

# ERK Phosphorylates p66shcA on Ser36 and Subsequently Regulates p27<sup>kip1</sup> Expression via the Akt-FOXO3a Pathway: Implication of p27<sup>kip1</sup> in Cell Response to Oxidative Stress<sup>D</sup>

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Mice deficient for p66shcA represent an animal model to link oxidative stress and aging. p66shcA is implicated in oxidative stress response and mitogenic signaling. Phosphorylation of p66shcA on Ser36 is critical for its function in oxidative stress response. Here we report the identification of ERK as the kinase phosphorylating p66shcA on Ser36. Activation of ERKs was necessary and sufficient for Ser36 phosphorylation. p66shcA interacted with ERK and was demonstrated to be a substrate for ERK, with Ser36 being the major phosphorylation site. Furthermore, in response to H<sub>2</sub>O<sub>2</sub>, inhibition of ERK activation repressed p66shcA-dependent phosphorylation of FOXO3a and the down-regulation of its target gene p27<sup>kip1</sup>. Down-regulation of p27 might promote cell survival, as p27 played a proapoptotic role in oxidative stress response. As a feedback regulation, Ser36 phosphorylated p66shcA attenuated H<sub>2</sub>O<sub>2</sub>-induced ERK activation, whereas p52/46shcA facilitated ERK activation, which required tyrosine phosphorylation of CH1 domain. p66shcA formed a complex with p52/46ShcA, which may provide a platform for efficient signal propagation. Taken together, the data suggest there exists an interplay between ERK and ShcA proteins, which modulates the expression of p27 and cell response to oxidative stress.

## INTRODUCTION

Reactive oxygen species (ROS) are implicated in the pathogenesis of degenerative diseases and in the process of aging (Finkel and Holbrook, 2000; Droge, 2002). They are produced upon exposure to  $\gamma$ -ray or UV light, during oxidative phosphorylation in mitochondria, or during inflammation by macrophages, and cause damage to DNA, proteins, and lipids, leading to cell death in the form of necrosis (high dosage) or apoptosis (low dosage). A connection between oxidative stress and aging has been established in *Drosophila* and *Caenorhabditis elegans* (Finkel and Holbrook, 2000). DAF-16 in *C. elegans*, encoding the forkhead related transcription factor (FOXO3a/FKHRL1 in mammals), promotes longevity in response to reduced insulin/IGF-1 signaling (Kenyon *et al.*, 1993). An analogous example in mammals is the p66shcA knockout mice. Deletion of p66shcA from mouse genome by gene targeting renders the mice resistant to oxidative stress, and a 30% increase in average lifespan of

the mice was observed. Mouse embryonic fibroblasts (MEFs) isolated from these mice are also resistant to the cytotoxic effects of H<sub>2</sub>O<sub>2</sub> (Migliaccio *et al.*, 1999). Oxidative stress leads to phosphorylation of p66shcA on Ser36. p66shcA carrying a S36A mutation, when reintroduced back into p66shcA null MEFs, could not correct the resistance of these cells to oxidative stress, suggesting that Ser36 phosphorylation is critical for the proapoptotic activity of p66shcA in response to oxidative stress (Migliaccio *et al.*, 1999). Recent studies have suggested a link between p66shcA and FOXO3a. In p66shcA-deficient MEFs, H<sub>2</sub>O<sub>2</sub>-induced phosphorylation/inactivation of FOXO3a is inhibited, correlated with increased activity of FOXO3a and up-regulation of target genes involved in free radical scavenging and oxidative stress resistance. Hence, the proapoptotic activity of p66shcA in response to oxidative stress might be mediated by FOXO3a (Nemoto and Finkel, 2002). Furthermore, phosphorylation of Ser36 on p66shcA was found to be critical for FOXO3a phosphorylation (Nemoto and Finkel, 2002). Hence, Ser36 phosphorylation is essential for p66shcA function in oxidative stress responses, in which Akt/FOXO3a play critical roles. However, the kinase responsible for this phosphorylation has not been identified.

p66shcA, p52shcA, and p46shcA constitute the ShcA family (reviewed in Luzi *et al.*, 2000; Ravichandran, 2001). All three isoforms contain a phospho-tyrosine binding domain (PTB), a collagen homology domain 1 (CH1), and a Src

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homology 2 domain (SH2), whereas p66shcA has a unique collagen homology domain 2 (CH2) at the N' terminus in which Ser36 is located (Luzi *et al.*, 2000). Working as adaptor proteins, p52 and p46 transmit signals from receptor tyrosine kinases (RTK) to the RAS-MAPK pathway (reviewed in Luzi *et al.*, 2000). Their SH2 and PTB domains are responsible for binding to the phosphorylated tyrosine residues of activated RTK, which subsequently phosphorylates the CH1 domain on tyrosine residues Y239/240 and/or Y317. Phosphorylated ShcA proteins form a complex with Grb2, which constitutively interacts with Sos (Rozakis-Adcock *et al.*, 1992). Recruitment of Grb2-Sos complex to the plasma membrane by p46/52 shcA leads to activation of Ras-ERKs pathway, and this promotes cell proliferation/transformation and cell survival (Ullrich and Schlessinger, 1990; Schlessinger, 2000). p66shcA is also phosphorylated after growth factor stimulation, but it competes with p52shcA for Grb2 binding (Migliaccio *et al.*, 1997). Overexpression of p66shcA markedly inhibits activation of ERK after epidermal growth factor (EGF) stimulation in Chinese hamster ovary cells (Okada *et al.*, 1997). Thus p66shcA and p52/46shcA have distinct functions in response to growth factor stimulation. Although p66shcA plays an important role in cell response to oxidative stress, the roles for p52shcA and p42shcA have not been explored.

In addition to their toxic and proapoptotic roles, ROS have been recently recognized as molecules with important physiological functions including acting as an oxygen sensor in the regulation of erythropoietin production, maintenance of redox homeostasis, and enhancement of cell signaling and amplification of cell responses to growth factors and antigens (Reth, 2002; Mikkelsen and Wardman, 2003). In some cases, ROS appear to function as second messengers in cell signaling, being produced in response to external stimuli and subsequently used for signal amplification (reviewed in Droge, 2002; Finkel, 2003). ROS can directly activate signaling molecules via modification, or indirectly activate signaling cascades by inhibiting protein tyrosine phosphatases. Yet the molecular mechanisms by which ROS function as a mitogen are still elusive. In an attempt to identify the kinase responsible for ShcA66 Ser36 phosphorylation upon oxidative stress, we found that 1) ERKs were responsible for H<sub>2</sub>O<sub>2</sub>-induced Ser36 phosphorylation of p66shcA; 2) ERKs acted upstream of p66shcA-Akt-FOXO3a pathway, which was delineated from studies on p66shcA<sup>-/-</sup> cells (Nemoto and Finkel, 2002); 3) one major target of ERK-ShcA66-FOXO3a pathway was p27, whose down-regulation by H<sub>2</sub>O<sub>2</sub> required Ser36 phosphorylation of p66shcA; 4) p27 participated in cell response to oxidative stress; 5) H<sub>2</sub>O<sub>2</sub>-induced ERK activation was facilitated by p52/46shcA but hindered by p66shcA, and that H<sub>2</sub>O<sub>2</sub> promoted the interaction between p66shcA and p52/46shcA. This suggests the existence of a negative feedback loop for regulation of ERK activation. The interaction between ShcA proteins may provide a novel mechanism by which ShcA proteins exert their diverse functions.

