Expression of p21Sdi1 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 p21Sdi1 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 PKCα and PKCβI. However, PKCα, but not PKCβI, regulated ROS generation and cell proliferation in senescent cells along with activation of cdk2, proven by siRNAs. PKCα-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 PKCα and its DN-mutant construct. These findings strongly suggest a new pathway to regulate senescence phenotypes by ROS through Sp1 phosphorylation between PKCα 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 p21Sdi1 expression. In summary, PKCα, 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 p21Sdi1 resulted in regulation of cellular senescence in primary human diploid fibroblast cells.

PKCα 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 isoforms, comprising α, βI, βII, and γ, are activated by Ca^{2+}, phosphatidylserine, diacylglycerol, or phorbol esters; the novel PKC isoforms, 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 G1/S transition (14, 16–18) and recently in the regulation of cancer cell senescence (19), whereas PKCβI has been shown to play a role in progression from G1 into M phase (15), and PKCd 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 vivo 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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of p21\textsuperscript{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 isoforms 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 isoforms 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\textsuperscript{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\textsuperscript{Waf1/Cip1} expression during cellular senescence, except transcriptional activation of the p21\textsuperscript{Waf1/Cip1} promoter by Sp1 after phosphorylation on the two threonine residues (Thr\textsuperscript{453} and Thr\textsuperscript{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 PKC\textalpha{}: PKC\textalpha{} was responsible for ROS generation and activation of Erk1/2 in senescent cells. SA-pErk1/2 enhanced the expression of p21\textsuperscript{Sdi1} via phosphorylation of Sp1 on Ser\textsuperscript{59} in vivo downstream of PKC\textalpha{}, resulting in induction of senescence phenotypes.

**EXPERIMENTAL PROCEDURES**

*Materials*—Anti-actin antibody, H\textsubscript{2}O\textsubscript{2}, TPA, N-acetylcytosine (NAC), 1,2-dioctanoylgerolid (DiC\textalpha{}), and GF109203X (PKC inhibitor) were purchased from Sigma. Anti-pErk1/2 antibody was from Cell Signaling; antibodies against PKCa, PKC\textbeta{}, PKC\textgamma{}, PKC\textdelta{}, and Sp1, and p21\textsuperscript{Sdi1} were from Santa Cruz; anti-p33 and α-tubulin antibodies were from Oncogene. 2′,7′-Dichlorodihydrofluorescein diacetate (H\textsubscript{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\([\text{A}/\text{BC}] \log\text{2}\); 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\textsubscript{2} incubator at 37°C.

*Cell Treatment*—Cells (3 × 10\textsuperscript{5} cells/60 mm dish) were plated 24 h before they were treated with various chemicals, such as H\textsubscript{2}O\textsubscript{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\textsubscript{2}-DCFDA, according to the method described elsewhere (22).

*siRNA Transfection*—Young and mid-old cells were transfected with siRNA against PKCa, PKC\textbeta{}, 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), 150 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 mM 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 isoforms, 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 mM 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-PKC\textalpha{}, anti-PKC\textbeta{}, 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-PKC\textalpha{}, anti-PKC\textbeta{}, 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-PKC\textalpha{}, anti-PKC\textbeta{}, anti-pErk1/2, anti-actin, anti-α-tubulin, anti-p21\textsuperscript{Sdi1}, anti-p53, anti-Sp1, anti-phosphothreonine, or anti-phosphoserine antibodies.
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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. 1A, PKCa, PKCb, and PKCe were detected in both young and old cells were activated by TPA treatment. Fig. 1B shows that there was no change in the expression of PKCa and PKCb 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. 1C). Furthermore, immunoblot analyses revealed that the inhibition of PKC activities clearly significantly reduced the expression of molecular markers of senescence, such as p21Sdi1, p53, and SA-pErk1/2 (Fig. 1D). These features are well accordant with a recent report that PKCa works as a mediator of G2/M arrest and senescence of non-small cell lung cancer cells, and that it induces p21Sdi1, an obligatory event for conferring the senescence phenotype (19).
PKCα and ROS are Concurrently Regulated during Cellular Senescence—Because of our earlier study that the level of H$_2$O$_2$ 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$_2$O$_2$ accumulated in the senescent cells was responsible for activation of PKCα and PKCβ. Therefore, in vitro kinase assay was performed with immunoprecipitates of PKCα and PKCβ obtained from young and old HDF cells after treatment with H$_2$O$_2$, TPA, or NAC. As shown in Fig. 2A, not only TPA (50 ng/ml) but H$_2$O$_2$ (1 mM) also increased the activities of PKCα and PKCβ 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 PKCβ-siRNA, and the changes of ROS levels
were measured by FACS using H₂DCF-DA. As already known, ROS levels of mid-old cells was higher than that of young cells (Fig. 2B), however, PKCα-siRNA, but not PKCβ-siRNA, significantly reduced the level in the mid-old cells (Fig. 2C). These findings show that H₂O₂ increased PKCα activity in the senescence process and PKCα regulated the H₂O₂ level in senescent cells. The observation can be supported by the reports that PKCα, -βII, -δ, and -ζ phosphorylate p47phox sites and activate NADPH oxidase (40, 41).

