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$p34^{SEI-1}$ Inhibits Doxorubicin-Induced Senescence through a Pathway Mediated by Protein Kinase C-δ and c-*Jun*-NH_2-Kinase 1 Activation in Human Breast Cancer MCF7 Cells

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Abstract

In this study, we describe a novel function of the $p34^{SEI-1}$ protein, which is both an oncogenic protein and a positive regulator of the cell cycle. The $p34^{SEI-1}$ protein was found to inhibit doxorubicin-induced senescence. We investigated the molecular mechanisms of the inhibitory effect of $p34^{SEI-1}$ on senescence. First, we found that the activation of protein kinase C-δ (PKC-δ), which is cleaved into a 38 kDa active form from a 78 kDa pro-form, induced after doxorubicin treatment, was inhibited by $p34^{SEI-1}$. Furthermore, $p34^{SEI-1}$ induced the ubiquitination of PKC-δ. Yet, there is no interaction between $p34^{SEI-1}$ and PKC-δ. We also found that the phosphorylation of c-*Jun*-NH_2-kinase 1 (JNK1) induced after doxorubicin treatment was suppressed by $p34^{SEI-1}$, but not in JNK2. Consistently, pharmacologic or genetic inactivation of either PKC-δ or JNK1 was found to inhibit doxorubicin-induced senescence. In addition, the genetic inactivation of PKC-δ by PKC-δ small interfering RNA resulted in an inhibition of JNK1 activation, but PKC-δ expression was not inactivated by JNK1 small interfering RNA, implying that the activation of JNK1 could be dependently induced by PKC-δ. Therefore, $p34^{SEI-1}$ inhibits senescence by inducing PKC-δ ubiquitination and preventing PKC-δ–dependent phosphorylation of JNK1. [Mol Cancer Res 2009;7(11):1845–53]

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Introduction

SEI-1 was recently identified as a cyclin-dependent kinase 4–binding protein (1), as well as a protein encoded in the chromosomal region 19q13, a region frequently amplified in patients with ovarian carcinomas, osteosarcomas, lung cancers, and pancreatic carcinomas (2-4), suggesting that *SEI-1* is potentially an oncogene. An oncogenic role for $p34^{SEI-1}$ has been shown because expression of the protein contributes to neoplastic cell expansion through genomic instability (5). Although the precise biochemical function of $p34^{SEI-1}$ is not fully understood, $p34^{SEI-1}$ exerts at least some of its oncogenic effect although positive regulation of the cell cycle and the resulting genomic instability (6, 7). Nude mice given $p34^{SEI-1}$–transfected NIH3T3 mouse fibroblast cells showed more anchorage-independent growth and tumor formation than did control mice receiving vector-transfected NIH3T3 cells only (5).

As with $p34^{SEI-1}$, proteins in the Bcl-2 family, the cell death antagonists such as Bcl-2, Bcl-x_L, and E1B-19K have oncogenic effects, with the exception of death agonists such as Bax, Bak, or Bid (8, 9). High levels of Bcl-2 expression are found in a wide variety of human cancers (10). Bcl-2 has been shown to protect cells against hydrogen peroxide–induced or thiol depletion–induced death, and to suppress lipid peroxidation (11). Bcl-2 therefore functions to prevent the generation of reactive oxygen species (ROS) induced by various environmental stresses. This ability of Bcl-2 was found to be involved in the inhibition of cellular senescence, in that Bcl-2 protected cells from ROS stress (12). These results suggest that the oncogenic potential of Bcl-2 lies in its ability to inhibit senescence by preventing increases in intracellular ROS levels.

Various cancer cells exposed to DNA-damaging agents undergo irreversible growth arrest and acquire a senescence-like phenotype (SLP; ref. 13). Doxorubicin is one of the most important DNA-damaging agents for the treatment of solid tumors (14). Cell death induced by doxorubicin is commonly effective only at high drug doses, with low doses showing merely cytostatic effects. A previous study found that various cancer cells treated with low doses of doxorubicin showed SLP resembling the replicative senescence of normal cells (15, 16). Senescence of cells involves the development of a morphology characterized by cell flattening and enlargement, and an increase in senescence-associated β-galactosidase (SA-β-Gal) activity (17).

