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Phosphorylated Extracellular Signal-regulated Protein Kinases 1 and 2 Phosphorylate Sp1 on Serine 59 and Regulate Cellular Senescence via Transcription of p21$^{Sdi1/Cip1/Waf1}$*[S]

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Expression of p21^{Sdi1} downstream of p53 is essential for induction of cellular senescence, although cancer cell senescence can also occur in the p53 null condition. We report herein that senescence-associated phosphorylated extracellular signal-regulated protein kinases 1 and 2 (SA-pErk1/2) enhanced p21^{Sdi1} transcription by phosphorylating Sp1 on Ser^{59} downstream of protein kinase C (PKC) *α*. Reactive oxygen species (ROS), which was increased in cellular senescence, significantly activated both PKC*α* and PKC*β*I. However, PKC*α*, but not PKC*β*I, regulated ROS generation and cell proliferation in senescent cells along with activation of cdk2, proven by siRNAs. PKC*α*-siRNA also reduced SA-pErk1/2 expression in old human diploid fibroblast cells, accompanied with changes of senescence phenotypes to young cell-like. Regulation of SA-pErk1/2 was also confirmed by using catalytically active PKC*α* and its DN-mutant construct. These findings strongly suggest a new pathway to regulate senescence phenotypes by ROS via Sp1 phosphorylation between PKC*α* and SA-pErk1/2: employing GST-Sp1 mutants and MEK inhibitor analyses, we found that SA-pErk1/2 regulated Sp1 phosphorylation on the Ser^{59} residue *in vivo*, but not threonine, in cellular senescence, which regulated transcription of p21^{Sdi1} expression. In summary, PKC*α*, which was activated in senescent cells by ROS strongly activated Erk1/2, and the SA-pErk1/2 in turn phosphorylated Sp1 on Ser^{59}. Sp1-enhanced transcription of p21^{Sdi1} resulted in regulation of cellular senescence in primary human diploid fibroblast cells.

PKC[3] comprises a family of serine/threonine kinase that modulates a variety of signal transduction pathways, leading to gene expression, cell proliferation, and differentiation. PKC isoforms are classified into three subgroups. The conventional PKC isozymes, comprising, α, βI, βII, and γ, are activated by Ca^{2+}, phosphatidylserine, diacylglycerol, or phorbol esters; the novel PKC isozymes, consisting of PKCδ, -ϵ, -η, -θ, and -μ, are activated by phosphatidylserine, diacylglycerol, or phorbol esters, but insensitive to Ca^{2+}; and the atypical isoforms (ζ and ι/λ) are dependent on phosphatidylserine for activation, but not affected by Ca^{2+}, diacylglycerol, or phorbol esters (1–3). Activated PKC translocates from cytosol to membranous organelles and/or to the nucleus (4, 5). In addition, oxidative stress has been reported to induce prolonged activation of PKC within the cells (6–9). The growth regulatory consequences of PKC activation suggest a link between PKC signaling and control of the cell cycle machinery. Activation of PKC has been shown to result in alterations of cell cycle progression in either stimulatory or inhibitory directions in several systems (10–15). Among the isoforms, PKCα has been implicated in the control of G_1/S transition (14, 16–18) and recently in the regulation of cancer cell senescence (19), whereas PKCβII has been shown to play a role in progression from G_2 into M phase (15), and PKCδ has been associated with the control of M phase (20).

Normal human diploid fibroblasts (HDFs) have a defined proliferative capacity in tissue culture. Hayflick and Moorhead (21) described in detail about the limited proliferative capacity of normal cells and postulated that it was an *in vitro* manifestation of human aging. Cellular senescence plays a key role in complex biological processes, including development, aging, and tumorigenesis. Hallmarks of cellular senescence are metabolically active but not responsive to mitogens, therefore, decreased cell growth, cell cycle arrest, and tumor suppressor effects are accompanied with flat and large cell shapes. Characteristic features of cellular senescence are cytoplasmic sequestration of phospho-extracellular signal-regulated protein kinase 1/2 (SA-pErk1/2), and increased levels of ROS (22). The phenomena are well corroborated by the results that activation of H-*ras* induces cellular senescence of mouse embryo fibroblasts (23) and primary culture of HDF cells (24), and that infection of HDF with virus carrying H-*ras* double mutants, G12V/T35S, G12V/E37G, and G12V/Y40C, accumulates SA-pErk1/2 with increased MEK activity. On the other hand, treatment of old HDF cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) reverses cellular senescence phenotypes (25): increased DNA synthesis, phosphorylation of pRB, reduced expressions

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[3] The abbreviations used are: PKC, protein kinase C; SA-pErk1/2, senescence-associated phosphorylated extracellular signal-regulated protein kinases 1 and 2; ROS, reactive oxygen species; HDF, human diploid fibroblast; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PMSF, phenylmethylsulfonyl fluoride; H_2-DCFDA, 2,7-dichlorodihydrofluorescein diacetate; MBP, myelin basic protein; NAC, *N*-acetyl-L-cysteine; DiC_8, 1,2-dioctanoylglycerol; FACS, fluorescence-activated cell sorter; siRNA, small interfering RNA; IP, immunoprecipitation; HA, hemagglutinin; GST, glutathione *S*-transferase; WT, wild type; EGF, epidermal growth factor; GFP, green fluorescent protein; DN, dominant negative; CA, catalytically active.

of $p21^{Sdi1}$ and SA-pErk1/2, as well as reduced expression of β-galactosidase along with morphological changes of the senescent cells. It has been known that TPA initially activates PKC, and then down-regulates its isoforms (26, 27). Therefore, PKC isozymes have strongly been suggested to be an important *in vivo* regulator of senescence phenotypes. However, there has been no report on the crosstalk between PKC isozymes and SA-pErk1/2 and the role of SA-pErk1/2 in the process of cellular senescence.

In addition to the above mentioned characteristics of cellular senescence, $p21^{Sdi1}$ has been well known as a key molecule to induce normal cell senescence (28, 29). However, there is no report on the induction mechanism of $p21^{Sdi1}$ expression during cellular senescence, except transcriptional activation of the $p21^{Waf1/Cip1}$ promoter by Sp1 after phosphorylation on the two threonine residues (Thr^{453} and Thr^{739}) by mitogen-activated protein kinase (30), but not serine residues (31). In this study, we investigated the role of SA-pErk1/2 in cellular senescence downstream of the PKCα; PKCα was responsible for ROS generation and activation of Erk1/2 in senescent cells. SA-pErk1/2 enhanced the expression of $p21^{Sdi1}$ via phosphorylation of Sp1 on Ser^{59} *in vivo* downstream of PKCα, resulting in induction of senescence phenotypes.

EXPERIMENTAL PROCEDURES

Materials—Anti-actin antibody, H_2O_2, TPA, *N*-acetylcysteine (NAC), 1,2-dioctanoylglycerol (DiC_8), and GF109203X (PKC inhibitor) were purchased from Sigma. Anti-pErk1/2 antibody was from Cell Signaling; antibodies against PKCα, PKCβI, PKCβII, PKCγ, PKCδ, PKCε, PKCη, PKCζ, Sp1, and $p21^{Sdi1}$ were from Santa Cruz; anti-p53 and α-tubulin antibodies were from Oncogene. 2′,7′-Dichlorodihydrofluorescein diacetate (H_2-DCFDA) was from Molecular Probes. All other reagents used were of molecular biology grade.

Cell Culture—Primary culture of HDF was prepared and maintained in our laboratory (22, 24) in Dulbecco's modified Eagle's medium (DMEM-high glucose, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). The number of population doublings of HDF was calculated based on the equation, PDs = log[*A*/*BC*]/log2; *A*, *B*, and C indicate the numbers of collected cells, plated cells, and the attachment efficiency, respectively. Doubling time of HDF cells was measured with PDs, and young cells used in this study represent cells with doubling time of around 24 h. Doubling time of mid-old and old cells were 7–10 and 14 days, respectively. All cells used in this study were maintained in a 5% CO_2 incubator at 37 °C.

Cell Treatment—Cells (3×10^5 cells/60 mm dish) were plated 24 h before they were treated with various chemicals, such as H_2O_2 (1 mM), TPA (50 ng/ml), GF109203X (4 μM), and NAC (10 mM) for the indicated times. Generation of ROS was measured by FACS using H_2-DCFDA, according to the method described elsewhere (22).

siRNA Transfection—Young and mid-old cells were transfected with siRNA against PKCα, PKCβI, and Sp1 (Santa Cruz) using Oligofectamine (Invitrogen) following the protocol provided by the manufacturer.