## MATERIALS AND METHODS

### Chemicals and Antibodies

Hydrogen peroxide was obtained from Acros Organics (Pittsburgh, PA). U0126 and wortmannin were purchased from Calbiochem (La Jolla, CA). JNK inhibitors were obtained from Calbiochem (L-form) and Alexis Biochemicals (D-form; San Diego, CA). Antibodies against active ERK, active p38 MAPK, active JNK, active Akt, Thr32 phosphorylated FOXO3a, Ser256 phosphorylated FOXO1, and ERK were obtained from Cell Signaling (Beverly, MA). Antibodies against p27, p21, and horseradish peroxidase-conjugated phosphotyrosine antibodies were obtained from BD Biosciences (San Diego, CA).

Antibodies against HA tag, Myc tag, and ShcA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Ser36 phosphorylated ShcA were obtained from Calbiochem.

### Cell Culture

MEFs were prepared following a standard protocol and these primary MEFs, NIH3T3, and immortalized *Jnk1<sup>-/-</sup>Jnk2<sup>-/-</sup>*, *ShcA<sup>+/+</sup>*, *ShcA<sup>-/-</sup>*, *Src<sup>+/+</sup>*, and *Src<sup>-/-</sup>* MEFs were cultured in DMEM (Life Technologies, Rockville, MD) containing 10% fetal calf serum (HyClone, Logan, UT) supplemented with glutamine and penicillin/streptomycin, and COS7 cells were cultured in DMEM containing 10% bovine calf serum (HyClone) supplemented with glutamine and penicillin/streptomycin. To generate *ShcA<sup>-/-</sup>* cells expressing p66shcA or p66shcA(S36A), *ShcA<sup>-/-</sup>* MEFs were transfected with constructs expressing these proteins or the empty vector, respectively, selected against puromycin (expressed by these constructs) for 2 wk. The expression of p66shcA or p66shcA(S36A) was analyzed by Western blot in comparison to *ShcA<sup>+/+</sup>* MEFs.

### Mutagenesis

Point mutations of ShcA were generated using a site-directed mutagenesis Kit from Invitrogen (Carlsbad, CA), following the manufacturer's protocol. Truncations of ShcA were synthesized by PCR and subsequently cloned into the expression vector pcDNA3.1. All the expression constructs were sequenced to verify the mutations.

### Immunoprecipitation, Western Blot, and Immunohistochemistry

Cells were subcultured the day before and then treated with H<sub>2</sub>O<sub>2</sub> for various durations of time. To test the effects of the kinase inhibitors, specific inhibitors were added to the cell cultures 1 h before the addition of H<sub>2</sub>O<sub>2</sub>. Cells were washed with phosphate-buffered saline (PBS) and lysed in TNEN buffer containing 50 mM Tris, pH 7.5, 100 mM KCl, 1 mM EDTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 10 mM NaF, 1 mM beta-glycerol phosphate, and 10 μg/ml each of aprotinin and leupeptin (Li *et al.*, 2002). Protein concentrations were determined by Bio-Rad protein quantitation assays (Richmond, CA). Equal amounts of protein (20 μg) were fractionated by electrophoresis on SDS-PAGE gels, transferred onto a nitrocellulose membrane (Millipore, Billerica, MA), probed with primary and secondary antibodies, and visualized using an ECL kit (Amersham, Piscataway, NJ). For immunoprecipitation (IP), antibodies against HA, myc, ShcA, or ERKs were added to the cell lysate (400 μg) for overnight incubation, followed by addition of protein A plus G agarose beads for two more hours. The beads were washed with TNEN buffer three times and once with PBS. The immunoprecipitated proteins were released from the beads by boiling in 1× sample buffer for 5 min and subsequently analyzing by Western blot. Total cell lysate (20 μg) was run next to the IP samples to assess the portions of proteins that were coprecipitated.

COS7 cells grown on coverslips were transfected with lipofectamine (Life Technologies, Gaithersburg, MD). After 24 h, the cells were permeabilized with methanol at -20°C for 3 min, washed three times with PBS containing 10% bovine calf serum, and incubated with the primary antibodies for 1 h at room temperature. 9E10 was used to detect p66shcA (myc-tagged), and polyclonal anti-ERK antibodies were used to detect ERKs. After interaction with the primary antibody, the slides were washed three times with PBS containing 10% bovine calf serum and then incubated with secondary anti-mouse antibodies conjugated with Texas Red (Sigma, St. Louis, MO) and anti-rabbit antibodies conjugated with FITC for 1 h at room temperature. The slides were then rinsed with PBS and examined under a confocal microscope.

### GST Fusion Protein Preparation

ShcA 46, ShcA52, ShcA66, and ShcA66(S36A) were each cloned into the pGEX vector that was then transformed into BL21 cells. The bacteria were cultured in 40 ml LB with 100 μg/ml ampicillin at 37°C overnight with vigorous shaking. On the second day, the cultures were used to inoculate 800 ml LB medium with 100 μg/ml ampicillin and shaken at 37°C until OD<sub>600</sub> reached ~1.0. IPTG was added to the culture to a final concentration of 0.15 mM. The cells were further cultured at 30°C for 6 h, harvested, resuspended in MTPBS buffer (150 mM NaCl, 16 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mg/ml lysozyme, 1% Triton X-100, 0.05 mM PMSF, 1 mM dithiothreitol [DTT]), and sonicated 30 s for five times. Insoluble debris was removed by centrifugation at 3000 ×g for 30 min at 4°C. The cell lysates were combined with 50% slurry of glutathione-agarose resin and then incubated for 30 min at 4°C. The resin was washed with PBS five times. Bound GST fusion protein was eluted using elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0).

### In Vitro Kinase Assay for ERK

ERK1 (HA-tagged) was expressed in COS7 cells, and one set of cells was treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 10 min. The other set was left untreated. ERK1 was immunoprecipitated from either set of cells using anti-HA antibody and protein A-agarose beads. Purified GST-ShcA66 or GST-ShcA66(S36A) protein

(3  $\mu$ g each) was mixed with protein A beads bound-ERK in 30  $\mu$ l kinase assay buffer (25 mM Tris-HCl, pH 7.4, 5 mM MgCl<sub>2</sub>, 0.5 mM EGTA, 1 mM DTT, 20  $\mu$ M ATP, 20  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP, 0.1 mM sodium orthovanadate) and incubated at room temperature for 20 min. The reaction was terminated by addition of 5 $\times$  SDS-PAGE sample buffer and boiling at 100°C for 5 min. Proteins were analyzed by SDS-PAGE, and the phosphorylated form of GST-ShcA66 or GST-ShcA66(S36A) was detected by auto-radiography.