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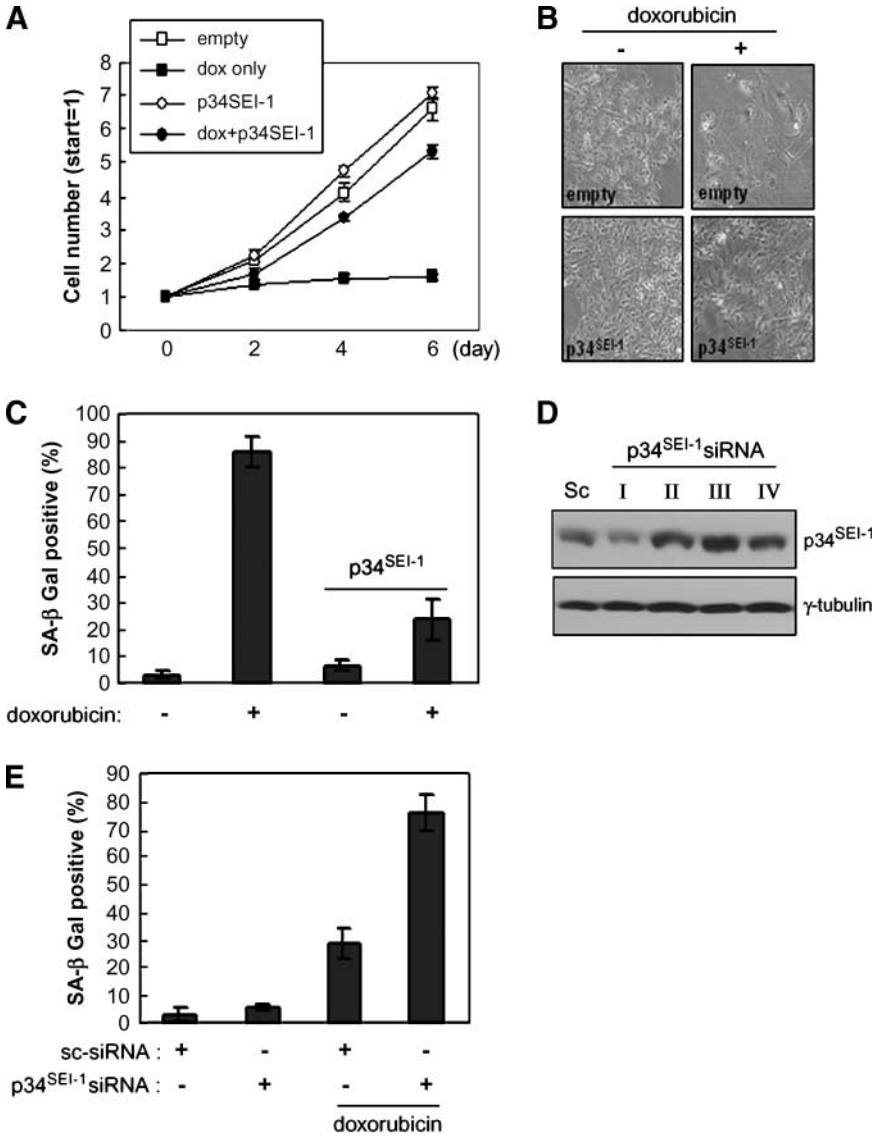

FIGURE 1. $p34^{SEI-1}$ protein inhibits cell senescence induced by doxorubicin. **A.** Either normally proliferating cells or $p34^{SEI-1}$–expressing MCF7 cells were treated with 38 nmol/L (0.02 μg/mL) of doxorubicin (□, ○) or untreated (■, ●) for the indicated times, and cell numbers were later counted. Points, means of three independent experiments; bars, SD. **B.** Cells were treated with 38 nmol/L (0.02 μg/mL) of doxorubicin for 6 d, and morphologic changes were then analyzed. **C.** SA-β-Gal–positive cells were counted 6 d posttreatment. **D.** $p34^{SEI-1}$ silencing sensitizes cells to doxorubicin. Cells treated with doxorubicin 24 h after transfection of either $p34^{SEI-1}$-siRNA I, II, III, or IV into cells. Four of the $p34^{SEI-1}$-siRNA sequences were mentioned in the Materials and Methods. **E.** SA-β-Gal staining at the indicated times after transfection with $p34^{SEI-1}$-siRNA I, following exposure to doxorubicin, were analyzed and SA-β-Gal–positive cells were counted.

Recently, it was shown that the presence of functional p53 and p21 proteins was important for the development of SLP after exposure to DNA-damaging agents (18-20). In addition, induction of p16 (21) and Rb protein (22) were required for SLP development, suggesting that replicative senescence, like apoptosis, is a programmed response of the organism to potentially oncogenic effects.

Other groups have recently reported that activation of p38 mitogen-activated protein kinase is necessary for senescence induced by cellular stressors such as UV light, oxidative stress, or DNA-damaging agents in U2OS osteosarcoma cells (23), whereas c-*Jun*-NH_2-kinase (JNK) has also been implicated in a human diploid fibroblast from foreskin (24). The activity of protein kinase C-δ (PKC-δ), an isoform of PKC, increased in aged Hs68 fibroblasts (25). These studies showed that activation of protein kinases could be induced by genotoxic stress. Protein kinases have multifunctional roles in cellular responses, including reversible growth arrest, proliferation, apoptosis, and tumor suppression (26). Although various studies have suggested that the activation of protein kinases can induce senescence in human tumor cells, the detailed mechanisms of senescence induction are not yet clear.

There is therefore a great deal of interest in genotoxic-induced senescence. In this study, we show that the potential oncogenic protein, $p34^{SEI-1}$, inhibits doxorubicin-induced senescence, and we explore the possible mechanism underlying this action.

Results

$p34^{SEI-1}$ Inhibits Doxorubicin-Induced Senescence

To examine whether $p34^{SEI-1}$ inhibits senescence induced by doxorubicin (15, 16, 27), we established a derivative of MCF7, a human breast cancer cell line stably expressing human *SEI-1* cDNA (Supplementary Fig. S1A). First, we determine the effect of 38 nmol/L of doxorubicin (0.02 μg/mL) on cell death.

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Almost no dead cells were found among the MCF7 cells treated or not treated with doxorubicin (Supplementary Fig. S1B). We next examined the effect of p34^{SEI-1} on cell proliferation. The proliferation of cells that expressed p34^{SEI-1} was not affected by 38 nmol/L of doxorubicin (0.02 μg/mL), and was almost the same as that of normally proliferating cells (Fig. 1A). We also measured SA-β-Gal activity (Fig. 1B and C), a marker for cellular senescence (17), and explored morphologic changes of cells, treated with doxorubicin in comparison with control MCF7 cells. SA-β-Gal activity by doxorubicin was time-dependently increased and its activity reached a maximum at 6 days after doxorubicin treatment (data not shown). The SLP induced by doxorubicin was not observed in p34^{SEI-1}–expressing MCF7 cells (Fig. 1B and C). Furthermore, the levels of various cytoplasmic marker proteins such as fibronectin (28) and promyelocytic leukemia (29, 30), which specifically decreases in senescent cells, were lower than control values in p34^{SEI-1}–expressing cells (Supplementary Fig. S1C), suggesting that p34^{SEI-1} inhibits doxorubicin-induced cellular senescence in MCF7, human breast cancer cells.