Subcellular Fractionation—Cytoplasmic and nuclear extracts were prepared as described previously (32) with minor modifications. Briefly, the cells were washed twice with ice-cold phosphate-buffered saline, and transferred to 1.5-ml Eppendorf tubes. The cells were then centrifuged at 300 × *g* for 4 min at 4 °C. After centrifugation, the pellet was resuspended in 400 μl of cold buffer A (10 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg/ml leupeptin). After incubation on ice for 15 min, 12.5 μl of 10% Nonidet P-40 was added, and the mixture vortexed briefly and incubated on ice for 10 min. The nuclei were pelleted by centrifugation at 1,500 × *g* for 5 min at 4 °C, whereas the supernatant (cytoplasmic extracts) was recovered by centrifugation at 13,000 × *g* for 15 min. Nuclei were washed twice with 1 ml of ice-cold buffer A, and then resuspended in 50 μl of ice-cold buffer B (20 mM HEPES-KOH (pH 7.5), 0.4 M NaCl, 1 mM dithiothreitol, 1 mM PMSF, and 1 μg/ml leupeptin), followed by incubation on ice for 30 min. The mixture was then centrifuged at 18,000 × *g* for 5 min, and the supernatant was collected as a nuclear extract. To determine subcellular distribution of PKC isozymes, soluble and particulate fractionation was performed as described previously with some modifications (33). For soluble and particulate fractions, cells were washed twice with ice-cold phosphate-buffered saline and scraped into a homogenization buffer containing 25 mM Tris/HCl (pH 7.4), 2 mM EDTA, 0.25 M sucrose, 1 μg/ml leupeptin, and 1 mM PMSF and then lysed twice by sonication (Sonic Dismembrator 550, Fisher) at level 2 for 10 s. The lysates were centrifuged at 500 × *g* for 5 min, and the low-speed supernatant was centrifuged at 100,000 × *g* for 1 h. The high-speed supernatant constituted the soluble fraction. The high-speed pellet was washed three times and extracted in ice-cold homogenization buffer containing 1% Triton X-100 for 30 min. The Triton-soluble component (particulate fraction) was separated from the Triton-insoluble material by centrifugation at 100,000 × *g* for 1 h.

Immunoprecipitation—Immunoprecipitation (IP) was performed with young and mid-old HDF cell lysates (500 μg or 1 mg) in the IP buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.5% deoxycholic acid, 1% Nonidet P-40, 1 mM PMSF, and 1 μg/ml leupeptin by the standard method. Whole cell lysates were pre-cleared with protein G-agarose beads (Invitrogen) for 1 h at 4 °C before precipitation overnight with anti-PKCα, anti-PKCβI, anti-cdk2, or anti-Sp1 antibody at 4 °C. The immunoprecipitates were washed 3 times with IP buffer, and then subjected to kinase assay or immunoblot analysis with anti-PKCα, anti-PKCβI, anti-cdk2, or anti-Sp1 antibody.

Immunoblot Analysis—Young and old HDF cells were solubilized with RIPA buffer (50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, 50 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 1 μg/ml leupeptin), and 40 μg of cell lysates were resolved on 8 to 12% SDS-PAGE in 25 mM Tris glycine buffer. The gel-resolved proteins were then transferred to nitrocellulose membrane. The membranes were blocked with 5% nonfat skim milk in phosphate-buffered saline containing 0.05% Tween 20 (PBST) for 1 h and then incubated with anti-PKCα, anti-PKCβI, anti-pErk1/2, anti-actin, anti-α-tubulin, anti-$p21^{Sdi1}$, anti-p53, anti-Sp1, anti-phosphothreonine, or anti-phosphoserine antibodies

at room temperature for 1 h or overnight at 4 °C. Nitrocellulose membranes were washed three times with PBST and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. ECL (Amersham Biosciences) kit was employed to visualize protein expression levels. Protein bands were quantitated by relative optical densities using Gel-Pro 4.0 computer software (Media Cybernetics).

Immunoprecipitation and in Vitro Kinase Assays—For the IP kinase assay, the immunoprecipitates of PKCα, PKCβI, and cdk2 were washed twice with IP buffer, and then twice with kinase buffer (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mM EGTA, 1 mM sodium fluoride, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate) before resuspended in 20 μl of kinase buffer. Kinase assay was initiated by adding 40 μl of kinase buffer containing 5 μg of MBP or histone H1 substrate and 5 μCi of [γ-^{32}P]ATP. For *in vitro* kinase assay, recombinant GST-hSp1 proteins (wild type and mutant) were used as substrates. The kinase assay was initiated by adding PKCα (Calbiochem) or Erk1 (Upstate) to 30 μl of kinase buffer containing 3 μg of GST-hSp1 and 5 μCi of [γ-^{32}P]ATP. The reactions were carried out at 30 °C for 30 min, and terminated by adding SDS sample buffer followed by boiling for 5 min. The reaction products were analyzed by SDS-PAGE and autoradiography.

Measurement of ROS by FACS Analysis—The level of H_2O_2 in the senescent cells was measured using FACScan (BD Biosciences) by measuring the fluorescence of DCF arising from oxidation of H_2-DCFDA.

Electroporation—An MP-100 Microporator (Digital Bio Technology) was used to electroporate HDF cells. Cells (2×10^5) were resuspended in 10 μl of microporation buffer (Digital Bio Technology) containing 1 μg of PKCα-DN or PKCα-CA DNA. The cells were pulsed by using a 1×30-ms protocol with 1400 V and then plated on 6-well plates. At 48 h after electroporation, cells were harvested for Western blot analysis. The pcDNA3-HA was used as a control.

Preparation of GST-Sp1 Mutant Proteins—Based on the Kinasephos program, potential phosphorylation sites of Sp1 by mitogen-activated protein kinase (Ser^{59}, Ser^{309}, Ser^{510}, Ser^{525}, Ser^{588}) were targeted. Therefore, the following GST-human Sp1 fusion proteins were prepared by PCR using the following primers: sense 5′-CGGAATTCCATGAGCGACCAAGATCACTCCATGGAT-3′ and antisense 5-CGAGTCGACTCGAGTCAGAAGCCATTGCCACTGATATTAAT-3′ for full-length human Sp1 (hSp1); sense primer of hSp1 and antisense 5′-GACTCGAGGATGATCTGCCAGCCATTGGC-3′ for Δ1-(1–110); sense 5′-CGGAATTCCTCAGGGACTACCATCAGTTCT-3′ and antisense 5′-GACTCGAGGTTGGTCCCTGATGATCCACT-3′ for Δ2-(301–350); and sense 5′-CGGAATTCCCCCATTGCCTCAGCTGCTTCC-3′ and antisense primer of hSp1 for Δ3-(504–785), respectively. To generate S59A and T739A point mutants of hSp1, "megaprimer" methods were prepared (34). For the S59A mutant megaprimer, sense primer of hSp1 and the antisense 5′-AGCCAAAGGGGCTGGCTGGGACT-3′ were used. For the T739A mutant megaprimer, sense primer 5′-GTGGCACTGCCGCTCCTTCAGCC-3′ and the antisense primer of hSp1 were used. PCR products were cloned into the EcoRI and XhoI sites of pGEX4T3 (GE Healthcare). GST or GST-hSp1 was purified from *Escherichia coli* strain BL21 transformed with pGEX4T3 or pGEX4T3-hSp1 using glutathione-Sepharose affinity column.

Plasmid Transfection and Promoter Analysis—NIH3T3 cells in 6-well plates (5×10^4 cells/well) were transiently transfected with 1 μg of a p21 promoter-driven luciferase reporter plasmid, pGL2-0.3 (35), together with 1 μg of pcDNA3-HA(vector), PKCα-DN, or PKCα-CA using Lipofectamine (Invitrogen). A *Renilla* luciferase expression vector (100 ng, pRL-TK, Promega) was co-transfected for normalization of transfection efficiency. Forty-eight hours later, cell extracts were subjected to luciferase determination using the Dual Luciferase Reporter Assay System (Promega). Results were expressed as the ratio between firefly and *Renilla* luciferase. Wild type (WT) and S59A point mutant (S59A) of full-length Sp1 were cloned into the EcoRI and SalI sites of p3xFLAG-CMV (Sigma). Huh7 cells in 6-well plates (5×10^4 cells/well) were transiently transfected with 500 ng of p21 promoter-driven luciferase reporter plasmid (pGL2-0.3) and WT-Sp1 or S59A-Sp1 (500 ng) using Lipofectamine. A *Renilla* luciferase expression vector (50 ng, pRL-TK) was co-transfected for normalization of transfection efficiency. Twenty-four hours later, serum was deprived for 1 day before stimulation with EGF for 6 h. And then, the cell extracts were subjected to promoter analysis by measuring the luminescence with TD-20/20 luminometer (Turner Designs).

Statistical Analysis—Data were presented as mean ± S.D. and analyzed by Student's *t* test. *p* value less than 0.05 was considered as statistically significant.

RESULTS

PKC Isozymes are Activated in the Replicatively Senescent Cells—Our previous study showed that treatment of senescent cells with TPA reverses senescence phenotypes to young cell-like (25). In the present study, we attempted to determine the PKC isozymes possibly involved in the process. Therefore, primary cultures of young and old HDF cells were treated with either TPA (50 ng/ml) or DMSO (0.01%) for 15 min, and the cell lysates were then separated into soluble (S) and particulate (P) fractions by centrifugation. Thirty μg each of the fractions were resolved on 8% SDS-PAGE and then hybridized with anti-PKC antibodies. As shown in Fig. 1*A*, PKCα, PKCβI, and PKCη in both young and old cells were activated by TPA treatment. Fig. 1*B* shows that there was no change in the expressions of PKCα and PKCβI proteins, whereas the kinase activities were significantly higher in the older cells than the younger cells. To investigate whether activation of PKC regulated cellular senescence or not, GF109203X was applied to senescent cells for 3 days, and the result showed that inhibition of PKC isozymes clearly changed the large and flat cells to small and slim fibroblasts (Fig. 1*C*). Furthermore, immunoblot analyses revealed that the inhibition of PKC activities with GF109203X significantly reduced expressions of molecular markers of senescence, such as $p21^{Sdi1}$, p53, and SA-pErk1/2 (Fig. 1*D*). These features are well accordant with a recent report that PKCα works as a mediator of G_2/M arrest and senescence of non-small cell lung cancer cells, and that it induces $p21^{Sdi1}$, an obligatory event for conferring the senescence phenotype (19).