### RT-PCR

To determine the mRNA levels of p27, we isolated total RNA from cells using Trizol. Reverse transcription reactions were carried out with 5  $\mu$ g of total RNA following the standard protocol supplied with the reverse transcriptase (Roche, Indianapolis, IN). The resulting cDNA was used for PCR and actin was used as a control. The primers used are: 5'-GTCAAACGTGAGAGTGTCTAAC-3'; 5'-GTTTACGTCTGGCGTCGAAGGC-3'. The primers for actin were as follows: forward, 5'-AGATGTGGATCAGCAAGCAG-3'; reverse, 5'-GCCAAGTTAGTGTTCATCA-3'.

### Cell Cycle and Cell Viability Analysis

Cells were serum-starved overnight, treated with increasing concentrations of H<sub>2</sub>O<sub>2</sub> for 24 h, trypsinized and fixed in 70% ethanol, and washed and analyzed by flow cytometry after PI staining. To measure cell death rates, cells were cultured in 96-well plates at 1  $\times$  10<sup>4</sup>/well, serum-starved for 24 h, and then treated with different concentrations of H<sub>2</sub>O<sub>2</sub> for 4 h. Cell proliferation reagent WST-1 (Roche) was added to each well, and the cells were incubated for 1 more hour at 37°C. The absorbance was measured against a background control by microplates (ELISA) reader at 430 nm. The reference wavelength is 650 nm. Trypan blue exclusion assays were used to confirm the results obtained from this quantitative assay.

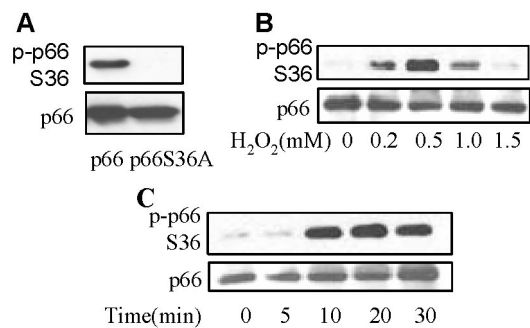
## RESULTS

### H<sub>2</sub>O<sub>2</sub>-induced Ser36 Phosphorylation of p66shcA

Phosphorylation of p66shcA at Ser/Thr residues has been reported in cells exposed to H<sub>2</sub>O<sub>2</sub>, UV, taxol, or endothelin (Yang and Horwitz, 2000; Le *et al.*, 2001; Foschi *et al.*, 2001). In these studies, a retarded migration of p66shcA on a Western blot was used as an indicator for Ser/Thr phosphorylation. It was discovered that H<sub>2</sub>O<sub>2</sub>-induced p66shcA phosphorylation occurred on Ser36 (Migliaccio *et al.*, 1999). Because p66shcA can be phosphorylated at multiple sites: Ser36, Ser138, and tyrosine residues on the CH1 domain (Faisal *et al.*, 2002), migration retardation may not necessarily represent phosphorylation at Ser36 (Supplementary Data S1). Instead we used a recently developed monoclonal antibody that specifically recognizes Ser36 phosphorylated p66shcA throughout our studies (Figure 1A). A dosage course study showed that as little as 0.2 mM of H<sub>2</sub>O<sub>2</sub> could induce Ser36 phosphorylation in serum-starved cells (Figure 1B). Ser36 phosphorylation reached the maximal level at 0.5 mM and then declined with higher doses of H<sub>2</sub>O<sub>2</sub>. A time course study revealed that Ser36 phosphorylation (close to the maximal level) occurred at 10 min after stimulation with 0.5 mM H<sub>2</sub>O<sub>2</sub> (Figure 1C).

### ERK1/2 Activation Was Necessary and Sufficient for Phosphorylation of p66shcA on Ser36

To identify the kinase responsible for p66shcA phosphorylation on Ser36, which is critical for its function in response to H<sub>2</sub>O<sub>2</sub>, we first tested MAPKs because JNK has been found necessary for Ser/Thr phosphorylation of p66shcA in human neuroblastoma cell line in response to UV irradiation (Le *et al.*, 2001), and ERK has been implicated in p66shcA phosphorylation in response to endothelin or taxol (Yang and Horwitz, 2000; Foschi *et al.*, 2001). As expected, JNKs were activated upon H<sub>2</sub>O<sub>2</sub> treatment in NIH3T3 cells (unpublished data). Yet pretreatment with 20  $\mu$ M of JNK specific inhibitor (L- or D-form) for 1 h did not affect H<sub>2</sub>O<sub>2</sub>-induced Ser36 phosphorylation (unpublished data). The JNK inhibitor, at 20  $\mu$ M, is sufficient to inhibit JNK activity (Le *et al.*, 2001). Furthermore, immortalized MEFs derived



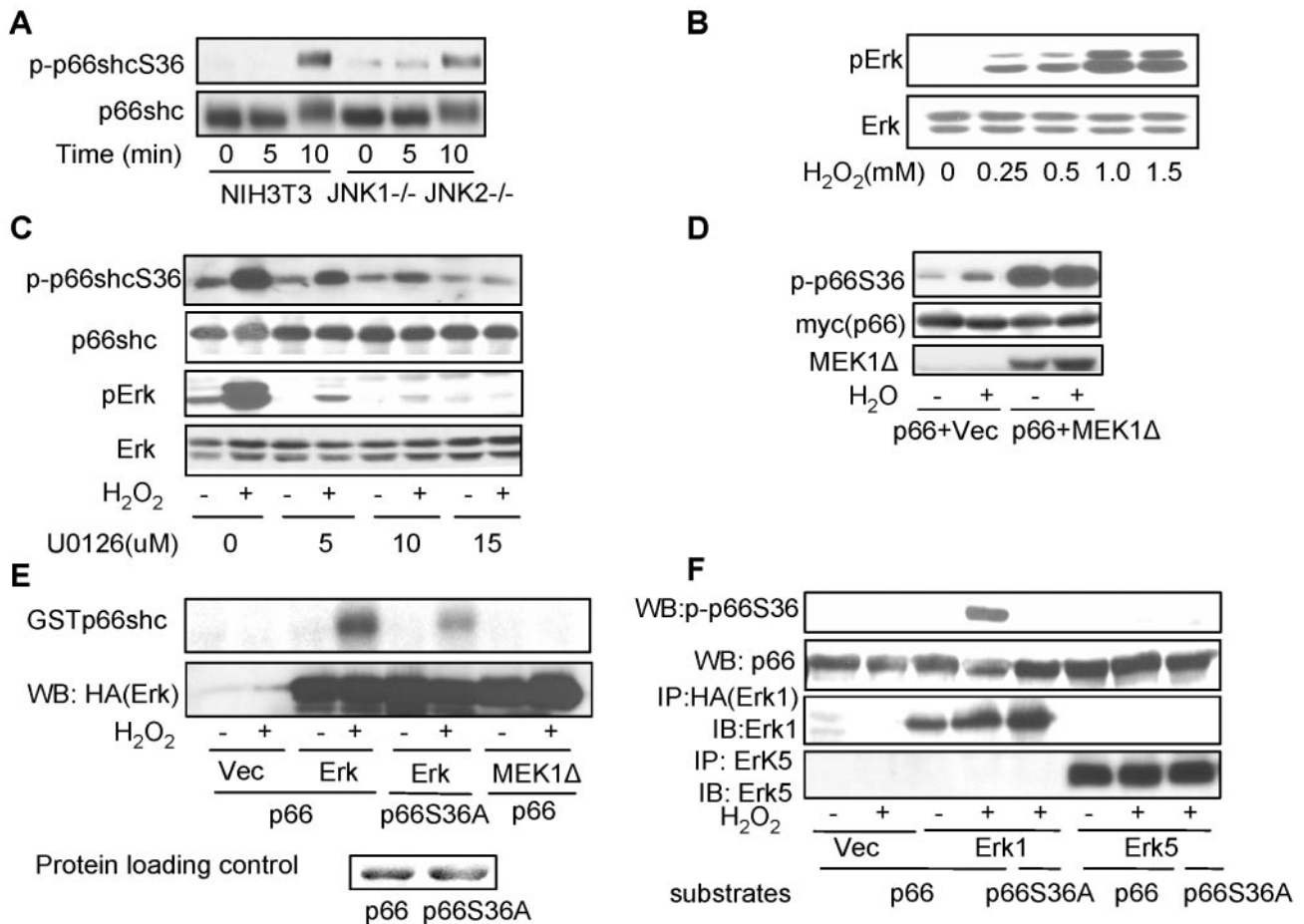
**Figure 1.** H<sub>2</sub>O<sub>2</sub> treatment induced phosphorylation of Ser36 of p66shcA. (A) Ser36 phosphorylation can be specifically recognized by the anti-phospho-Ser36 antibodies. NIH3T3 cells were transfected with constructs expressing p66shcA or mutant p66shcA (S36A), serum-starved, and then stimulated with H<sub>2</sub>O<sub>2</sub> for 10 min. Normal and mutant p66shcA proteins were immunoprecipitated and analyzed by Western blot using specific antibodies. (B) Dosage studies of phosphorylation of p66shcA on Ser36 and tyrosine phosphorylation. NIH3T3 cells were treated with increasing concentrations of H<sub>2</sub>O<sub>2</sub> for 10 min, collected, and lysed. p66shcA proteins were immunoprecipitated with anti-p66shcA antibodies. Antibodies against Ser36 phosphorylated p66shcA were used to detect Ser36 phosphorylation. (C) Cells were treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for different periods of time. p66shcA was immunoprecipitated and Ser36 phosphorylation was determined as in B.