Also, we examined whether knockdown of p34^{SEI-1} by small interfering RNA (siRNA) sensitizes cells to doxorubicin (Fig. 1D and E). Endogenous p34^{SEI-1} protein levels were decreased in p34^{SEI-1}-siRNA–treated cells (Fig. 1D). Cells treated with p34^{SEI-1}-siRNA were compared for SA-β-Gal activity with those treated using scrambled RNA. SA-β-Gal activity of p34^{SEI-1}-siRNA–treated cells that were stained 4 days after doxorubicin treatment was increased as much as control cells that were stained at 6 days, suggesting that p34^{SEI-1} silencing sensitizes cells to doxorubicin.

The Cleavage of PKC-δ Is Induced in Doxorubicin-Induced Senescence

To address the question of how p34^{SEI-1} inhibits doxorubicin-induced senescence. We observed the changes of various protein kinases in either control cells or p34^{SEI-1}–expressing cells, followed by a low dose (0.02 μg/mL) of doxorubicin, which did not induce cell death. Surprisingly, we found that the cleavage of PKC-δ in control MCF7 cells lack caspase-3, which induces the cleaved form of PKC-δ, but not in p34^{SEI-1}–expressing cells following doxorubicin treatment (Fig. 2A). Therefore, we have focused on the change of PKC-δ induced by doxorubicin. First, we determined whether the cleaved form of PKC-δ is necessary for the induction of senescence. Consistent with SA-β-Gal activity in p34SEI-1–expressing cells, doxorubicin-induced senescence was significantly inhibited by a pharmaceutical inhibitor of PKC-δ, rottlerin (Fig. 2B). We further confirmed these results using a siRNA silencing

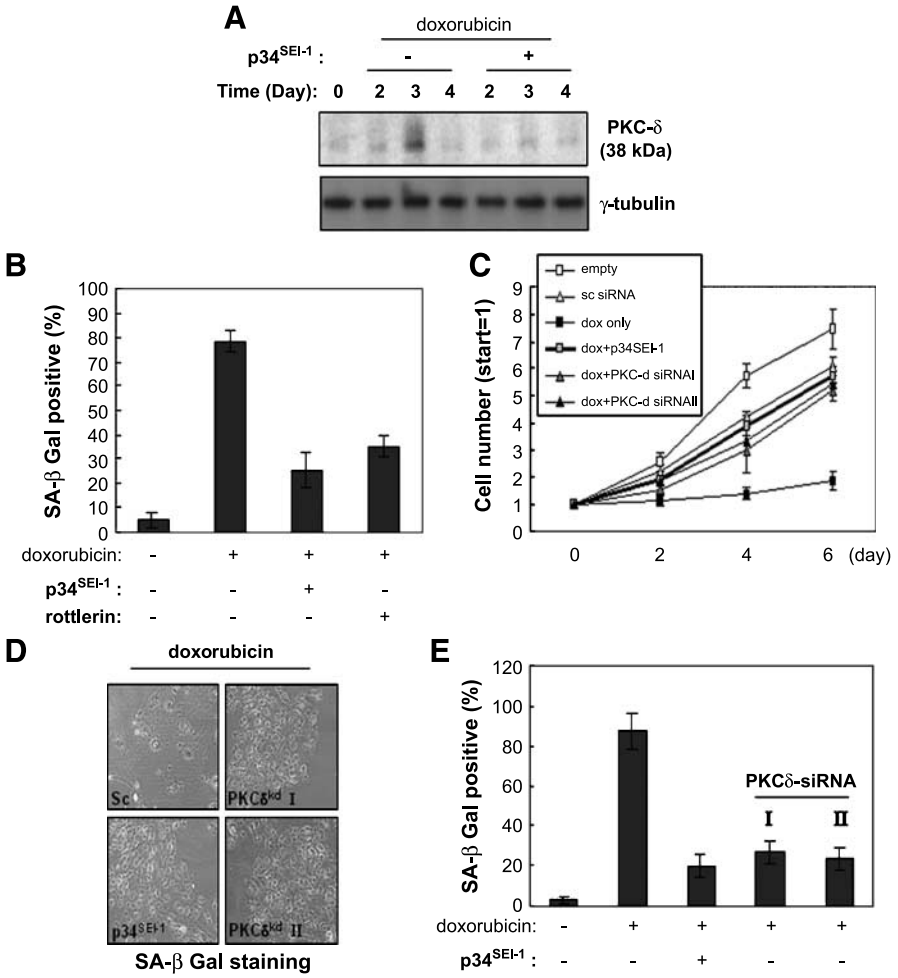

FIGURE 2. p34^{SEI-1} protein inhibits the cleavage of PKC-δ induced by doxorubicin. **A.** Activation of PKC-δ by doxorubicin and the inhibitory effect of p34^{SEI-1} on PKC-δ activity. p34^{SEI-1}–expressing cells were exposed to doxorubicin, and the level of cleaved PKC-δ were analyzed by immunoblotting at the indicated times. **B.** SA-β-Gal activity levels were analyzed after treatment with 10 μmol/L of rottlerin following exposure to doxorubicin. Columns, means of three independent experiments; bars, SD. **C.** Cells treated with doxorubicin 24 h after transfection of either PKC-δ-siRNA I or PKC-δ-siRNA II into MCF7-p34^{SEI-1} cells, respectively. Proliferation of PKC-δ-siRNA–treated cells was measured at the indicated times after doxorubicin treatment. Points, means of three independent experiments; bars, SD. **D.** SA-β-Gal staining and cell morphology 6 d after transfection with PKC-δ siRNA I and PKC-δ siRNA II following exposure to doxorubicin were analyzed. **E.** SA-β-Gal–positive cells were counted. Columns, means of three independent experiments; bars, SD.