**FIGURE 2. Regulation of ROS level by PKCα, but not PKCβ, 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 H₂O₂ (1 μM) 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 PKCβ activities, whereas NAC, a specific inhibitor of H₂O₂, 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 H₂DCF-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 PKCβ-siRNA (20 nM) for 2 days failed to change the ROS level in the old cells (lower panel).

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Down-regulation of PKCα Inhibits 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 PKCβ-siRNA were introduced into HDF cells, immunoblot analyses revealed reduced expressions of PKCα (Fig. 3A) and PKCβ (Fig. 3D), respectively. Moreover, transfection of PKCα-siRNA released growth arrest of the senescent cells (Fig. 3C), however, there was no change in young cells (Fig. 3B). 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. 3F), but rather decreased the proliferation in young cells (Fig. 3E). 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 G1 to S phase in addition to G2/M was more than 2-fold higher in the PKCα-siRNA-treated cells than GFP control and no treatment control (Fig. 3G). 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. 3H). Indeed, down-regulation of PKCα induced G1 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 p21Sdi1, p53, and SA-pErk1/2, as compared with those of the GFP-siRNA (Fig. 3I). 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. 3J) and after (Fig. 3K) 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 PKCa-siRNA, as compared with GFP-siRNA. Here, lamin B was used as a loading control and a marker of nuclear proteins.

PKCa Regulates Expressions of SA-pErk1/2 and p21Sdi1—To investigate whether the expression of SA-pErk1/2 was regulated by PKCa or not, pcDNA3-HA (vector), dominant negative PKCa (PKCa-DN), or catalytically active PKCa (PKCa-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. 4A, PKCa-CA significantly induced expressions of SA-pErk1/2 and p21Sdi1, whereas PKCa-DN down-regulated expression of SA-pErk1/2, as compared with that of pcDNA3-HA. Moreover, DiC8 (20 μg/ml), a PKC activator, also increased expression of SA-pErk1/2 and p21Sdi1 in young HDF cells (Fig. 4B) with a concomitant decrease of cell growth (Fig. 4C). These findings strongly suggested SA-pErk1/2 as a downstream effector of PKCa in the process of cellular senescence. To confirm the regulation of the phenotypes by active PKCa, p21Sdi1 promoter-driven luciferase assay was performed in NIH3T3 cells (Fig. 4D); cells were co-transfected with p21Sdi1 promoter-luciferase (pGL2–0.3) together with pcDNA3-HA, PKCa-DN, or PKCa-CA. In 48 h, the cell lysates were subjected to luciferase (Luc) assay. As expected, luciferase activity was markedly increased after transfection with PKCa-CA, indicating that transcription of p21Sdi1 was increased only by PKCa-CA, but not PKCa-DN or the vector alone. Taken together, PKCa regulated transcription of p21Sdi1 in addition to the induction of SA-pErk1/2 expression.

Not Only PKCa but Also Active Erk1 Phosphorylate GST-Sp1—Because Sp1 has been shown to be important for p21Sdi1 transcription
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by PKCα 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\textsuperscript{Sdi1} expression in the replicative senescence of HDF cells. Therefore, GST- Sp1 fusion proteins were prepared (Fig. 5A). 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. 5B). Employing the prepared proteins as substrates and active PKCα and pErk1 as enzymes, \textit{in vitro} kinase assays were performed. As shown in Fig. 5C, 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. 5C), whereas GST-Δ1 was a slightly better substrate for Erk1 than GST-Δ3 (Fig. 5D). The data strongly supported the contention that not only PKCα but also SA-pErk1/2 could regulate p21\textsuperscript{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. 5E) and threonine (Fig. 5F) residues, respectively.