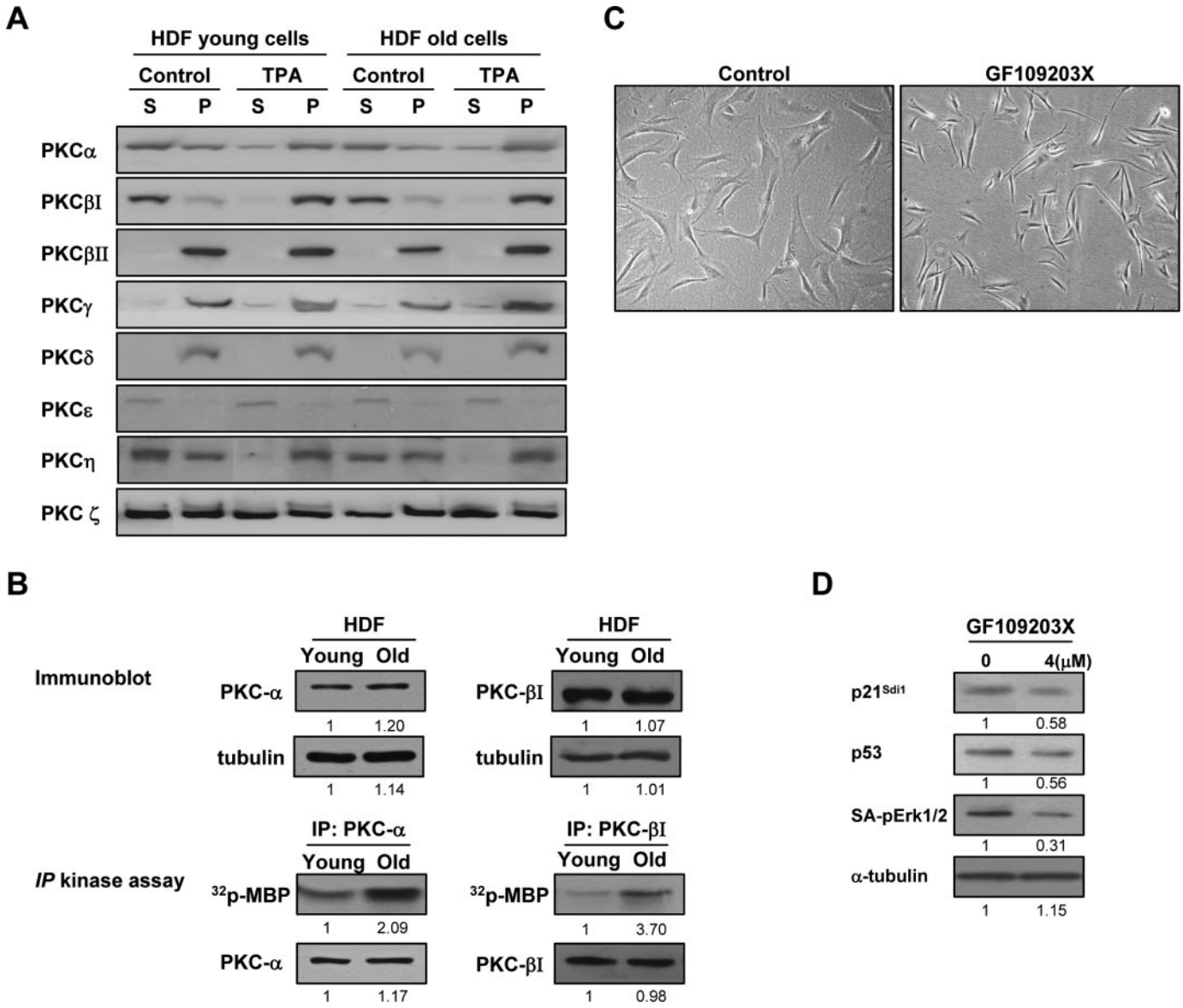


FIGURE 1. **Activation of PKCα and PKCβI during replicative senescence.** *A*, primary culture of HDF, young and old, cells were treated with either TPA (50 ng/ml) or DMSO (0.01%) for 15 min, and the cell lysates were fractionated into soluble (*S*) and particulate (*P*) fractions by centrifugation. Thirty μg of the fractions were resolved on 8% SDS-PAGE and then hybridized with anti-PKC antibodies. Note activations of PKCα, PKCβI, and PKCη after treatment with TPA in both the young and old cells. *B*, to evaluate expression and activity of the PKC isozymes in the cells, immunoblot analyses and *in vitro* kinase assays were performed with cell lysates obtained from the young and old HDF cells using anti-PKC antibodies and myelin basic protein (*MBP*) as a substrate, respectively. There was no significant change in the expressions of PKCα and PKCβI, however, the activities of PKCα and PKCβI were significantly higher in the old cells than that of the young cells. For activity assay, cell lysates were immunoprecipitated with either anti-PKCα or anti-PKCβI antibodies. The precipitates were washed twice with precipitation and kinase buffers, and then resuspended in 20 μl of kinase buffer. Kinase assay was performed by adding 40 μl of the buffer containing 5 μg of MBP and 5 μCi of [γ-^{32}P]ATP at 30 °C for 30 min, and then terminated by adding SDS sample buffer and boiling for 5 min. The reaction products were analyzed by SDS-PAGE and autoradiography. *C*, to investigate whether activation of PKC regulates cellular senescence or not, PKC inhibitor, GF109203X (4 μM), was applied to mid-old cells for 3 days, and the treatment was found to clearly change senescent cells to young cell-like; cells were changed from large with flat cytoplasm to small and slim. *D*, molecular changes of cellular senescence were also evaluated by immunoblot analyses after inhibiting PKC activities. Note reduced expressions of senescence markers, such as p21^{Sdi1}, p53, and SA-pErk1/2, after treatment of senescent cells with PKC inhibitor, GF109203X.

PKCα and ROS are Concurrently Regulated during Cellular Senescence—Because of our earlier study that the level of H_2O_2 is much higher in the senescent HDF than the young cells (22) and the reports that ROS stimulates PKC activity (36–38), we assessed whether the H_2O_2 accumulated in the senescent cells was responsible for activation of PKCα and PKCβI. Therefore, *in vitro* kinase assay was performed with immunoprecipitates of PKCα and PKCβI obtained from young and old HDF cells after treatment with H_2O_2, TPA, or NAC. As shown in Fig. 2*A*, not only TPA (50 ng/ml) but H_2O_2 (1 mM) also increased the activities of PKCα and PKCβI in young cells, whereas treatment of old cells with NAC significantly reduced the PKC activities. These data are well supported by the study that ROS regulates PKC activity in human granulocytes according to age (39).

To investigate which PKC isozyme can regulate ROS levels in cellular senescence, senescent cells were treated with either PKCα-siRNA or PKCβI-siRNA, and the changes of ROS levels