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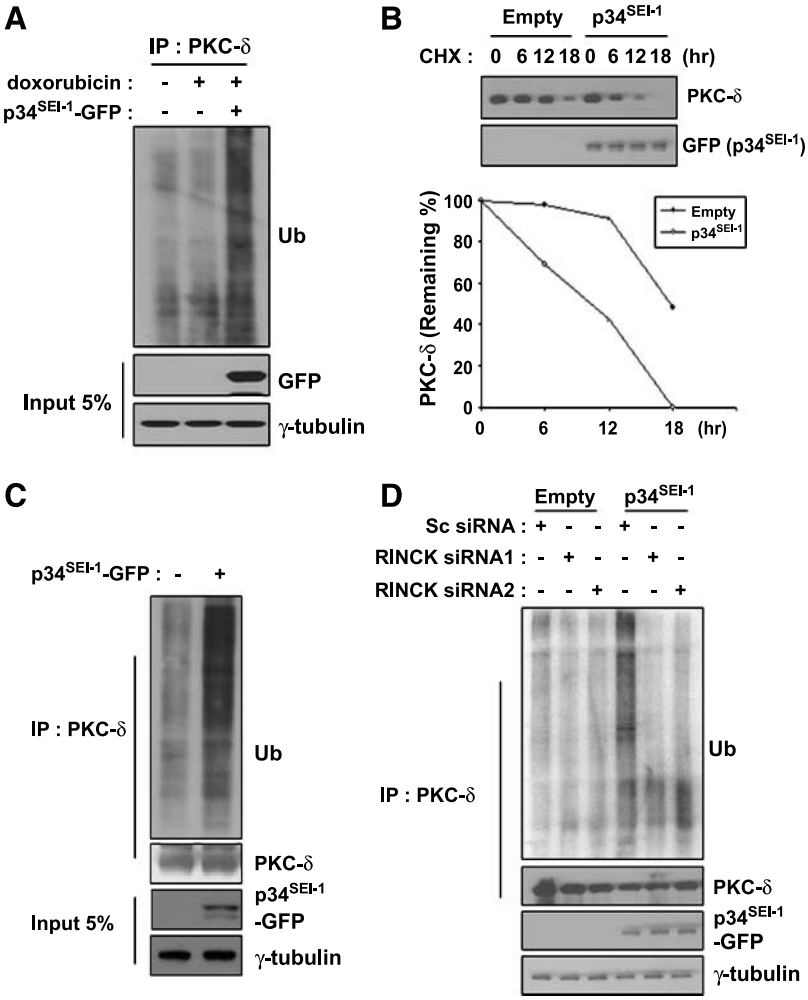

FIGURE 3. p34^{SEI-1} induces the proteasomal degradation of PKC-δ. **A.** After the expression of p34^{SEI-1}, cells were treated with doxorubicin (0.02 μg/mL). Cell lysates were prepared 3 d after doxorubicin treatment and then were immunoprecipitated with anti–PKC-δ. PKC-δ ubiquitination was confirmed by immunoblotting using antiubiquitin. **B.** MCF7 cells were transfected with either GFP-tagging p34^{SEI-1} plasmid or control vector for 18 h, and then treated with cycloheximide (50 μg/mL) for the indicated times. The levels of PKC-δ and GFP-tagging p34^{SEI-1} were confirmed by Western blotting using anti–PKC-δ or anti-GFP, respectively. **C.** Cell lysates prepared as in Fig. 4B were immunoprecipitated with anti–PKC-δ, and then immunoblotted with anti–PKC-δ and anti-GFP. γ-Tubulin was used as a loading control. **D.** Either MCF7 control cells or MCF7 p34^{SEI-1}–expressing cells were transfected with RINCK-1-siRNA I, RINCK-1-siRNA II, or scrambled siRNA at 20 pmol for 24 h, and then were immunoprecipitated with anti–PKC-δ. PKC-δ ubiquitination was confirmed by immunoblotting using antiubiquitin. The levels of PKC-δ and GFP (p34^{SEI-1}) were determined by immunoblotting using anti–PKC-δ or anti-GFP. γ-Tubulin was used as a loading control.

the expression of PKC-δ. Proliferation of cells was observed (Fig. 2C), and growth arrest was not induced in PKC-δ-siRNA–treated cells following doxorubicin treatment (Fig. 2C), in line with the inhibitory effect of p34^{SEI-1} on doxorubicin-induced senescence. Also, the development of SA-β-Gal activity and senescence-specific morphologic changes were blocked by PKC-δ silencing using siRNA, as was also seen in the expression of p34^{SEI-1} (Fig. 2D and E). In addition, it was recently reported that cellular senescence was induced after 1 μmol/L of doxorubicin treatment of 2-hour durations (27). We confirmed that a similar activation of PKC-δ occurred in our system. After 1 μmol/L of doxorubicin treatment for 2 hours, the cleavage of PKC-δ was induced (Supplementary Fig. S2), indicating that the cleavage of PKC-δ is induced during doxorubicin-induced senescence.