Ser\textsuperscript{59} and Thr\textsuperscript{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\textsuperscript{59} to alanine (Fig. 6A) and Thr\textsuperscript{739} to alanine (Fig. 6C). 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\textsuperscript{59} in GST-Δ1 and Thr\textsuperscript{739} in GST-Δ3 as \textit{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 \textit{in vivo} or not, Sp1 was isolated from cell lysates by immunoprecipitation after

**FIGURE 4.** Increased expression of SA-pErk1/2 and transcription of p21\textsuperscript{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\textsuperscript{Sdi1} by PKCα-CA, as opposed to down-regulation of pErk1/2 expression by PKCα-DN, B, treatment of HDF young cells with diC\textsubscript{8} (20 μg/ml), a PKC activator, for 7 days also induced p21\textsuperscript{Sdi1} and SA-pErk1/2 expressions, and concomitantly decreased cell growth (C). To prove regulations of the above mentioned phenotypes by PKCα activity, p21\textsuperscript{Sdi1} promoter-driven luciferase assay was performed (D); NIH3T3 cells were co-transfected with the reporter plasmid, p21\textsuperscript{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 significantly the activity of the p21\textsuperscript{Sdi1} transcription by PKCα-CA, but not PKCα-DN. All of the data indicate mean ± S.D. from three independent experiments.
treatment of old HDF cells with GFP-siRNA, PKC-H9251-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 PKC-H9251-siRNA (Fig. 7A) and NAC (Fig. 7B). To confirm in vivo phosphorylation of Sp1 by 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.

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-32P]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 phosphorylations of serine and threonine residues in the GST-Δ1 and GST-Δ3, respectively, by active Erk1.

In Vivo Phosphorylation of Sp1 on Ser59 Regulates the Expression of p21Sdi1—To investigate whether phosphorylation of Sp1 on Ser59 regulates p21Sdi1 expression, mid-old HDF cells were treated with siRNAs for 2 days, and then expressions of Sp1 and p21Sdi1 were determined by immunoblot analyses. As shown in Fig. 7D, Sp1 expression was almost completely down-regulated (0.08) with concurrent reduction of p21Sdi1 (0.42) expression when treated with Sp1-siRNAs, as compared with control scrambled siRNA. To confirm whether Ser59 in Sp1 affects
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. 7E). 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 PKCa, pErk1/2, and ROS in cellular senescence. Indeed, ROS and PKCa were concurrently regulated in HDF cells, and this was confirmed by treatment of senescent HDF cells with PKCa-siRNA. Activation of PKCa 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 G1 phase, leading to growth arrest of senescent cells (Fig. 8).

On the other hand, down-regulation of PKCa by treating old cells with PKCa-siRNA significantly reduced SA-pErk1/2 in both nuclear and cytoplasmic fractions of old cells, and released senescent cells from G1 arrest of the cell division cycle with activation of cdk2. Taken together, p21^Sdi1 expression seemed to be regulated via Sp1 phosphorylation on Ser^59 by SA-pErk1/2 downstream of PKCa in the replicative senescence process.

**DISCUSSION**

Reproducible 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 PKCa, 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 PKCa induces senescence of human lung cancer cells (19).

We found that stimulation of HDF cells with TPA significantly increased activities of PKCa, 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 > PKCa > PKCβ > PKCε. PKCη is normally expressed in the more differentiated cells of epithelial tissues. However, expression of PKCη is reduced in 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). PKCa was responsible for generation of ROS in senescent cells (Fig. 2), and PKCa-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 PKCa and PKCβI (Figs. 1A and 24), and down-regulation of PKCa and PKCβI occurred in 8 and 4 h, respectively (supplemental Fig. S2). Nevertheless, growth arrest was regulated by PKCa only, not PKCβI (Fig. 3). The kinetics of PKCa reduction are well correlated with reversal of senescence morphology in 8 h of TPA treatment (25). Furthermore, down-regulation of PKCa was accompanied...
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 PKCe 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
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During cellular senescence