from mice deficient for both JNK1 and JNK2 was compared with NIH3T3 cells and we found that phosphorylation of p66shcA on Ser36 was not significantly inhibited in the absence of JNK1/2 (Figure 2A). Because JNK3 is not expressed in MEFs (Tournier *et al.*, 2000), we conclude that activation of JNKs is not an absolute requirement for H<sub>2</sub>O<sub>2</sub>-induced phosphorylation of Ser36. We observed that the knockout cells showed a slight increase in the basal level of phosphorylated p66shcA. This may reflect a compensation mechanism occurred to cells deficient for all three JNKs, e.g., inactivation of phosphatases involved in terminating JNK signaling. Similarly, inhibition of p38 MAPK activation by SB25083 showed no effect on Ser36 phosphorylation (unpublished data).

We next tested whether ERKs, which were also activated by H<sub>2</sub>O<sub>2</sub> (Figure 2B), played a role in phosphorylation of p66shcA on Ser36 in response to oxidative stress. We used a widely used inhibitor U0126 to inhibit MEK1, a kinase that directly phosphorylates and activates ERK1/2. We found that Ser36 phosphorylation of p66shcA was inhibited in a dosage-dependent manner when cells were pretreated with U0126 (Figure 2C). ERK1/2 activation was indeed suppressed by the inhibitor in a dosage-dependent manner, suggesting that ERK1/2 activation was required for Ser36 phosphorylation.

To further confirm this conclusion, a constitutively active form of MEK1 was coexpressed with p66shcA and phosphorylation of Ser36 was tested on immunoprecipitated p66shcA (Mansour *et al.*, 1994). It was found that activated MEK1 led to Ser36 phosphorylation even without H<sub>2</sub>O<sub>2</sub> treatment and to much higher levels (Figure 2D), suggesting that ERK activation was sufficient for Ser36 phosphorylation.

To demonstrate that p66shcA is a substrate for ERKs, an *in vitro* kinase assay was carried out. p66shcA was fused to GST and the fusion protein was expressed and purified. A mutant GST-p66shcA, Ser36 to Ala (S36A), was also generated by site-directed mutagenesis, expressed in bacteria, and