p34^{SEI-1} Inhibits Senescence through RINCK-Dependent Ubiquitination of PKC-δ

As shown in Fig. 2A, the cleavage of PKC-δ induced after doxorubicin treatment was not observed in p34^{SEI-1}–expressing cells. We therefore investigated the inhibitory mechanisms of p34^{SEI-1} on PKC-δ. We first examined whether the inhibitory effect of p34^{SEI-1} on PKC-δ was related with the proteasomal degradation of the PKC-δ pro-form. The ubiquitination of PKC-δ in cells expressing p34^{SEI-1} with MG132, the proteasome inhibitor, was induced, but not in control cells (Fig. 3A). We further studied whether PKC-δ turnover is affected by p34^{SEI-1} in the presence of cycloheximide, the protein synthesis inhibitor. PKC-δ levels were significantly decreased in cells expressing p34^{SEI-1}, whereas its levels were gradually decreased in control cells (Fig. 3B). We did *in vivo* ubiquitination assays to further confirm the induction of PKC-δ ubiquitination by p34^{SEI-1}. PKC-δ ubiquitination was clearly detected upon the expression of p34^{SEI-1} (Fig. 3C). In addition, we also confirmed PKC-δ ubiquitination by p34^{SEI-1} in 293 cells cotransfected with green fluorescent protein (GFP)-tagged p34^{SEI-1} and hemagglutinin (HA)-tagged PKC-δ (data not shown). However, there is no direct interaction between p34^{SEI-1} and PKC-δ, suggesting that p34^{SEI-1} indirectly induces PKC-δ ubiquitination.

It was recently reported that RINCK is an E3 ligase of PKC-δ (31). We therefore investigated whether ubiquitination of PKC-δ by p34^{SEI-1} is dependent on RINCK (Fig. 3D). PKC-δ ubiquitination induced by the expression of p34^{SEI-1} was remarkably decreased by RINCK silencing using siRNA (Supplementary Fig. S4A), indicating that p34^{SEI-1} induces the ubiquitination of PKC-δ through RINCK.

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The Phosphorylation of JNK1 Is Essential for Doxorubicin-Induced Senescence

It was recently reported that the activity of JNK/stress-activated protein kinase (SAPK) is induced in human diploid fibroblasts from the foreskin (24). JNK/SAPK is involved in cell proliferation, apoptosis, a stress-induced signal pathway, and senescence (29). It remains unclear, however, whether JNK/SAPK is activated during doxorubicin-induced senescence. We therefore examined whether the activity of JNK/SAPK is induced in this senescence system and sought an inhibitory effect of p34^{SEI-1} on such an induction. After treatment with 38 nmol/L of doxorubicin (0.02 μg/mL) in control cells, JNK1 was phosphorylated, but JNK2 was not, whereas the phosphorylation of this kinase was weakly observed in p34^{SEI-1}–expressing cells compared with control cells (Fig. 4A); a similar effect was seen after 1 μmol/L of doxorubicin treatment for 2 hours (Supplementary Fig. S2). We next determined whether the phosphorylation of JNK1 was necessary for the induction of senescence. Senescence induced by doxorubicin was significantly inhibited by a chemical inhibitor of JNK/SAPK, SP600125, in a manner similar to that shown by p34^{SEI-1} (Fig. 4B).

Above, we described the effect of siRNA targeting PKC-δ expression on senescence induced by doxorubicin (Fig. 2). We next examined the effect of silencing of JNKs siRNA on senescence (Supplementary Fig. S4B). The proliferation of doxorubicin-treated cells transfected with JNK1-siRNA or JNK1/2-siRNA significantly increased from control levels, and cell numbers dramatically increased (Fig. 4C), but JNK2 siRNA had no effect. Furthermore, senescence-specific