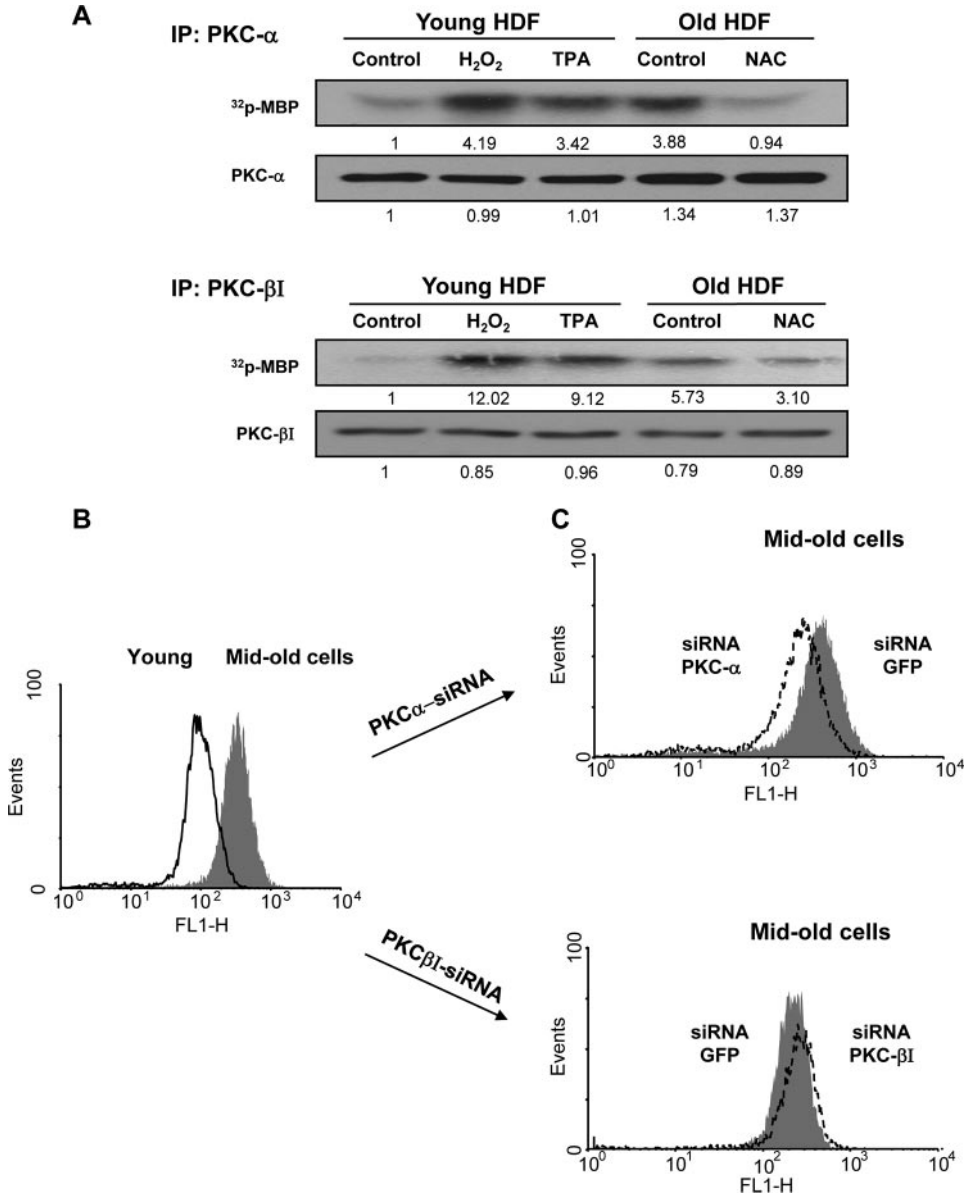


FIGURE 2. **Regulation of ROS level by PKCα, but not PKCβI, in senescent cells.** *A*, treatment of old HDF cells with NAC significantly reduced PKC activities. To evaluate whether the increased PKC activity was due to ROS accumulated in senescent cells, young cells were treated with H_2O_2 (1 mM) and TPA (50 ng/ml), whereas old cells were incubated with NAC (10 mM) for 12 h. The cell lysates of the young cells were shown to have markedly increased PKCα and PKCβI activities, whereas NAC, a specific inhibitor of H_2O_2, significantly reduced PKCα activity in the old cells. *B*, difference of ROS levels between the young and mid-old HDF cells. Cells were incubated with H_2DCF-DA for 10 min, and then changes of DCF-DA fluorescence were measured by FACS analysis. Note increased ROS levels in the senescent cells compared with the young cells. *C*, when the old cells were treated with PKCα-siRNA (20 nM) for 2 days, the level of ROS was significantly reduced compared with that of the GFP-siRNA (20 nM) treated cells (*upper panel*), however, treatment with PKCβI-siRNA (20 nM) for 2 days failed to change the ROS level in the old cells (*lower panel*).

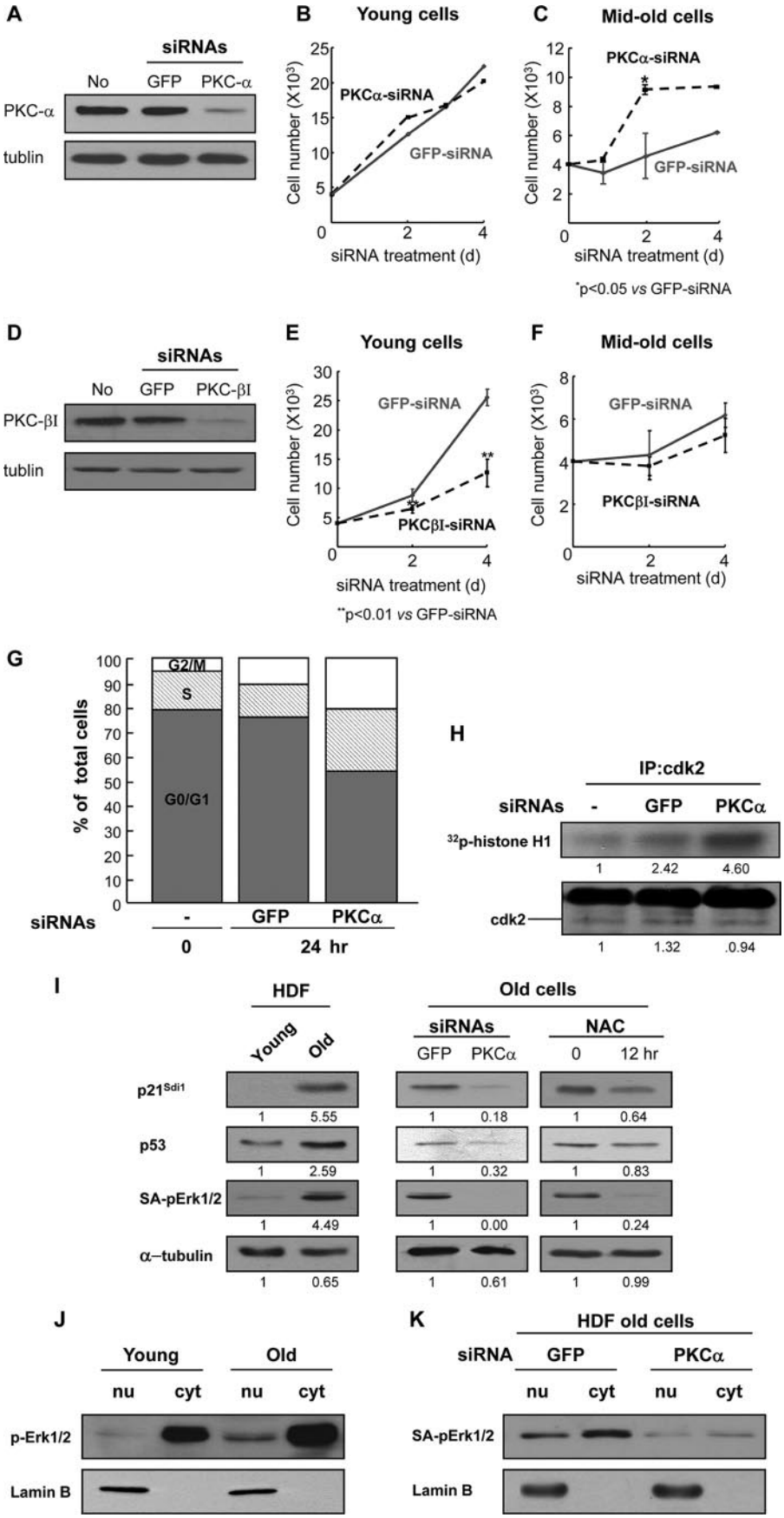
were measured by FACS using H_2DCF-DA. As already known, ROS levels of mid-old cells was higher than that of young cells (Fig. 2*B*), however, PKCα-siRNA, but not PKCβI-siRNA, significantly reduced the level in the mid-old cells (Fig. 2*C*). These findings show that H_2O_2 increased PKCα activity in the senescence process and PKCα regulated the H_2O_2 level in senescent cells. The observation can be supported by the reports that PKCα, -βII, -δ, and -ζ phosphorylate $p47^{phox}$ sites and activate NADPH oxidase (40, 41).

Down-regulation of PKCα Inhibits Its Growth Arrest and Senescence Phenotypes—Because PKCα-siRNA reduced ROS accumulation in senescent cells, regulation of senescence phenotypes by PKCα was investigated. When PKCα-siRNA and PKCβI-siRNA were introduced into HDF cells, immunoblot analyses revealed reduced expressions of PKCα (Fig. 3*A*) and PKCβI (Fig. 3*D*), respectively. Moreover, transfection of PKCα-siRNA released growth arrest of the senescent cells (Fig. 3*C*), however, there was no change in young cells (Fig. 3*B*). Here, GFP-siRNAs were employed for a control experiment. On the other hand, down-regulation of PKC-βI failed to induce proliferation of senescent cells (Fig. 3*F*), but rather decreased the proliferation in young cells (Fig. 3*E*). Therefore, to confirm the effect of PKCα on cell proliferation, we assessed cell cycle profiles using siRNAs against GFP or PKCα. In 24 h of treatment, progression of G_1 to S phase in addition to G_2/M was more than 2-fold higher in the PKCα-siRNA-treated cells than GFP control and no treatment control (Fig. 3*G*). PKCα-mediated progression of cell division cycle was further proved by an increase of cdk2 activity in the PKCα-siRNA-treated cells, but not the control, evaluated by the IP kinase assay with histone H1 as substrate (Fig. 3*H*). Indeed, down-regulation of PKCα induced G_1 to S transition and multiplication of the replicatively senescent HDF cells. To investigate whether PKCα also regulated molecular markers of senescence, old HDF cells were treated with PKCα-siRNA for 2 days, and a few markers were measured by immunoblot analyses while employing treatment with NAC as positive control. As expected, PKCα-siRNA significantly reduced the expressions of $p21^{Sdi1}$, p53, and SA-pErk1/2, as compared with those of the GFP-siRNA (Fig. 3*I*). It should be noted particularly that down-regulation of PKCα significantly reduced the expression of SA-pErk1/2. To evaluate distribution of SA-pErk1/2, immunoblot analyses were performed with nuclear and cytoplasmic fractions of the young and old HDF cells before (Fig. 3*J*) and after (Fig. 3*K*) transfection with siRNAs. As shown in the figures, the expression of SA-pErk1/2

was significantly reduced in both fractions of the old cells treated with PKCα-siRNA, as compared with GFP-siRNA. Here, lamin B was used as a loading control and a marker of nuclear proteins.