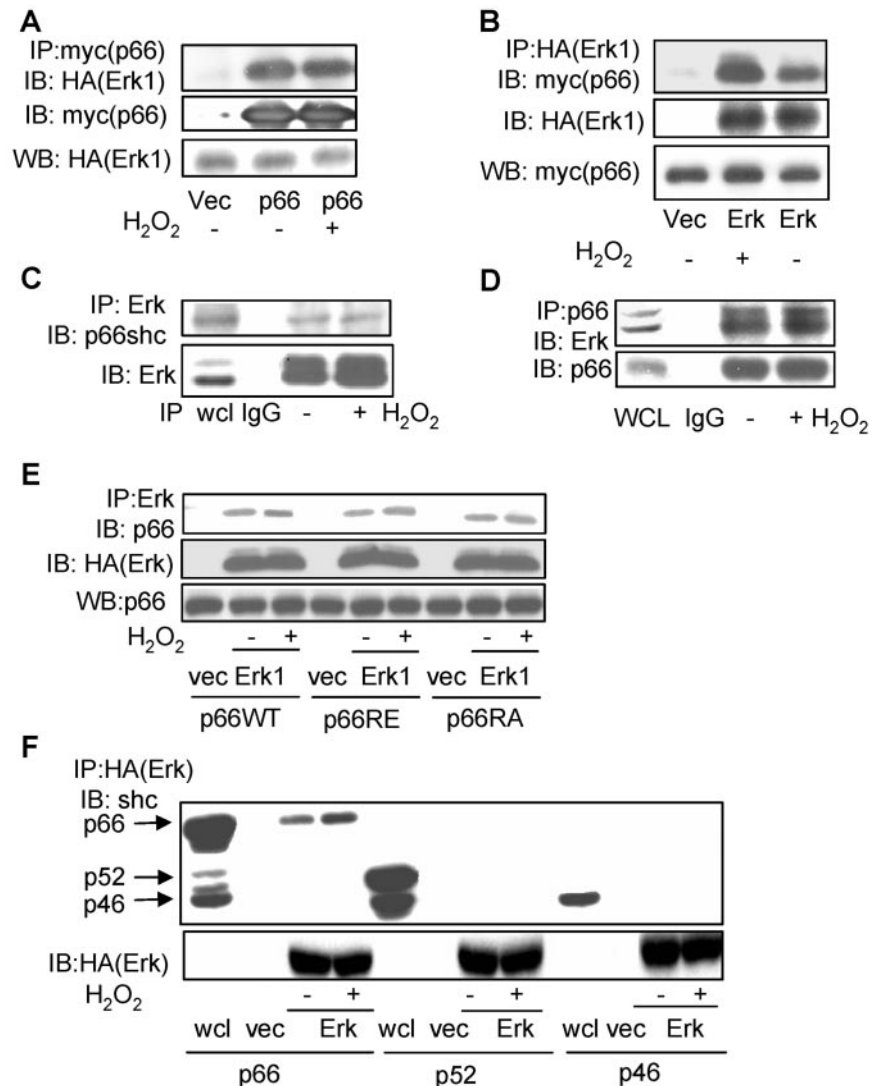


**Figure 2.** Activation of ERK, but not JNKs, was sufficient and required for phosphorylation of p66shcA on Ser36. (A) H<sub>2</sub>O<sub>2</sub>-induced p66shcA phosphorylation on Ser36 was not reduced by JNK1/JNK2 deficiency. NIH3T3 and immortalized MEFs isolated from *Jnk1*<sup>-/-</sup> *Jnk2*<sup>-/-</sup> mice were starved and treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 5 or 10 min. Endogenous p66shcA was immunoprecipitated, and its phosphorylation on Ser36 was analyzed by Western blot. (B) H<sub>2</sub>O<sub>2</sub> treatment led to ERK activation in a dosage-dependent manner. Cells were treated with increasing concentrations of H<sub>2</sub>O<sub>2</sub> for 10 min, and ERK activation was determined by Western blot using antibodies specifically recognizing activated ERKs. (C) Inhibition of ERK MAPKs with a specific inhibitor U0126 suppressed Ser36 phosphorylation. NIH3T3 cells were pretreated with U0126 (5, 10, 15 μM) for 1 h before H<sub>2</sub>O<sub>2</sub> was added. Phosphorylation of p66shcA on Ser36 was determined by Western blot analysis. (D) Coexpression of the constitutively active MEK1 (MEK1Δ) led to massive phosphorylation of p66shcA on Ser36. The mutant form of MEK1Δ and p66shcA were used to transfect NIH3T3 cells, which were serum-starved for 20 h and stimulated with H<sub>2</sub>O<sub>2</sub> for 10 min. p66shcA was immunoprecipitated and its Ser36 phosphorylation was determined by Western blot. (E) ERK1 was able to phosphorylate p66shcA in vitro in a Ser36-dependent manner. ERK1 and MEK1Δ were immunoprecipitated from COS7 cells treated or untreated with H<sub>2</sub>O<sub>2</sub> and in vitro kinase assays were performed using GST-p66shcA and mutant GST-p66shcA(S36A) as the substrate. (F) ERK1, but not ERK5, was able to phosphorylate p66shcA at Ser36. The experiment was carried out as in E, except that cold ATP was used and the phosphorylation was analyzed by Western blot using the specific anti-phospho-p66shcA antibodies.

purified. ERK1 was expressed in COS7 cells and immunoprecipitated. The kinase assay was carried out using p66shcA or p66shcA (S36A) as a substrate. It was observed that activated ERK1 but not activated MEK1, which were isolated from H<sub>2</sub>O<sub>2</sub>-treated cells, was able to phosphorylate p66shcA (Figure 2E). Phosphorylation of p66shcA (S36A) was considerably less, indicating that Ser36 was a major site for ERK1. Furthermore, Western blot analysis with the specific anti-phospho-p66shcA antibodies confirmed that the Ser36 was indeed phosphorylated in the in vitro assay (Figure 2F). In the same settings, ERK5, another member of the MAPK family that can be activated by oxidative stress (Abe *et al.*, 1996), failed to phosphorylate p66shcA at Ser36 (Figure 2F). The results indicate that p66shcA is a bona fide substrate for ERK1/2, but not for ERK5. In the p66shcA protein sequence, Ser36 is followed by Pro37, constituting a MAP kinase recognition motif (Kolch, 2000; Sharrocks *et al.*, 2000).

#### ERK1 Formed a Complex with p66shcA

Having shown that in response to H<sub>2</sub>O<sub>2</sub> treatment, ERK1/2 phosphorylated p66shcA on Ser36, we next questioned whether ERKs physically interact with p66shcA. To test whether ERK1 and p66shcA interact, we first used COS7 cells to do co-IP assays. COS7 cells were transfected with constructs expressing either HA-tagged ERK1, or ERK1 and myc-tagged p66shcA. Ectopically expressed p66shcA, along with its associated proteins, was precipitated with anti-myc antibodies, and fractionated onto an 8% SDS-PAGE gel. ERK1 was detected with anti-HA antibodies. We found that ERK1 (~20% of expressed ERKs) coprecipitated with p66shcA, but was not pulled down when p66shcA was not expressed (Figure 3A). Treatment of COS7 cells with H<sub>2</sub>O<sub>2</sub> for 10 min did not increase the formation of ShcA-ERK1 complex. Reciprocally, COS7 cells were transfected with