PKCa → Down-regulation of PKCa 

ROS → SA-pErk1/2 → p-Sp1Ser59 → Transcription of p21Sdi1 → G1 → Transition from G1 to S

Growth arrest

FIGURE 8. Phosphorylation of Sp1 on Ser59 by SA-pErk1/2 downstream of PKCa, and induction of p21Sdi1 expression. ROS and PKCa concurrently stimulated each other during cellular senescence. Accumulated ROS stimulates the activity of PKCa, and the active PKCa in turn stimulates ROS generation during cellular senescence, demonstrated by treatment of the old cells with PKCa-siRNA. Moreover, activated PKCa regulates the expression of senescence-associated pErk1/2, which in turn increases transcription of p21Sdi1 via in vivo phosphorylation of Sp1 on Ser59. Elevated p21Sdi1 induces cell-cycle arrest of the actively growing cells at G1 phase, leading to growth arrest of senescent cells. On the other hand, down-regulation of PKCa expression by treatment of old cells with PKCa-siRNA significantly reduces SA-pErk1/2 in both nuclear and cytoplasmic fractions of the old cells, and inhibits Sp1 phosphorylation on Ser59, but not Thr739, and transcription of p21Sdi1 in vitro, resulting in the release of senescent cells from G1 arrest. In summary, in vivo regulation of p21Sdi1 expression occurs via Sp1 phosphorylation on Ser59 by pErk1/2 downstream of PKCa in the process of cellular senescence.

and 2A dephosphorylate. Furthermore, PKCa was constitutively activated in senescent cells without any change of expression levels (Fig. 1B). One possible cause of PKCa activation might be ROS accumulated in senescent cells (22). ROS such as H$_2$O$_2$ can activate PKC by receptor tyrosine kinase (36–38). In accord with this notion, NAC reduced PKCa activity in senescent cells (Fig. 2A). 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 PKCa using PKCa-siRNA and the catalytic subunit of PKCa, respectively, regulated senescence phenotypes oppositely (Figs. 3 and 4). Furthermore, it has been reported that Sp1 is phosphorylated on Ser59, Ser101, Ser131, Thr278, Thr355, Thr453, Ser641, Thr668, Ser670, Thr681, and Thr739 (56–65). When phosphorylation of Sp1 by PKCa and active Erk1 was investigated in vitro with deletion mutants of human Sp1 proteins, PKCa and active Erk1 were found to phosphorylate Sp1 on both serine and threonine residues, however, Sp1–Δ1 was a weaker substrate for PKCa 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 PKCa. When phosphorylation of the Sp1–Δ1 S59A mutant by PKCa was examined, phosphorylation was found to be significantly reduced with some residual phosphorylation (1.00 versus 0.17; supplemental Fig. S3), strongly suggesting that PKCa phosphorylates Sp1–Δ1 not only on Ser59 but also with some additional target site. In contrast, Ser59 may be the only target for Erk1 (Fig. 5D). Employing GST-Sp1 mutant proteins (S59A and T739A), we confirmed indeed that both Ser59 and Thr739 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 Ser59 by pErk1/2 occurs both in vitro (Fig. 6A) and in vivo (Fig. 7C) in senescent cells, thereby increasing transcription of p21Sdi1 by phosphorylated Sp1 on the Ser59 residue in response to growth factor signals (Fig. 7E). In the present study, it seems that Ser59 of Sp1 is a major phosphorylation residue by Erk1/2 for inducing transcription of p21Sdi1, although PKCa also phosphorylates Sp1 on Ser59 directly. However, we need to explain why in vivo phosphorylation of Sp1 occurred only on Ser59, but not on Thr739, in the senescent cells. It has been reported that Sp1 is phosphorylated on Thr453 and Thr739 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 p21Sdi1 as a strong candidate of the Sp1-regulated gene downstream of PKCa and SA-pErk1/2 in the cellular senescence process.

In conclusion, we presented a novel pathway of p21Sdi1 that was induced in a senescence process by Sp1, which was phosphorylated on Ser59 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 p21Sdi1, thus inducing growth arrest and senescence phenotypes in tumor tissue.

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