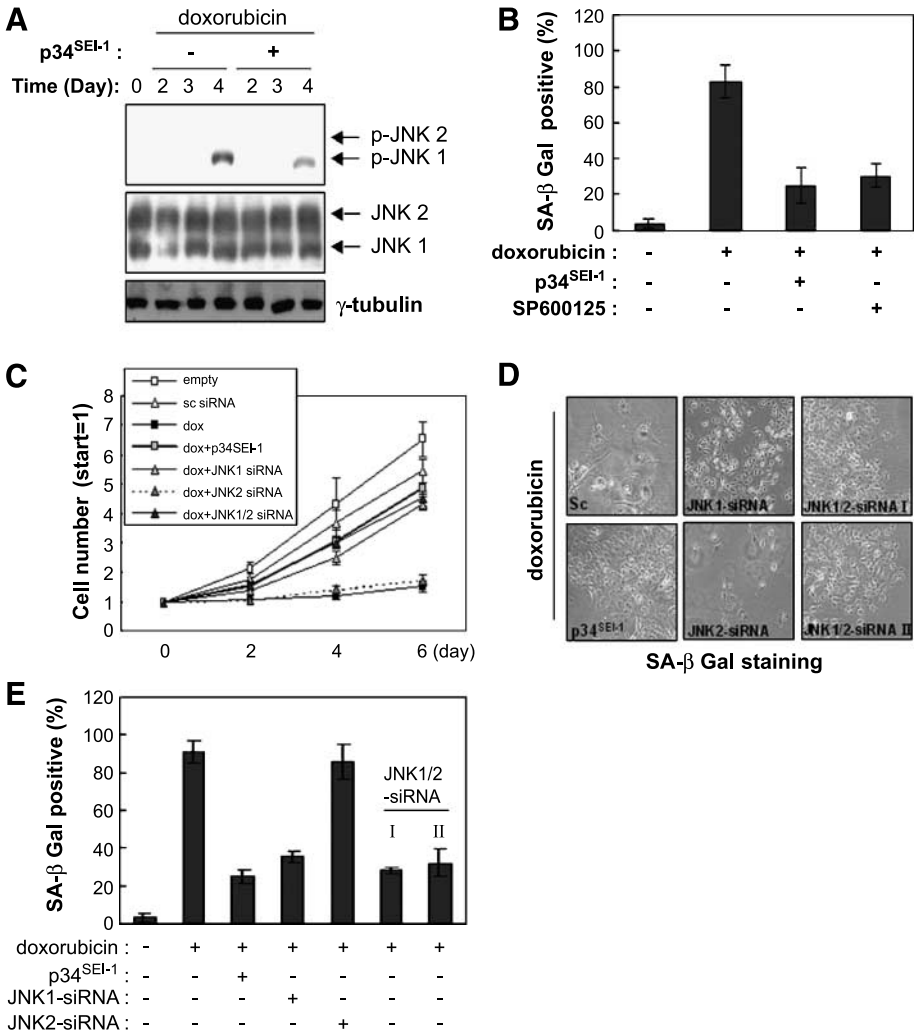

FIGURE 4. p34^{SEI-1} protein inhibits the activation of JNK1 induced by doxorubicin. **A.** Activation of JNK1 by doxorubicin and the inhibitory effect of p34^{SEI-1} on JNK1 activity. Either control MCF7 cells or MCF7 cells stably expressing 34^{SEI-1} were treated with 38 nmol/L of doxorubicin. The phosphorylation of JNK1/2 was confirmed by Western blot analysis. γ-Tubulin was used as a loading control. **B.** Cells were pretreated with 10 μmol/L of SP600125 for 1 h and then treated with 38 nmol/L of doxorubicin. Senescent cells were counted by SA-β-Gal staining. Three independent experiments were each done in duplicate. **C.** Either MCF7 control cells or MCF7 p34^{SEI-1}–expressing cells were transfected with JNK1-siRNA, JNK2-siRNA, JNK1/2-siRNA I, JNK1/2-siRNA II, or scrambled siRNA at 200 pmol for 24 h, followed by treatment with 38 nmol/L of doxorubicin. Cell growth was analyzed by cell counting at the indicated times following doxorubicin treatment. Points, mean of three independent experiments; bars, SD. Morphologic changes (**D**) and SA-β-Gal activities (**E**) 6 d after treatment with 38 nmol/L of doxorubicin. Columns, means of three independent experiments; bars, SD.

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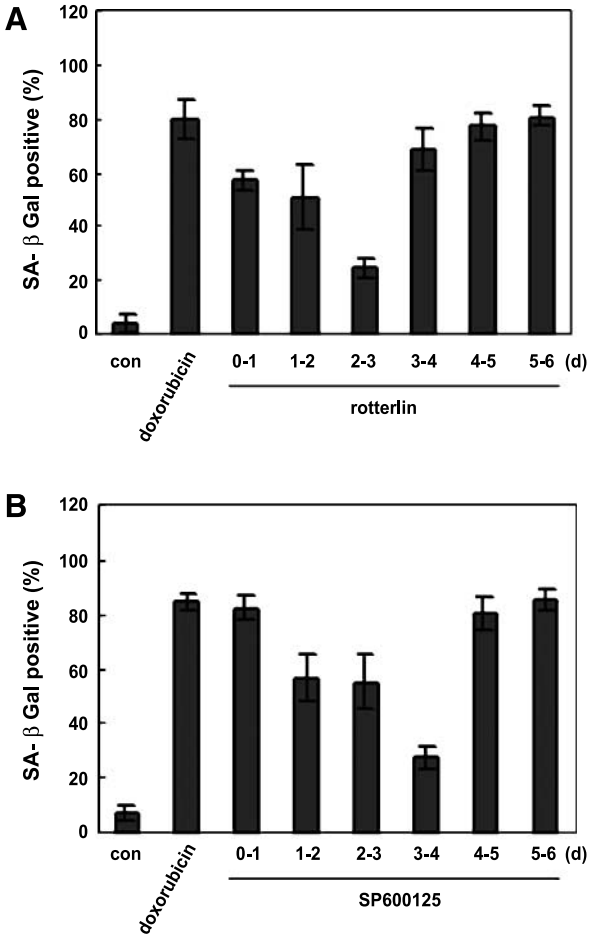

FIGURE 5. The activation of PKC-δ and JNK1 is essential for doxorubicin-induced senescence. MCF7 cells were treated with rottlerin or SP600125 at the indicated times following doxorubicin treatment, and SA-β-Gal activity was analyzed 6 d later. **A.** Intervals when rottlerin affected doxorubicin-induced senescence. **B.** Intervals when SP600125 affected doxorubicin-induced senescence.

morphologic changes were not observed in JNK1-siRNA–treated cells or JNK1/2-siRNA–treated cells (Fig. 4D), whereas SA-β-Gal activity also decreased in such cells (Fig. 4E), implying that JNK1 phosphorylation is specifically required for doxorubicin-induced senescence, and that $p34^{SEI-1}$ may inhibit senescence through the suppression of this signaling pathway.

$p34^{SEI-1}$ Protein Prevents Senescence Induction through Suppression of PKC-δ–Dependent Activation of JNK1

Above, we showed that the cleavage of PKC-δ was increased 3 days after doxorubicin treatment and that the phosphorylation of JNK1 was significantly increased 4 days after such treatment (Figs. 2A and 4A). Furthermore, $p34^{SEI-1}$ suppressed the activities of PKC-δ and JNK1 (Figs. 2A and 4A). We next examined the functional relationship between PKC-δ and JNK1 during doxorubicin-induced senescence. First, we confirmed the times at which PKC-δ or JNK1 were activated, as knowledge of these kinetics is important in developing an understanding of senescence induction. To identify the intervals during which either rottlerin or SP600125 inhibited doxorubicin-induced senescence, cells were exposed to either drug for 24 hours at the indicated times (Fig. 5). Rottlerin dramatically blocked SA-β-Gal activity when cells were treated for 2 or 3 days after doxorubicin treatment (Fig. 5A) and SP600125 significantly prevented senescence induction when present 3 or 4 days after drug treatment (Fig. 5B), but the two drugs were not active when present at other times, implying that the activity of JNK1 might be PKC-δ–dependently induced during doxorubicin-induced senescence.