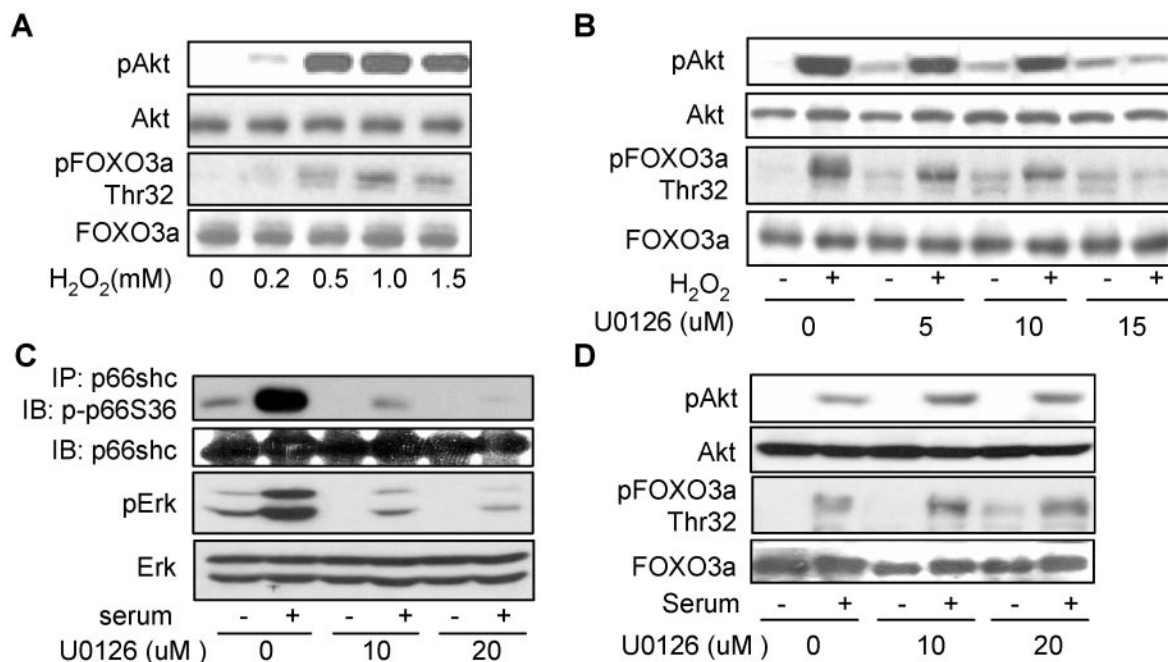


**Figure 3.** Interaction between ERK1 and p66shcA. p66shcA (myc tagged) and ERK1 (HA tagged) were coexpressed in COS7 cells and co-IP experiments were carried out. (A) p66shcA and associated proteins were precipitated with anti-myc antibody, and ERK1 was detected with anti-HA. Cells transfected with DNA expressing ERK1 alone were used as control. (B) ERK1 and associated proteins were precipitated with anti-HA, and p66shcA was detected with anti-myc. Cells transfected with p66shcA alone were used as a control. (C) Co-IP of endogenous p66shcA with ERK1. Cell lysates were incubated with anti-ERK1 antibodies conjugated with agarose beads overnight, washed with lysis buffer, and analyzed by Western blot using anti-p66shcA antibodies. Rabbit IgG conjugated to agarose beads was used as a control in the assays. One twentieth of the cell lysate used for IP experiment was loaded as a control. (D) Co-IP of endogenous ERK1 with p66shcA. Cell lysates were incubated with anti-p66shcA antibodies and protein A beads, washed with lysis buffer, and analyzed by Western blot using anti-ERK1 antibodies. Rabbit IgG was used as a control in the assays. One twentieth of the cell lysate used for IP experiment was loaded as a control. (E) Mutations of putative docking sites of SH2 domain did not affect the interaction between ERK and p66shcA. The experiments were carried out as in B, except that mutant p66shcA proteins were used as well (RR to AA, p66RA; RR to EE, p66RE). (F) p52shcA or p46shcA did not interact with ERK1. The experiments were carried out as described in B.

DNA expressing p66shcA, or DNA expressing p66shcA and ERK1. ERK1, along with its associated proteins, was precipitated from cell lysate with anti-HA antibodies and fractionated on 8% SDS-PAGE gel. p66shcA was detected with anti-myc antibodies by Western blot. p66shcA (~15% of the expressed p66shcA) was pulled down from the lysate by ERK1, but was not pulled down with anti-HA antibodies when ERK1 was not coexpressed (Figure 3B). Treatment of COS7 cells with H<sub>2</sub>O<sub>2</sub> for 10 min slightly increased formation of ShcA-ERK1 complex. Together these results indicate that p66shcA interacts with ERK1 and formation of a complex may facilitate p66shcA phosphorylation by ERK1. Furthermore, co-IP assays confirmed the interaction between endogenous p66shcA and ERKs (Figure 3C), as p66shcA was precipitated by anti-ERK antibodies conjugated to agarose beads. Similarly, ERK1/2 could be precipitated with anti-p66shcA antibodies (Figure 3D). The fact that H<sub>2</sub>O<sub>2</sub> was not found to increase ERK-p66shcA complex formation consistently could be due to technical difficulties, especially with proteins like p66shcA that is expressed at very low levels and have many interacting partners (see below). Alternatively, it could be that the increase in complex formation is mediated by phosphorylation, which is removed quickly in the signaling cascade. For example, tyrosine phosphoryla-

tion of ShcA proteins reached the maximal level after 5 min of stimulation with H<sub>2</sub>O<sub>2</sub> and started to decline after 10 min (see below).

Alignment of ShcA against the consensus MAPK docking domains revealed that ShcA proteins do have a docking site in the SH2 domain (479 LQGEFWFHGKLSRREAEALLQL 500; residues in bold are highly conserved). Although no canonical ERK binding site (Phe-Xaa-Phe-Pro) was found, the CH2 domain of p66shcA did contain a Phe-Phe-Promotif (24 aa downstream of Ser36). Together with Ser36-Pro37, it may constitute an ERK docking site (reviewed in Kolch, 2000; Sharrocks *et al.*, 2000). To determine whether this putative docking site was required for the interaction between ERK and p66shcA, we generated two point mutations in the docking sites of p66shcA (Arg491Arg492 to AlaAla and ArgArg to GluGlu) and tested their influence on ERK-p66shcA interaction in co-IP experiments. It was found that these two mutants could still bind to ERK in a manner similar to that of the wild-type p66shcA (Figure 3E). Furthermore, we found that neither p52 nor p46shcA, which contains the docking site, was able to interact with ERK in the co-IP experiments, suggesting that this docking site is not important in this interaction (Figure 3F). Rather, these



**Figure 4.** Phosphorylation of FOXO3a on Thr32 induced by H<sub>2</sub>O<sub>2</sub> involved ERKs activation. (A) H<sub>2</sub>O<sub>2</sub> treatment led to Akt activation and FOXO3a phosphorylation on Thr32. Cells were treated with increasing concentrations of H<sub>2</sub>O<sub>2</sub> for 10 min. Akt activation and FOXO3a phosphorylation were analyzed by Western blot using anti-activated Akt antibodies and anti-phospho-FOXO3a antibodies, respectively. (B) Inhibition of ERK activation compromised H<sub>2</sub>O<sub>2</sub>-induced Akt activation and FOXO3a phosphorylation. NIH3T3 cells were pretreated with different concentrations of U0126 before H<sub>2</sub>O<sub>2</sub> was added. Activation of Akt and phosphorylation of FOXO3a were analyzed by Western blot as described in Figure 3A. (C) Serum stimulation induced Ser36 phosphorylation required ERK activation. Cells were starved and pretreated with U0126 for 1 h before serum was added. Endogenous p66shcA was immunoprecipitated and Ser36 phosphorylation was determined by Western blot. (D) Serum-induced activation of AKT and FOXO3a phosphorylation were not affected by inhibition of ERKs activation. The experiments were done as described in B, except that 10% serum was used to treat cells.

results indicate that the CH2 domain of p66shcA plays an essential role in this interaction.

We also tested whether ERK and p66shcA colocalize in fibroblasts. COS7 cells were transfected with ERK1 and myc-tagged p66shcA, starved for 20 h, stimulated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 10 min, and fixed and stained with anti-myc antibody and anti-ERK antibodies. It appears that these two proteins colocalize in the cell, especially in the cytoplasmic region (unpublished data). A proportion of the ERK1 was also found in the nucleus, whereas no significant amount of p66shcA was detected in the nucleus. H<sub>2</sub>O<sub>2</sub> treatment did not induce nuclear accumulation/translocation of ERK1. The results indicate that p66shcA is a cytoplasmic substrate for ERKs.

