoxide anion in CKIIα-downregulated cells (Fig. 4E). Taken together, these results suggest that triciribine reduces p53 stabilization through mTOR inactivation, contributing to suppression of senescence in cells with downregulated CKII.

4. Discussion

It has been shown that PI3K is an important driver of oncogenesis [3,4]. However, paradoxically, hyperactivated PI3K can actually promote cellular senescence. Inactivation of PTEN, a negative regulator of the PI3K-AKT pathway, mediates senescence in mouse prostate epithelium and embryonic fibroblasts, indicating that the PI3K-AKT pathway is involved in cellular senescence [18]. Activation of AKT, a downstream mediator of PI3K signaling, also induces premature senescence in mouse embryonic fibroblasts by increasing intracellular ROS levels through elevation of oxygen consumption and inhibition of FoxO transcription factors [19,20]. Here, we showed that CKII inhibition induced senescence through activation of the PI3K-AKT-mTOR pathway. CKII inhibition stimulated phosphorylation of AKT, mTOR, and p70S6K in HCT116 cells. Treatment with the PI3K inhibitor wortmannin, AKT inhibitor triciribine, and mTOR inhibitor rapamycin all blocked this phosphorylation event, indicating that CKII inhibition activates PI3K-AKT-mTOR signaling. Consistent with this, hyperactivation of CKII by CKIIα overexpression reduced levels of phospho-AKT, phospho-mTOR, and phospho-p70S6K. Most importantly, all of these inhibitors attenuated CKII inhibition-mediated senescence. Therefore, this study suggests that the PI3K-AKT-mTOR pathway is involved in CKII inhibition-mediated senescence (Fig. 4F).

ROS production is a major upstream trigger of CKII inhibition-mediated senescence, and CKII inhibition-induced senescence is inhibited by the antioxidant N-acetylcysteine. Further, CKII inhibition elevates cellular ROS production via NADPH oxidase activity based on the observation that the NADPH oxidase inhibitor apocynin as well as knockdown of $p22^{phox}$, NADPH oxidase subunit, suppresses CKII inhibition-mediated senescence [12]. Consistent with this, we showed that other antioxidants, including vitamin C and vitamin E, also attenuate CKII inhibition-mediated senescence in HCT116 cells. In addition, the present study demonstrated that inhibitors of PI3K, AKT, or mTOR suppress CKII inhibition-mediated ROS production as well as senescence. Therefore, the PI3K-AKT-mTOR pathway exerts its effects on senescence by elevating intracellular ROS (Fig. 4F).

In summary, the present study, for the first time, demonstrated that activation of the PI3K-AKT-mTOR pathway is required for CKII inhibition-induced ROS generation, p53 activation, and senescence in HCT116 cells. This finding suggests that CKII inhibition activates the mTOR pathway through upregulation of PI3K-AKT activity, leading to ROS generation in HCT116 cells. Since oncogenic and oxidative stress-induced senescence attenuates tumorigenesis, this study provides not only a new understanding of cellular senescence but also novel therapeutic options for the treatment of various tumors.

Acknowledgments

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