We therefore sought to determine whether PKC-δ affects the activity of JNK1 using siRNA of PKC-δ. In parallel with PKC-δ silencing, the phosphorylation of JNK1 was decreased in PKC-δ-siRNA–treated cells following doxorubicin treatment, consistent with the effect of $p34^{SEI-1}$ on JNK1 activity. This indicated that the activity of PKC-δ was indispensable for the activation of JNK1 (Fig. 6A; Supplementary Fig. S3A). In contrast, silencing of JNK1 by siRNA did not affect the activity of PKC-δ (Fig. 6C; Supplementary Fig. S3B), indicating that the phosphorylation of JNK1 is PKC-δ–dependently induced in doxorubicin-caused senescence. These results suggest that PKC-δ–dependent JNK1 phosphorylation is necessary for senescence induction by doxorubicin and that the activities of both protein kinases are suppressed by $p34^{SEI-1}$ protein.

Discussion

In the present study, we first showed the inhibitory effect of $p34^{SEI-1}$ on cellular senescence induced by activation of both PKC-δ and JNK1 in doxorubicin-treated cells. A recent report indicated that p130, a member of the Rb family, regulates doxorubicin-induced senescence (27). The assemblage of factors responsible for doxorubicin-induced senescence remains to be fully described. We found that PKC-δ–dependent activation of JNK1 is necessary for doxorubicin-induced senescence and that $p34^{SEI-1}$ inhibits senescence by inducing PKC-δ ubiquitination and preventing PKC-dependent JNK1 phosphorylation in human breast cancer MCF7 cells.

Previous studies have shown that $p34^{SEI-1}$ has an oncogenic role, and positively regulates the cell division cycle by binding to cyclin-dependent kinase 4 (1-5). Our group recently showed that one oncogene, Bcl-xL, could inhibit cellular senescence (12). We have therefore focused on the effect of $p34^{SEI-1}$ on cellular senescence. It has been reported that treatment with a low dose of doxorubicin, which did not cause cell death, induced a rapid onset of senescence in human tumor cells (15, 16). This encouraged us to investigate the role of $p34^{SEI-1}$ in doxorubicin-induced senescence. Here, we report that $p34^{SEI-1}$ inhibits doxorubicin-induced cellular senescence (Fig. 1). These results suggest that $p34^{SEI-1}$ suppresses the cellular commitment to senescence.

To study the molecular mechanisms by which $p34^{SEI-1}$ inhibits doxorubicin-induced senescence, we focused on protein kinases that are activated during senescence. Recent studies have shown that PKC-δ activity in old human diploid fibroblasts was higher than in young cells (25), and that the same was true for JNK/SAPK (24). The present study shows that activation of both PKC-δ and JNK1 is essential for the induction of senescence by doxorubicin, and that $p34^{SEI-1}$ inhibits

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doxorubicin-induced senescence through the suppression of both PKC-δ and JNK1 activation (Figs. 2–4). Consistently, a chemical inhibitor of PKC-δ (rotterlin) or JNK (SP600125) was found to inhibit doxorubicin-induced senescence (Figs. 2–4). Moreover, the time intervals during which the inhibitors of either PKC-δ or JNK1 effectively inhibited doxorubicin-induced senescence were found to be in accord with the intervals during which activation of either kinase was apparent (Fig. 5). We suggest that the 3- to 4-day intervals during which kinase activations are noted represent commitment points to senescence induction. In addition, we examined whether p34^{SEI-1} regulates PKC-δ and JNK1 in normal cells, such as human dermal fibroblasts (HDF; Supplementary Fig. S5). Surprisingly, both PKC-δ and JNK1 were not activated at late population doublings of HDFs with p34^{SEI-1} expression vector. These observations indicated that p34^{SEI-1} inhibits senescence through downregulation of PKC-δ and JNK1.

Thus, senescence induced by doxorubicin was controlled through the correlation between p34^{SEI-1} and two kinases. Consequently, we sought to further study the link between these proteins. At first, we confirmed that JNK1 activation is dependent on PKC-δ (Fig. 6). We did not, however, obtain evidence for direct binding between PKC-δ and JNK1. Our results thus suggest that PKC-δ–dependent activation of JNK1 is necessary for doxorubicin-induced senescence. Second, we observed the changes of PKC-δ in cells expressing p34^{SEI-1}. Interestingly, PKC-δ was ubiquitinated after transfection with GFP-tagged p34^{SEI-1} (Fig. 3). Recent reports have shown that RINCK-1 is an E3 ligase of PKC-δ (31). Our report also showed that ubiquitination of PKC-δ upon p34^{SEI-1} was not induced in cells treated with RINCK-1-siRNA (Fig. 3D), implying that p34^{SEI-1} indirectly induces the enzymatic activity of RINCK-1 and subsequent to the induction of PKC-δ ubiquitination (Fig. 3). However, further studies are necessary to elucidate these mechanisms. Also, the detailed molecular mechanisms by which the cleavage of PKC-δ is induced in MCF7 cells lacking caspase-3 after doxorubicin treatment needs to be elucidated.

In addition, we examined the effect of p34^{SEI-1} on the intracellular levels of ROS, which plays a key role in senescence (32, 33). Interestingly, p34^{SEI-1} inhibited an increase in ROS levels by doxorubicin in MCF7 cells (Supplementary Fig. S6). Thus, p34^{SEI-1} may prevent both PKC-δ and JNK1 activity through the inhibition of ROS production.

Finally, our studies suggest that p34^{SEI-1} has a new function, one in which p34^{SEI-1} is involved in the inhibition of cellular senescence.