PKCα Regulates Expressions of SA-pErk1/2 and p21^{Sdi1}—To investigate whether the expression of SA-pErk1/2 was regulated by PKCα or not, pcDNA3-HA (vector), dominant negative PKCα (PKCα-DN), or catalytically active PKCα (PKCα-CA) was transfected to HDF young cells by electroporation, and whole cell lysates (30 μg) were subjected to immunoblot analyses in 2 days. As shown in Fig. 4*A*, PKCα-CA significantly induced expressions of SA-pErk1/2 and p21^{Sdi1}, whereas PKCα-DN down-regulated expression of SA-pErk1/2, as compared with that of pcDNA3-HA. Moreover, DiC_8 (20 μg/ml), a PKC activator, also increased expression of SA-pErk1/2 and p21^{Sdi1} in young HDF cells (Fig. 4*B*) with a concomitant decrease of cell growth (Fig. 4*C*). These findings strongly suggested SA-pErk1/2 as a downstream effector of PKCα in the process of cellular senescence. To confirm the regulation of the phenotypes by active PKCα, p21^{Sdi1} promoter-driven luciferase assay was performed in NIH3T3 cells (Fig. 4*D*); cells were co-transfected with p21^{Sdi1} promoter-luciferase (pGL2–0.3) together with pcDNA3-HA, PKCα-DN, or PKCα-CA. In 48 h, the cell lysates were subjected to luciferase (Luc) assay. As expected, luciferase activity was markedly increased after transfection with PKCα-CA, indicating that transcription of p21^{Sdi1} was increased only by PKCα-CA, but not PKCα-DN or the vector alone. Taken together, PKCα regulated transcription of p21^{Sdi1} in addition to the induction of SA-pErk1/2 expression.

Not Only PKCα but Also Active Erk1 Phosphorylate GST-Sp1—Because Sp1 has been shown to be important for p21^{Sdi1} transcription



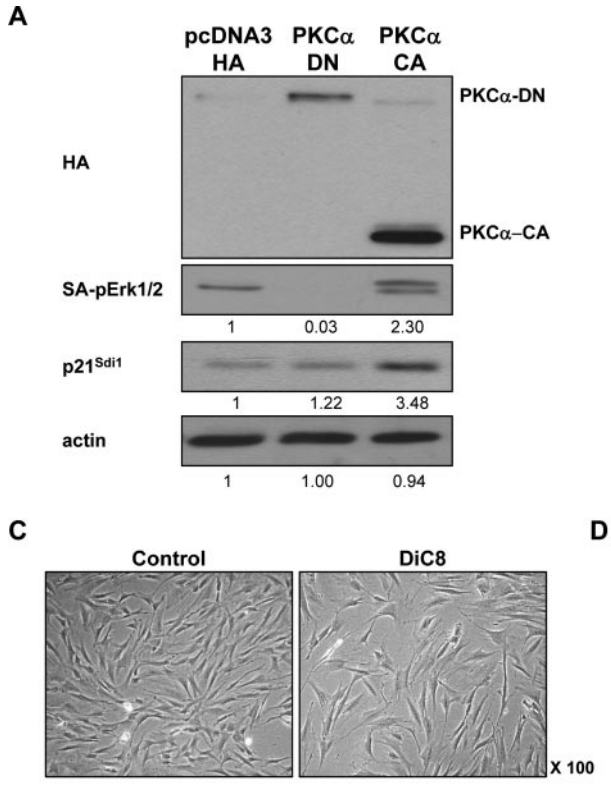


FIGURE 4. **Increased expression of SA-pErk1/2 and transcription of p21^{Sdi1} by active PKCα.** *A*, pcDNA3-HA (*vector*), dominant negative PKC-α (PKCα-DN), or catalytically active PKC-α (*PKCα-CA*) were introduced into HDF young cells by electroporation, and the whole cell lysates (30 μg) were subjected to immunoblot analyses in 2 days. Note significant inductions of SA-pErk1/2 and p21^{Sdi1} by PKCα-CA, as opposed to down-regulation of pErk1/2 expression by PKCα-DN. *B*, treatment of HDF young cells with DiC_8 (20 μg/ml), a PKC activator, for 7 days also increased p21^{Sdi1} and SA-pErk1/2 expressions, and concomitantly decreased cell growth (*C*). To prove regulations of the above mentioned phenotypes by PKCα activity, p21^{Sdi1} promoter-driven luciferase assay was performed (*D*); NIH3T3 cells were co-transfected with the reporter plasmid, p21^{Sdi1} promoter-luciferase (pGL2-0.3), along with pcDNA3-HA (vector), PKCα-DN, or PKCα-CA for 48 h. The cells were homogenized and then subjected to luciferase (*Luc*) assay. Note the significant activity of the p21^{Sdi1} transcription by PKCα-CA, but not PKCα-DN. All of the data indicate mean ± S.D. from three independent experiments.

by PKCε and ROS in human T-cells and monocytic leukemia cells in response to TPA treatment (42–45), we investigated the source of the upstream kinase of Sp1 regulating p21^{Sdi1} expression in the replicative senescence of HDF cells. Therefore, GST-Sp1 fusion proteins were prepared (Fig. 5*A*). In addition to wild type Sp1 (GST-Sp1), three deletion mutant proteins, GST-Δ1 (37 kDa), GST-Δ2 (31 kDa), and GST-Δ3 (57 kDa), were prepared in bacteria and visualized by Coomassie Blue stain after SDS-PAGE (Fig. 5*B*). Employing the prepared proteins as substrates and active PKCα and pErk1 as enzymes, *in vitro* kinase assays were performed. As shown in Fig. 5*C*, GST-Sp1, GST-Δ1, and GST-Δ3, but not GST-Δ2, could be strongly phosphorylated by PKCα. Interestingly, a similar result was also observed with active Erk1, although GST-Δ1 was a weaker substrate for PKCα than GST-Δ3 (Fig. 5*C*), whereas GST-Δ1 was a slightly better substrate for Erk1 than GST-Δ3 (Fig. 5*D*). The data strongly supported the contention that not only PKCα but also SA-pErk1/2 could regulate p21^{Sdi1} expression via Sp1 phosphorylation. We examined residues phosphorylated in Sp1 by active Erk1 and found that GST-Δ1 and GST-Δ3 were phosphorylated only on serine (Fig. 5*E*) and threonine (Fig. 5*F*) residues, respectively.

Ser^{59} and Thr^{739} in Sp1 are Phosphorylated by Active Erk1—To unequivocally establish the sites in Sp1 phosphorylated by active Erk1, site-directed mutagenesis of the GST-Δ1 and GST-Δ3 proteins was performed by point mutation of Ser^{59} to alanine (Fig. 6*A*) and Thr^{739} to alanine (Fig. 6*C*). As shown in Fig. 6, *B* and *D*, active Erk1 failed to phosphorylate GST-Δ1 S59A and GST-Δ3 T739A mutant proteins, as opposed to phosphorylation of the wild types, thus revealing Ser^{59} in GST-Δ1 and Thr^{739} in GST-Δ3 as *in vitro* targets of active Erk1.

PKCα and SA-pErk1/2 Phosphorylate Sp1 on Serine, but Not Threonine, in Senescent Cells—To investigate whether PKCα and SA-pErk1/2 regulate Sp1 phosphorylation *in vivo* or not, Sp1 was isolated from cell lysates by immunoprecipitation after

FIGURE 3. **Regulations of G$_1$ arrest and other senescent phenotypes by PKCα, but not PKCβI.** Young and old HDF cells (4×10^3) were cultured in 12-well plates for 16 h, and then treated with GFP-siRNA, PKCα-siRNA, or PKCβI-siRNA for the indicated times. Down-regulation of PKCα (*A*) significantly increased proliferation of senescent cells from 2 days after treatment with PKCα-siRNA (*C*), as compared with that of the GFP-siRNA treated. However, there was no effect of PKCα-siRNA in the young cells (*B*). Data indicate mean ± S.D., *, $p < 0.05$ (paired *t* test). On the other hand, PKCβI-siRNA failed to regain cell proliferation of the mid-old cells (*F*). Instead, down-regulation of PKC-βI (*D*) significantly reduced growth of the young cells from 2 days after treatment (*E*), **, $p < 0.01$ (paired *t* test). When mid-old cells were treated with PKCα-siRNA, cells in G$_1$ phase were significantly reduced in 24 h, as compared with that of the GFP-siRNA-treated cells (*G*), indicating release of senescent cells from G$_1$ arrest by PKCα-siRNA. *H*, down-regulation of PKCα significantly increased cdk2 activity in senescent cells. Mid-old cells were treated with siRNAs for 24 h, and the cell lysates (200 μg) were immunoprecipitated with anti-cdk2 antibody to perform an *in vitro* kinase assay using histone H1 as a substrate. Treatment of senescent cells with PKCα-siRNA significantly induced ckd2 activity, but not GFP-siRNA. *I*, to confirm the reversal of the senescence program by regulating PKCα activity, markers of cellular senescence in the old cells were evaluated. Note the significant expressions of p21^{Sdi1}, p53, and SA-pErk1/2 in the old cells compared with those of the young cells (*left panel*). GFP-siRNA and PKCα-siRNA were transfected into old HDF cells and the changes of senescent markers were measured by immunoblot analyses. Note significantly reduced expressions of p21^{Sdi1}, p53, and SA-pErk1/2 by treating the cells with PKCα-siRNA, as compared with those of the GFP-siRNA-treated cells (*middle panel*). NAC also reduced the cellular senescence markers (*right panel*). Expression of tubulin indicates loading control between the lanes. *J*, young and old HDF cells were fractionated into nuclear (*Nu*) and cytosolic (*Cyt*) fractions as described under "Experimental Procedures." Thirty μg of proteins were resolved on 10% SDS-PAGE and then hybridized with anti-pErk1/2 and anti-lamin B, a marker for nuclear fraction, antibodies. It shows increased expression of pErk1/2 in the old compared with the young cells. *K*, note significantly reduced SA-pErk1/2 in both the cytoplasm and nuclear fractions of the old cells, when treated with PKCα-siRNA, compared with those of the GFP-siRNA-treated cells.