#### *Inhibition of ERKs Diminished FOXO3a Phosphorylation*

p66shcA plays a proapoptotic role in response to oxidative stress, by modulating FOXO3a activity, a member of forkhead-related transcription factors (Nemoto and Finkel, 2002). The activity of FOXO3a is dependent on its Thr32 phosphorylation status: the nonphosphorylated form can enter the nucleus and is transcriptionally active, whereas the phosphorylated form remains in the cytoplasm. Furthermore, phosphorylation of FOXO proteins can also affect their DNA-binding activities and transactivation activities (Van Der Heide *et al.*, 2004). We did observe Akt activation, FOXO3a phosphorylation at Thr32, and a significant translocation of FOXO3a from the nucleus to the cytoplasm in response to H<sub>2</sub>O<sub>2</sub> (Figure 4A and Supplementary Data S2). It was also found that the majority of the FOXO3a proteins

were already in the cytoplasm, likely due to the fact that the cells were serum-starved (Supplementary Data S2). Nonetheless, these results indicate that H<sub>2</sub>O<sub>2</sub> treatment could inactivate FOXO3a via cytoplasm sequestration. Thr32 phosphorylation is a function of Akt kinase and requires Ser36 phosphorylation of p66shcA in response to oxidative stress, because H<sub>2</sub>O<sub>2</sub>-induced Thr32 phosphorylation of FOXO3a is dramatically reduced in p66shcA-deficient cells (Nemoto and Finkel, 2002). Because ERK1/2 were upstream of p66shcA and responsible for Ser36 phosphorylation, we predicted that interference with ERK1/2 would also affect the Akt activity and FOXO3a phosphorylation. As expected, H<sub>2</sub>O<sub>2</sub>-induced activation of Akt1 was inhibited by U0126 in a dosage-dependent manner, and so was phosphorylation of FOXO3a on Thr32 (Figure 4B). These results further support the conclusion that ERK1/2 act as positive regulators of p66shcA in response to oxidative stress. Comparison of Akt activation and p66shcA phosphorylation (Figure 1B vs. 4A) revealed that p66shcA phosphorylation, but not Akt activation, declined in the presence of a higher concentration of H<sub>2</sub>O<sub>2</sub>. Activation of Akt at high concentrations of H<sub>2</sub>O<sub>2</sub> may be mediated by a pathway independent of p66shcA, e.g., phosphoinositide-dependent kinase 1 (PDK1). It has been reported that PDK1 could be activated by H<sub>2</sub>O<sub>2</sub> in fibroblasts via tyrosine phosphorylation, especially at higher concentrations (>1.0 mM; Prasad *et al.*, 2000). Furthermore, inhibition of Akt activation by wortmannin abrogated the FOXO3a phosphorylation (unpublished data), confirming the observation that Akt is the major kinase responsible for FOXO phosphorylation in response to oxidative stress.

We also analyzed another member of the forkhead family, FOXO1, in response to H<sub>2</sub>O<sub>2</sub> treatment. We found that phosphorylation of FOXO1 on Thr28 (corresponding to Thr32 of FOXO3a, which can be recognized by the same antibody) was barely detectable in presence of H<sub>2</sub>O<sub>2</sub>, suggesting that Thr28 phosphorylation of FOXO1 may not be involved in oxidative stress response (unpublished data). On the contrary, phosphorylation of Ser256 on FOXO1, also a function of Akt, was strongly stimulated by H<sub>2</sub>O<sub>2</sub>. Inhibition of ERK also diminished Ser256 phosphorylation in dosage-dependent manner (unpublished data).

#### **Serum Stimulation Led to p66shcA Phosphorylation on Ser36 in an ERK-dependent Manner**

Because serum stimulation also leads to ERK activation, we tested whether it induces p66shcA phosphorylation on Ser36. NIH3T3 cells were serum-starved for 20 h and thereafter stimulated with 10% serum for 10 min. Endogenous p66shcA was immunoprecipitated, and its phosphorylation on Ser36 was analyzed by Western blot with specific antibodies. We found that serum greatly induced Ser36 phosphorylation, which was blocked by ERK inhibition (Figure 4C), suggesting that ERKs are also responsible for serum-induced Ser36 phosphorylation. As expected, Akt activation was also observed and so was the phosphorylation of FOXO3a. However, inhibition of ERK activation showed no effect on Akt activation or FOXO3a phosphorylation, suggesting that the ERK-p66shcA-Akt pathway is not a main pathway in serum-induced Akt activation (Figure 4D). The results excluded the possibility that U0126 may somehow inhibit Akt activation in an unspecific manner.

#### **H<sub>2</sub>O<sub>2</sub> Treatment Down-regulated FOXO Target Gene p27**

FOXO3a controls cell cycle, cell death, and oxidative stress response through transactivating different sets of genes (Tran *et al.*, 2003). Expression profiles of a couple of known FOXO3a target genes such as MnSOD and catalase were tested in response to H<sub>2</sub>O<sub>2</sub> treatment (Kops *et al.*, 2002). In our experimental settings, MnSOD and catalase levels did not show any significant change after H<sub>2</sub>O<sub>2</sub> exposure. Instead, H<sub>2</sub>O<sub>2</sub> exposure at 0.2–0.5 mM from 2 to 20 h was able to reduce the protein levels of p27<sup>kip1</sup> (Figure 5A and Supplementary Data S3), a cyclin-dependent kinase inhibitor and a target gene for FOXO3a (Dijkers *et al.*, 2000; Medema *et al.*, 2000; Stahl *et al.*, 2002), whereas p21 and cyclin D was not affected (unpublished data). p27 has FOXO3a-binding sites in its promoter region, and its expression requires activation of FOXO3a (Tran *et al.*, 2003). The reduction of p27 on H<sub>2</sub>O<sub>2</sub> exposure could be a consequence of inactivation of FOXO3a through the p66shcA-Akt pathway because it was accompanied by a translocation of FOXO3a from the nucleus to the cytoplasm (Supplementary Data S2). More interestingly, inhibition of ERK activation, which also compromised Akt activation and inactivation of FOXO3a, was able to suppress the down-regulation of p27 (Figure 5A). RT-PCR assays confirmed that H<sub>2</sub>O<sub>2</sub> treatment down-regulated p27 mRNA levels in several cell types tested, including NIH3T3 and MEFs, and that inhibition of ERKs abolished the down-regulation (Figure 5B). These results indicate that H<sub>2</sub>O<sub>2</sub> treatment also leads to a decrease in the levels of a CDK inhibitor and might facilitate the cellular events involving p27 and that ERK activation negatively regulates p27 expression at the mRNA levels. More importantly, MEFs lacking p66shcA did not show this down-regulation of p27 (Figure 5C), which was restored by reconstitution of p66shcA in the knockout cells (Figure 5D). But the MEFs expressing p66shcA (S36A) behaved like the knockout mutant (Figure

5D). These results indicate that p27 down-regulation is also dependent on the phosphorylation of Ser36 in p66shcA, similar to the activation of Akt and phosphorylation of FOXO3a in response to H<sub>2</sub>O<sub>2</sub> (Nemoto and Finkel, 2002).