Materials and Methods

Cell Culture and DNA Transfection

MCF7 human breast cancer cells were cultured in 5% CO_2 at 37°C in RPMI (Life Technologies) containing high glucose and supplemented with 10% fetal bovine serum (Hyclone), penicillin (100 units/mL), and streptomycin (100 μg/mL). To establish MCF7 cells expressing p34^{SEI-1}, we constructed a retroviral expression system for human p34^{SEI-1}(pBabe-Puro-p34^{SEI-1}). *SEI-1* cDNA was provided by Dr. Rikiro Fukunaga (Osaka University, Osaka, Japan). We transfected cells with pBabe-Puro-p34^{SEI-1} and selected individual clones of stable transfectants (designated MCF7-p34^{SEI-1}) for further analysis. HDF, which was provided by Dr. In-Chul Park (Institute of Radiological and Medical Sciences, Seoul, Korea), were prepared from the dermis of neonatal human foreskin. HDF was cultured in DMEM (Life Technologies) containing 10% fetal bovine serum. To calculate population doublings, cell number was counted for subcultures and divided by the number of seeded cells (34, 35). Young HDF have <20 to 25 population doublings, and old (senescent) HDF have >50 population doublings.

RNA Interference

LipofectAMINE 2000 (Invitrogen) was used for transfection of p34^{SEI-1}-siRNA I (5′-CCGAAUUGGACUACCUCAU-3′), p34^{SEI-1}-siRNA II (5′-GCAAGGGUCUGAAGCGGAA-3′), p34^{SEI-1}-siRNA III (5′-GGAAACGGGAGGAGGAGGA-3′), p34^{SEI-1}-siRNA IV (5′-AGGAGGAGGAGGAGAAGGA-3′), JNK1 (5′-GGGCCUACAGAGAGCUAGUUCUUAU-3′) siRNA (36), JNK2 (5′-GCCCAAGGGAUUGUUUGUGCUG-CAU-3′) siRNA (36), JNK1/2 (I, 5′-AAAAAGAAUGUCCUAC-CUUCU-3′; II, 5′-UGUCUGGUAUGAUCCUUCUdTdT-3′) siRNA (37, 38), or PKC-δ (I, 5′-CGAGAAGAUCAUCGGCA-GATT-3′; II, 5′-UCUGCCGAUGAUCUUGUCGTT-3′) siRNA (39). Scrambled RNA as control siRNA was obtained from Proligo LLC.

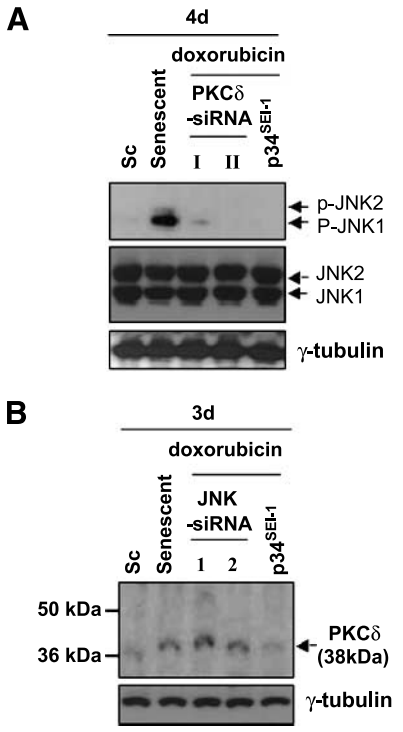

FIGURE 6. p34^{SEI-1} protein inhibits senescence induced by doxorubicin involving the PKC-δ–dependent activation of JNK1. **A.** MCF7 cells were transfected with either PKC-δ-siRNA I, PKC-δ-siRNA II, or wild-type p34^{SEI-1}, and cells were then lysed after 4 d for the detection of JNKs. **B.** Cells were transfected with JNK1-siRNA or JNK2-siRNA and then cell lysates were prepared 4 d later. The cleavage of PKC-δ was analyzed by Western blotting. γ-Tubulin was used as a loading control.

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SA-β-Gal Staining

Cells were washed in PBS, and fixed with 0.25% glutaraldehyde in PBS/2 mmol/L $MgCl_2$ for 20 min at room temperature. SA-β-Gal activity at pH 6.0 was detected as reported (17).

Analysis of Cell Proliferation

MCF7 or MCF7-p34^{SEI-1} cells (2×10^5 cells) were plated in 60 mm culture dishes. Wells transfected with scrambled siRNA, JNK1, JNK2, JNK1/2 siRNA, or PKC-δ siRNA were treated with 38 nmol/L of doxorubicin (0.02 μg/mL), and then cell proliferation was analyzed by cell counting at the indicated times.

Measurement of ROS Levels

Cells were incubated with 10 μmol/L of H_2DCF-DA (Molecular Probe) for 30 min, then washed with PBS, trypsinized, and collected in 1 mL of PBS. Fluorescence-stained cells were transferred to polystyrene tubes with cell-strainer caps (Falcon) and subjected to fluorescence-activated cell sorter (Becton Dickinson FACScan) using Cell Quest 3.2 (Becton Dickinson) software for analysis (12).

Immunoblot Analysis

Cells were lysed in EBC lysis buffer. Protein quantification was done using the protein assay kit (Bio-Rad). Approximately 40 μg of total cell protein per sample was subjected to SDS-PAGE and transferred to an Immobilon polyvinylidene difluoride filter (Millipore). The filter was then blocked in 5% nonfat dry milk/0.1% Tween/TBS followed by incubation with each antibody and immunodetection using the ECL System (Amersham Pharmacia Biotech). p34^{SEI-1} (Axxora LLC), fibronectin (BD PharMingen), p-JNK1/2, and JNK1/2 (Cell Signaling Technology), promyelocytic leukemia, PKC-δ, and γ-tubulin (Santa Cruz Biotechnology), respectively.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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