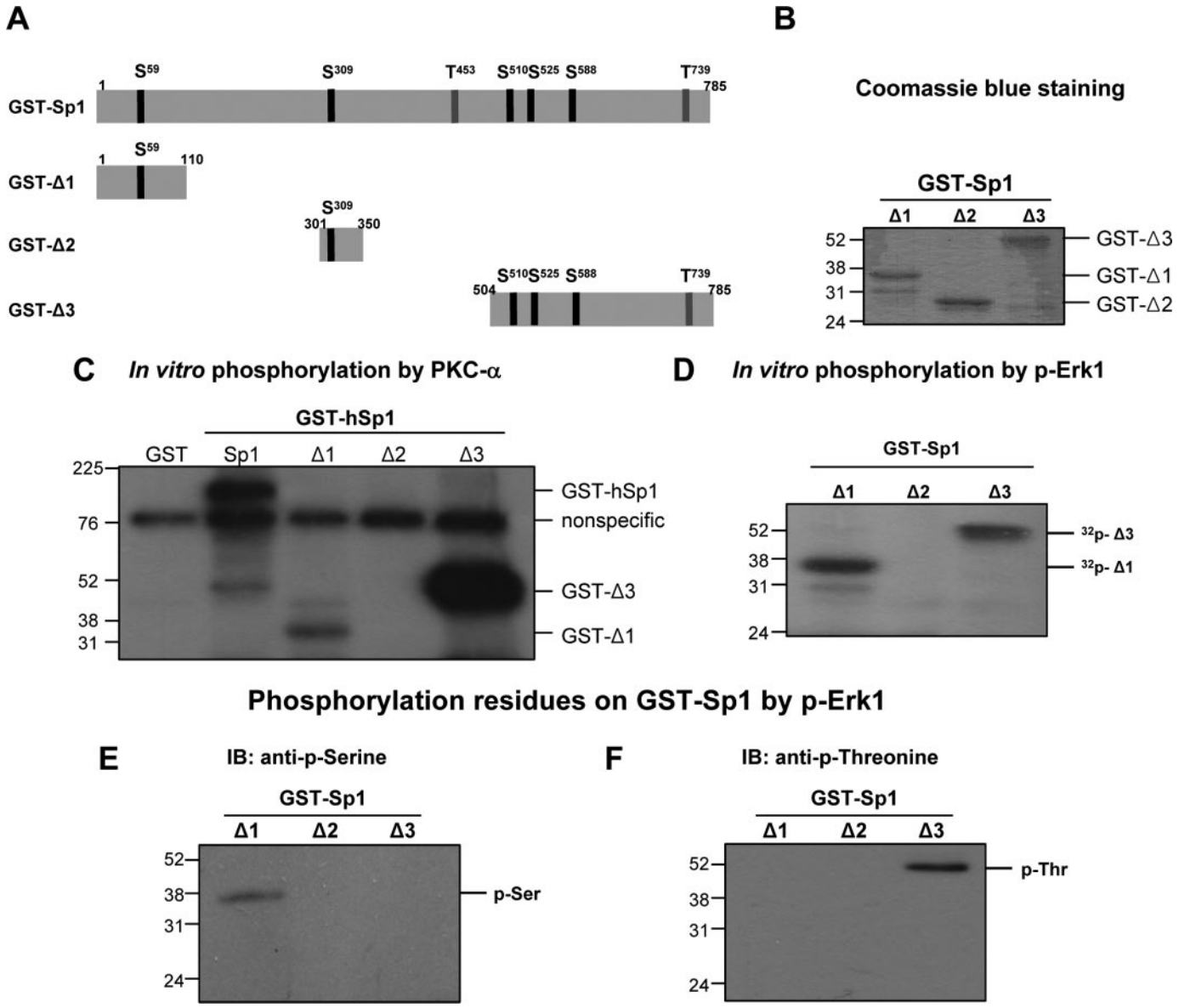


FIGURE 5. ***In vitro* phosphorylation of GST-Sp1 by PKCα and Erk1.** To investigate whether phosphorylation of Sp1 was regulated directly by PKCα or SA-pErk1/2, a human Sp1 fusion construct (*GST-Sp1*) and its deletion mutants (*GST-Δ1*, 1–110 residues of Sp1; *GST-Δ2*, 301–350 residues of Sp1; *GST-Δ3*, 504–785 residues of Sp1) were prepared as described under "Experimental Procedures," focusing on the potential phosphorylation sites (*A*). The recombinant proteins were expressed in *E. coli* and confirmed by Coomassie Blue stain after SDS-PAGE (*B*). *In vitro* phosphorylations of Sp1 by PKCα (*C*) and active Erk1 (*D*) were evaluated by an *in vitro* kinase assay. The recombinant proteins were subjected to kinase assay in the presence of 5 μCi of [γ-^{32}P]ATP and 10 times excess amount of unlabeled ATP. The reaction mixture was separated by SDS-PAGE and then visualized by autoradiography. Note phosphorylated GST-Δ1 and GST-Δ3, but not GST-Δ2, in addition to the GST-Sp1 full sequence by active PKCα and active Erk1. To identify phosphorylated residues in the recombinant proteins, immunoblot (*IB*) analyses were performed with anti-Ser(P) (*E*) and anti-Thr(P) (*F*) antibodies. Note the phosphorylations of serine and threonine residues in the GST-Δ1 and GST-Δ3, respectively, by active Erk1.

treatment of old HDF cells with GFP-siRNA, PKCα-siRNA, or NAC. The precipitates were then analyzed by immunoblotting with anti-Ser(P) and anti-Thr(P) antibodies. Unexpectedly, Sp1 was phosphorylated only on serine, but not threonine, and was regulated by treatment with PKCα-siRNA (Fig. 7*A*) and NAC (Fig. 7*B*). To confirm *in vivo* phosphorylation of Sp1 by SA-pErk1/2, mid-old HDF cells were treated with U0126 for 1 h, and the Sp1 protein was extracted by immunoprecipitation. As shown in Fig. 7*C*, U0126 significantly reduced the expressions of pErk1/2 (80%) and Ser(P) (50%) in Sp1 as compared with the untreated control. At the same time, threonine phosphorylation was not found in the Sp1 molecules. These data strongly indicate serine residue as the *in vivo* phosphorylation site of Sp1 by not only PKC-α but also SA-pErk1/2, but not threonine, in the process of cellular senescence.

In Vivo Phosphorylation of Sp1 on Ser^{59} Regulates the Expression of p21^{Sdi1}—To investigate whether phosphorylation of Sp1 on Ser^{59} regulates p21^{Sdi1} expression, mid-old HDF cells were treated with siRNAs for 2 days, and then expressions of Sp1 and p21^{Sdi1} were determined by immunoblot analyses. As shown in Fig. 7*D*, Sp1 expression was almost completely down-regulated (0.08) with concurrent reduction of p21^{Sdi1} (0.42) expression when treated with Sp1-siRNAs, as compared with control scrambled siRNA. To confirm whether Ser(P)^{59} in Sp1 affects

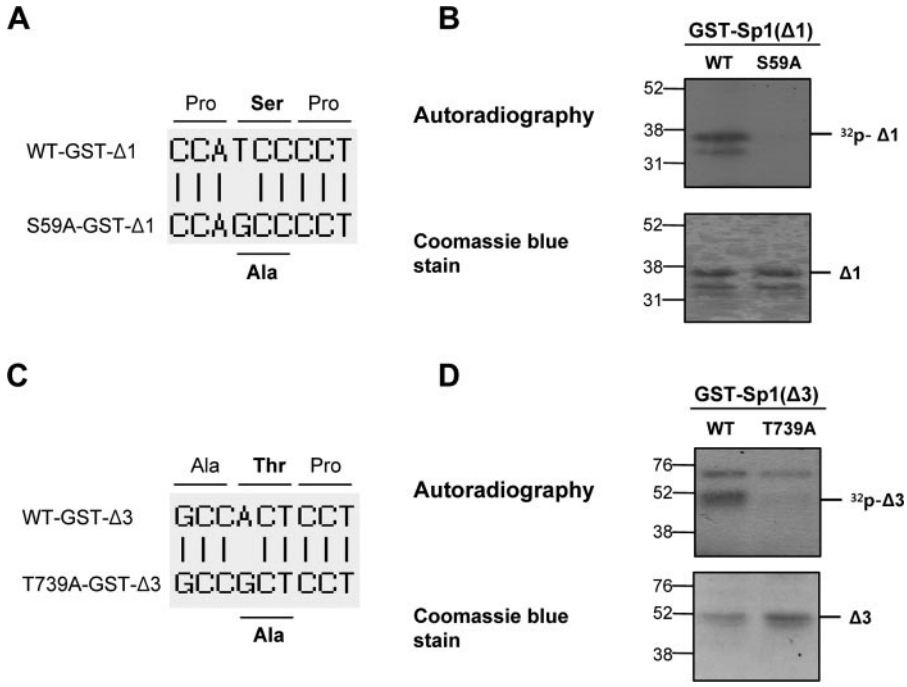


FIGURE 6. ***In vitro* phosphorylation of Sp1 on Ser^{59} and Thr^{739} by active Erk1.** Based on the above data, serine and threonine residues were suspected as the phosphorylation sites in the GST-Δ1 and GST-Δ3, respectively. Therefore, Ser^{59} to Ala (*A*) and Thr^{739} to Ala (*C*) mutant constructs were prepared by site-directed mutagenesis. Employing kinase analyses, we confirmed that active Erk1 phosphorylated GST-Δ1 and GST-Δ3 proteins as expected, however, it failed in GST-Δ1 and GST-Δ3 proteins with the Ser^{59} to Ala mutant (*B*) and the Thr^{739} to Ala mutant (*D*), indicating the Ser^{59} and Thr^{739} residues as *in vitro* phosphorylation sites of active Erk1.

transcriptional activity of p21^{Sdi1} or not, Huh7 cells were transfected with p21^{Sdi1}-luciferase together with either WT-Sp1 or the S59A-Sp1 construct for 24 h, and then serum-starved for 24 h before treatment with EGF for 6 h. As expected, luciferase activity was significantly increased by EGF treatment in the WT-Sp1-transfected cells, as compared with the untreated control. However, the effect of EGF was not found in the S59A-Sp1-transfected cells (Fig. 7*E*). The data clearly indicate the role of Ser(P)^{59} Sp1 for regulation of p21^{Sdi1} transcription in response to EGF treatment.

The cellular response to ROS was not confined to PKC activation, but also induced expression of SA-pErk1/2 (supplemental Fig. S1). The findings strongly suggest concurrent regulations of PKCα, pErk1/2, and ROS in cellular senescence. Indeed, ROS and PKCα were concurrently regulated in HDF cells, and this was confirmed by treatment of senescent HDF cells with PKCα-siRNA. Activation of PKCα increased the expression of pErk1/2 in senescent cells, and SA-pErk1/2 phosphorylated Sp1 only on Ser^{59} *in vivo*, consequently enhancing transcription of p21^{Sdi1}. Elevated p21^{Sdi1} inhibited the cell cycle at the G_1 phase, leading to growth arrest of senescent cells (Fig. 8). On the other hand, down-regulation of PKCα by treating old cells with PKCα-siRNA significantly reduced SA-pErk1/2 in both nuclear and cytoplasmic fractions of old cells, and released senescent cells from G_1 arrest of the cell division cycle with activation of ckd2. Taken together, p21^{Sdi1} expression seemed to be regulated via Sp1 phosphorylation on Ser^{59} by SA-pErk1/2 downstream of PKCα in the replicative senescence process.

DISCUSSION

Replicative senescence of human diploid fibroblast has frequently been used as an aging model *in vitro* (46). One of the significant phenotypes of replicative senescence is the SA-pErk1/2 due to persistent activation of H-*ras* (23, 24). On the other hand, the role of SA-pErk1/2 in cellular senescence has not yet been reported, although the expression is constitutively higher in the cytoplasm of senescent cells (22). To the best of our knowledge, therefore, this is the first study to confirm that SA-pErk1/2 activates the transcription factor, Sp1, via Ser^{59} phosphorylation downstream of PKCα, leading to transcription of p21^{Sdi1} and resulting in replicative senescence of HDF cells. We do not yet know why SA-pErk1/2 phosphorylated only Ser^{59} *in vivo*, but not Thr^{739}, despite *in vitro* phosphorylation of both of these residues by active Erk1 (Fig. 6). Nevertheless, the present study is well supported by the recently proposed mechanism of TPA-induced Erk1/2 regulation during early events of ML-1 cell differentiation to macrophage via induction of p21^{Sdi1} (47) and also the observation that PKCα induces senescence of human lung cancer cells (19).

We found that stimulation of HDF cells with TPA significantly increased activities of PKCα, PKCβI, and PKCη (Fig. 1). At present, there are no reports on the role of PKCη in senescence of HDF, whereas there are a few reports that may suggest a role of PKCη for cellular senescence. Normal colon mucosa expresses mRNAs of the following isoforms of PKC, in decreasing order of abundance: PKCδ > PKCη > PKCα > PKCβ > PKCϵ. PKCη is normally expressed in the more differentiated cells of epithelial tissues. However, expression of PKCη is reduced in colon cancer and returns to normal levels in the more differentiated epithelial cells (48). Inhibition of PKCη expression significantly reduces keratinocyte growth, suggesting a potential role of PKCη in keratinocytes (49). Induced differentiation of neoplastic keratinocytes accompanies an increase of PKCη expression (50). PKCα was responsible for generation of ROS in senescent cells (Fig. 2), and PKCα-siRNA, but not PKCβI-siRNA, released senescent cells from growth arrest (Fig. 3) accompanied with reversal of the senescence phenotypes (Fig. 4).

Treatment of HDF cells with TPA initially activated PKCα and PKCβI (Figs. 1*A* and 2*A*), and down-regulation of PKCα and PKCβI occurred in 8 and 4 h, respectively (supplemental Fig. S2). Nevertheless, growth arrest was regulated by PKCα only, not PKCβI (Fig. 3). The kinetics of PKCα reduction are well correlated with reversal of senescence morphology in 8 h of TPA treatment (25). Furthermore, down-regulation of PKCα was accompanied

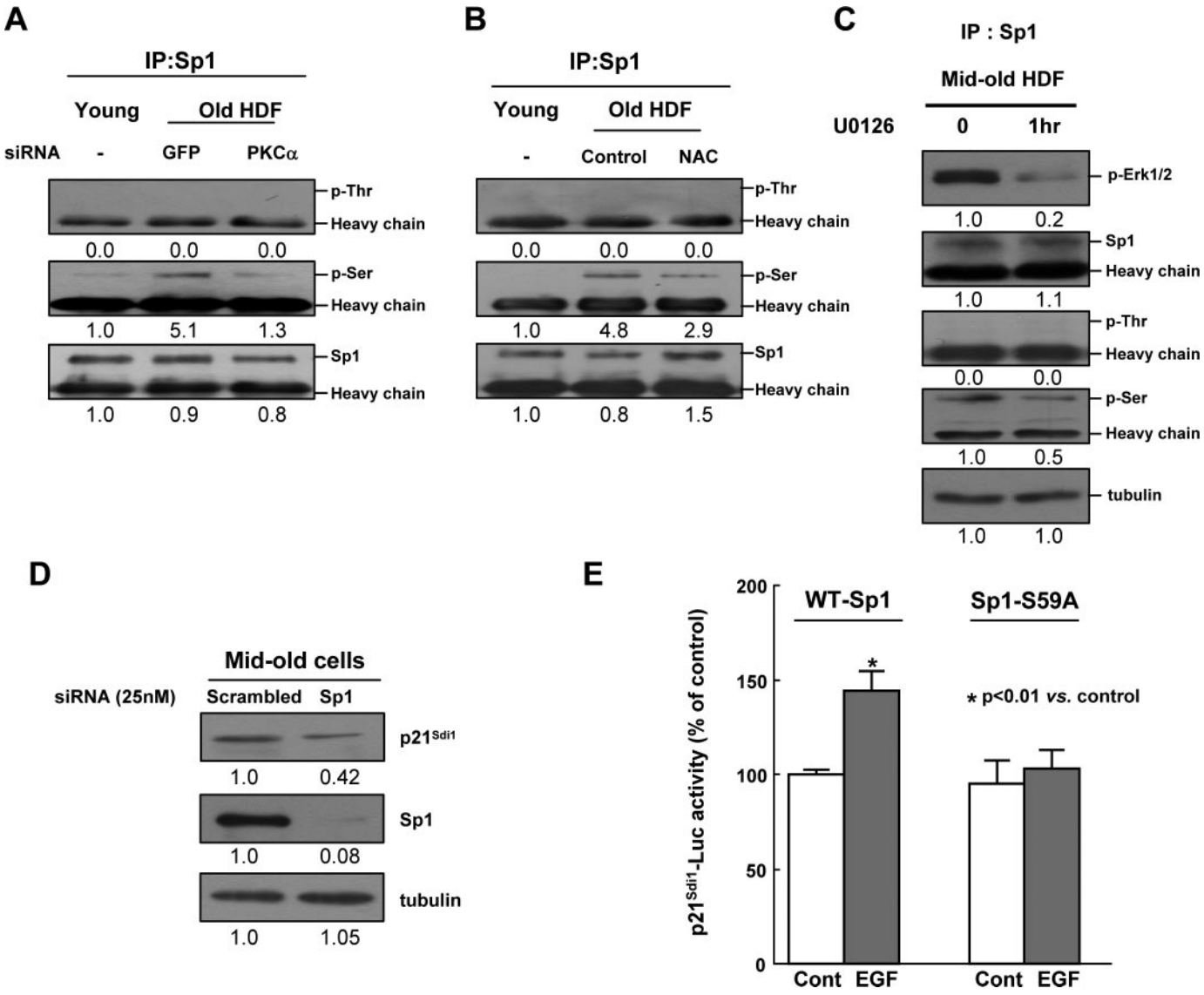


FIGURE 7. ***In vivo* phosphorylation of Sp1 on Ser[59] by SA-pErk1/2 and regulation of p21[Sdi1] expression.** To evaluate *in vivo* phosphorylation of Sp1 by PKC-α, HDF old cells were treated with either GFP-siRNA or PKCα-siRNA for 2 days (*A*), or treated with either vehicle or 10 mM NAC for 12 h (*B*). The cell lysates (500 μg) were incubated with anti-Sp1 antibody and protein G-Sepharose beads for 8 h at 4 °C with continuous agitation. Immunoprecipitated complexes were separated by SDS-PAGE, and subjected to immunoblot analyses with anti-Thr(P), anti-Ser(P), or anti-Sp1 specific antibodies. Note serine phosphorylation of Sp1 in the cells, but not with threonine. Moreover, the degree of Ser(P) in Sp1 was significantly reduced by treatment of the cells with PKCα-siRNA and NAC. These findings indicate that Sp1 was *in vivo* phosphorylated only on the serine residue, but not on threonine. *C*, at the same time, to evaluate *in vivo* regulation of Sp1 phosphorylation by SA-pErk1/2, mid-old HDF cells were treated with U0126 for 1 h, and Sp1 protein was extracted by immunoprecipitation. U0126 significantly reduced expressions of pErk1/2 (0.2) and Ser(P) in Sp1 (0.5) than the untreated control. Moreover, threonine phosphorylation in the Sp1 molecule by pErk1/2 was not found. These experiments clearly indicate Sp1 phosphorylation on the serine residue by not only PKC-α but also SA-pErk1/2, but not threonine, in the process of cellular senescence. *D*, to investigate whether phosphorylation of Sp1 on Ser[59] regulates p21[Sdi1] expression, mid-old HDF cells were treated with scrambled-siRNA or Sp1-siRNA for 2 days, and then expressions of Sp1 and p21[Sdi1] were determined by immunoblot analyses. Expression of Sp1 was almost completely down-regulated with concurrently reduced p21[Sdi1] expression when treated with Sp1-siRNAs. *E*, to confirm whether Ser(P)[59] in Sp1 affects transcription of p21[Sdi1] or not, Huh7 cells were transfected with p21[Sdi1]-luciferase along with either WT-Sp1 or the S59A-Sp1 construct for 24 h, and then serum-starved for 24 h before treatment with EGF for 6 h. As expected, luciferase activity was significantly increased by EGF treatment in the WT-Sp1-transfected cells, as compared with the control (*, $p < 0.01$). However, the effect of EGF was not found in the S59A-Sp1-transfected cells, indicating the role of Ser(P)[59]-Sp1 in regulation of p21[Sdi1] transcription after EGF treatment.

with reduced expression of senescence markers, especially SA-pErk1/2 (Fig. 3, *I* and *K*). These findings strongly support SA-pErk1/2 as a downstream mediator of PKCα, which is activated in response to ROS in cellular senescence.

In the present study, SA-pErk1/2 was found to be the kinase of Sp1 on Ser[59], resulting in transcription of p21[Sdi1], in good agreement with reports that activation of the H_2O_2-mediated ERK signaling pathway is required for p21[Sdi1] expression by TGF-β1, and that elimination of ROS with either antioxidant or catalase is accompanied by the inhibition of ERK activation by TGF-β1, resulting in attenuation of p21[Sdi1] expression without any alteration of nuclear translocation of Smads (31, 51, 52). Some studies have implicated pErk1/2 as a mediator of p53-independent p21[Sdi1] induction (53, 54). These observations are in good accordance with another report that PKCα and PKCϵ stimulate p21[Sdi1] expression via Sp1 (42). DNA-PK, PKA, PKCζ, casein kinase II, Erk1/2, and cyclin-dependent kinase are responsible for Sp1 activation, whereas protein phosphatase 1

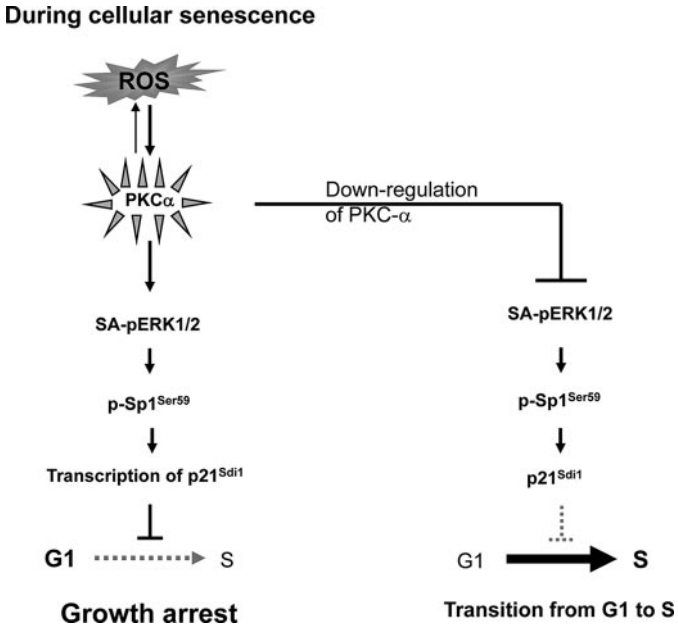


FIGURE 8. **Phosphorylation of Sp1 on Ser^{59} by SA-pErk1/2 downstream of PKCα, and induction of p21^{Sdi1} expression.** ROS and PKCα concurrently stimulated each other during cellular senescence. Accumulated ROS stimulates the activity of PKCα, and the active PKCα in turn stimulates ROS generation during cellular senescence, demonstrated by treatment of the old cells with PKCα-siRNA. Moreover, activated PKCα regulates the expression of senescence-associated pErk1/2 in the cytoplasm (*SA-pErk1/2*), which in turn increases transcription of p21^{Sdi1} via *in vivo* phosphorylation of Sp1 on Ser^{59}. Elevated p21^{Sdi1} induces cell-cycle arrest of the actively growing cells at G_1 phase, leading to growth arrest of senescent cells. On the other hand, down-regulation of PKCα expression by treatment of old cells with PKCα-siRNA significantly reduces SA-pErk1/2 in both nuclear and cytoplasmic fractions of the old cells, and inhibits Sp1 phosphorylation on Ser^{59}, but not Thr^{739}, and transcription of p21^{Sdi1}, resulting in the release of senescent cells from G_1 arrest. In summary, *in vivo* regulation of p21^{Sdi1} expression occurs via Sp1 phosphorylation on Ser^{59} by pErk1/2 downstream of PKCα in the process of cellular senescence.

and 2A dephosphorylate. Furthermore, PKCα was constitutively activated in senescent cells without any change of expression levels (Fig. 1*B*). One possible cause of PKCα activation might be ROS accumulated in senescent cells (22). ROS such as H_2O_2 can activate PKC by receptor tyrosine kinase (36–38). In accordant with this notion, NAC reduced PKCα activity in senescent cells (Fig. 2*A*). Another cause of PKC activation might be higher levels of diacylglycerol, a PKC activator, in senescent cells than in young cells (55). Indeed, down-regulation or activation of PKCα using PKCα-siRNA and the catalytic subunit of PKCα, respectively, regulated senescence phenotypes oppositely (Figs. 3 and 4). Furthermore, it has been reported that Sp1 is phosphorylated on Ser^{59}, Ser^{101}, Ser^{131}, Thr^{278}, Thr^{355}, Thr^{453}, Ser^{641}, Thr^{668}, Ser^{670}, Thr^{681}, and Thr^{739} (56–65).

When phosphorylation of Sp1 by PKCα and active Erk1 was investigated *in vitro* with deletion mutants of human Sp1 proteins, PKCα and active Erk1 were found to phosphorylate Sp1 on both serine and threonine residues, however, Sp1-Δ1 was a weaker substrate for PKCα than Sp1-Δ3. In contrast, however, Sp1-Δ1 was a slightly better substrate for Erk1 than Sp1-Δ3 (Fig. 5, *C versus D*). As Sp1-Δ3 has more sites phosphorylated by PKC (64, 65) than by Erk (59, 63), it might be a better substrate for PKCα. When phosphorylation of the Sp1-Δ1 S59A mutant by PKCα was examined, phosphorylation was found to be significantly reduced with some residual phosphorylation (1.00 *versus* 0.17; supplemental Fig. S3), strongly suggesting that PKCα phosphorylates Sp1-Δ1 not only on Ser^{59} but also with some additional target site. In contrast, Ser^{59} may be the only target for Erk1 (Fig. 5*D*). Employing GST-Sp1 mutant proteins (S59A and T739A), we confirmed indeed that both Ser^{59} and Thr^{739} residues of Sp1 were *in vitro* phosphorylated by active Erk1 (Fig. 6), however, Sp1 immunoprecipitates isolated from senescent HDF cells contained only Ser(P) (Fig. 7, *A–C*). The result indicates that phosphorylation of Ser^{59} by pErk1/2 occurs both *in vitro* (Fig. 6*A*) and *in vivo* (Fig. 7*C*) in senescent cells, thereby increasing transcription of p21^{Sdi1} by phosphorylated Sp1 on the Ser^{59} residue in response to growth factor signals (Fig. 7*E*). In the present study, it seems that Ser^{59} of Sp1 is a major phosphorylation residue by Erk1/2 for inducing transcription of p21^{Sdi}, although PKCα also phosphorylates Sp1 on Ser^{59} directly. However, we need to explain why *in vivo* phosphorylation of Sp1 occurred only on Ser^{59}, but not on Thr^{739}, in the senescent cells. It has been reported that Sp1 is phosphorylated on Thr^{453} and Thr^{739} residues by Erk1/2 in SL2 *Drosophila* cells, and Sp1 has been suggested as a key molecular link between activation of RAS and vascular endothelial growth factor transcription in tumor cells (59). Therefore, we suggest p21^{Sdi1} as a strong candidate of the Sp1-regulated gene downstream of PKCα and SA-pErk1/2 in the cellular senescence process.

In conclusion, we presented a novel pathway of p21^{Sdi1} that was induced in a senescence process by Sp1, which was phosphorylated on Ser^{59} by SA-pErk1/2 in response to the ROS signal and the constitutive activation of RAS. Our data may also explain an interesting report that hepatocellular carcinoma patients with activation of RAS/MAPK pathway, assessed by positive staining for pErk1/2, have a longer time to cancer progression (178 *versus* 46 days) (66), which strongly suggests that patients with higher pErk1/2 may express more p21^{Sdi1}, thus inducing growth arrest and senescence phenotypes in tumor tissue.

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