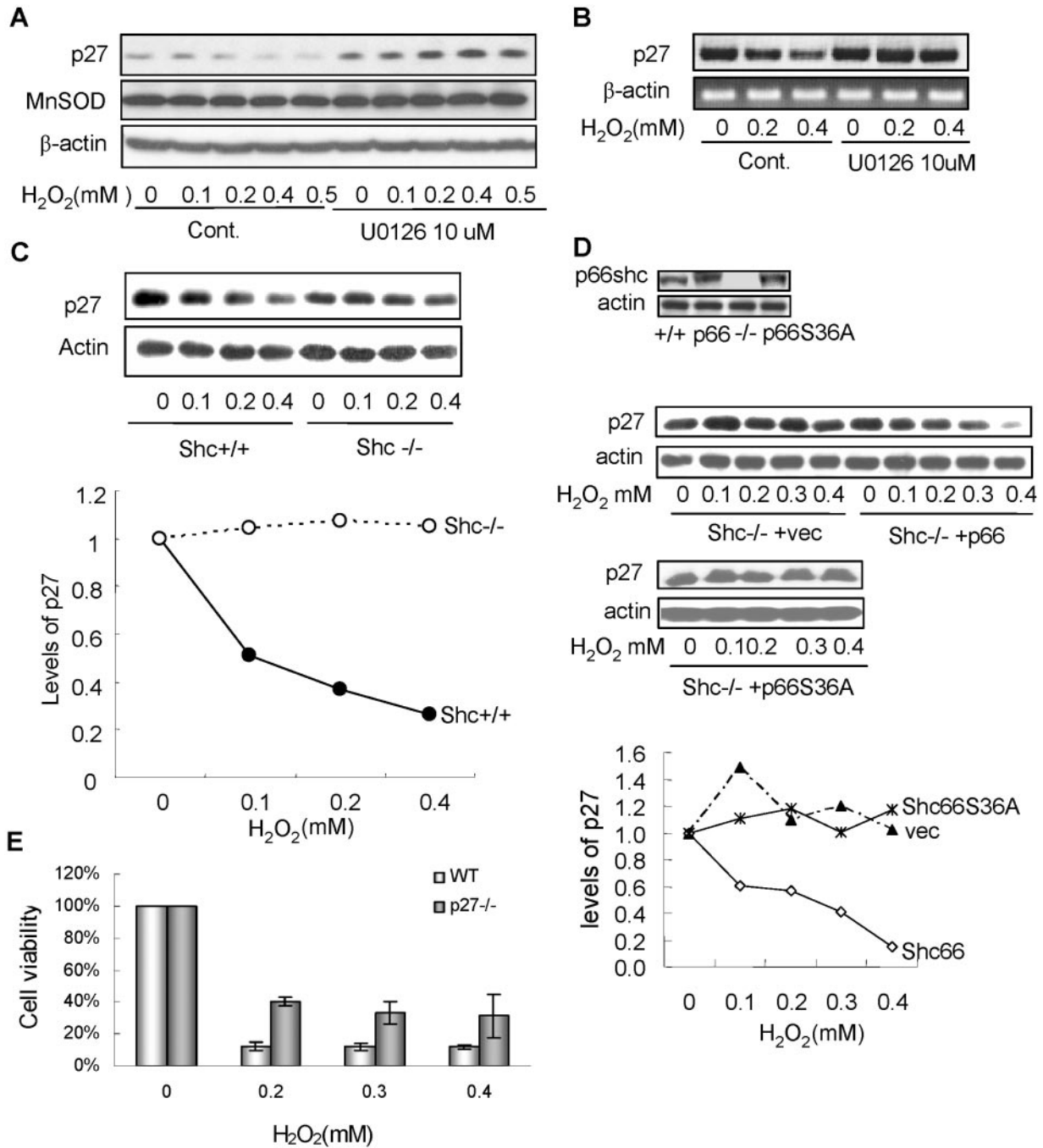
#### **Participation of p27 in Cell Responses to Oxidative Stress**

What is the biological function of reduced expression of p27 in cell response to oxidative stress? The observation that CDK inhibitor p27 was down-regulated in response to H<sub>2</sub>O<sub>2</sub> and the fact that low doses of ROS have been reported to have mitogenic effect (Reth, 2002; Mikkelsen and Wardman, 2003) prompted us to test whether H<sub>2</sub>O<sub>2</sub> could support cell proliferation in our experimental settings. Serum-starved cells were treated with increasing concentrations of H<sub>2</sub>O<sub>2</sub> for 20 h (or 2 h), and their cell cycle profiles were analyzed by FACS analysis. Only a marginal increase was observed in the percentage of cells in G<sub>2</sub>/M phase (unpublished data). Bromodeoxyuridine (BrdU) incorporation assays did not reveal an increase in the BrdU-positive cells in the presence of H<sub>2</sub>O<sub>2</sub>, suggesting that the mitogenic effect is minimal. One interpretation for failure to detect a mitogenic effect for H<sub>2</sub>O<sub>2</sub> is because H<sub>2</sub>O<sub>2</sub> also has a proapoptotic effect. Therefore, the cells have to balance life and death under this condition, and the ultimate cellular response to H<sub>2</sub>O<sub>2</sub> is apoptosis in the experimental settings. In fact, p27 is suitable for such a role because it not only regulates cell cycle progression but also apoptosis (Coqueret, 2003). p27 can be proapoptotic or antiapoptotic, depending on the cell types used and the factors used for apoptosis induction (Katayose *et al.*, 1997; Hiro-mura *et al.*, 1999; Dijkers *et al.*, 2000). To study the role for p27 in oxidative stress-induced apoptosis, MEFs were prepared from p27-deficient and control wild-type mice. These primary MEFs were challenged with different concentrations of H<sub>2</sub>O<sub>2</sub> for 5 h, and the cell death rates and cell survival rates were measured by trypan blue exclusion method and a cell proliferation/survival kit, respectively. It was found that p27<sup>-/-</sup> MEFs survived better than control wild-type cells in both assays (Figure 5E, unpublished data for trypan blue exclusion method). These results indicate that p27 possesses a proapoptotic function in oxidative stress response. Therefore, down-regulation of p27 by H<sub>2</sub>O<sub>2</sub> might normally protect the cells against oxidative stress. A similar role for p27 and FOXO3a has been found in cytokine-mediated survival of hematopoietic cells (Dijkers *et al.*, 2000).

#### **Tyrosine Phosphorylation of p52/46shcA and Their Role in H<sub>2</sub>O<sub>2</sub>-induced ERK Activation**

It is known that ShcA proteins regulate ERK activation in response to growth factors. Having shown that ERK could phosphorylate p66shcA in the presence of H<sub>2</sub>O<sub>2</sub>, we intended to study whether ShcA proteins have an influence on ERK activation in cell response to oxidative stress. During the studies of Ser36 phosphorylation, it was found that H<sub>2</sub>O<sub>2</sub> also induced tyrosine phosphorylation of p66shcA, as well as p52 and p46shcA (Figure 6A). For all three isoforms, tyrosine phosphorylation was detectable at 0.2 mM and reached the maximal level at 1.0 mM H<sub>2</sub>O<sub>2</sub>. A time course study revealed that tyrosine phosphorylation of p66shcA occurred at 5 min after treatment with 0.5 mM H<sub>2</sub>O<sub>2</sub> (Figure 6B), indicating that tyrosine phosphorylation precedes Ser36 phosphorylation (Figure 1C). Hence, H<sub>2</sub>O<sub>2</sub> treatment leads to successive phosphorylation of all three ShcA proteins on tyrosine residues and of p66shcA on Ser36 in murine fibroblasts.

What is the function of ShcA tyrosine phosphorylation in response to H<sub>2</sub>O<sub>2</sub>? We first tried to determine whether these

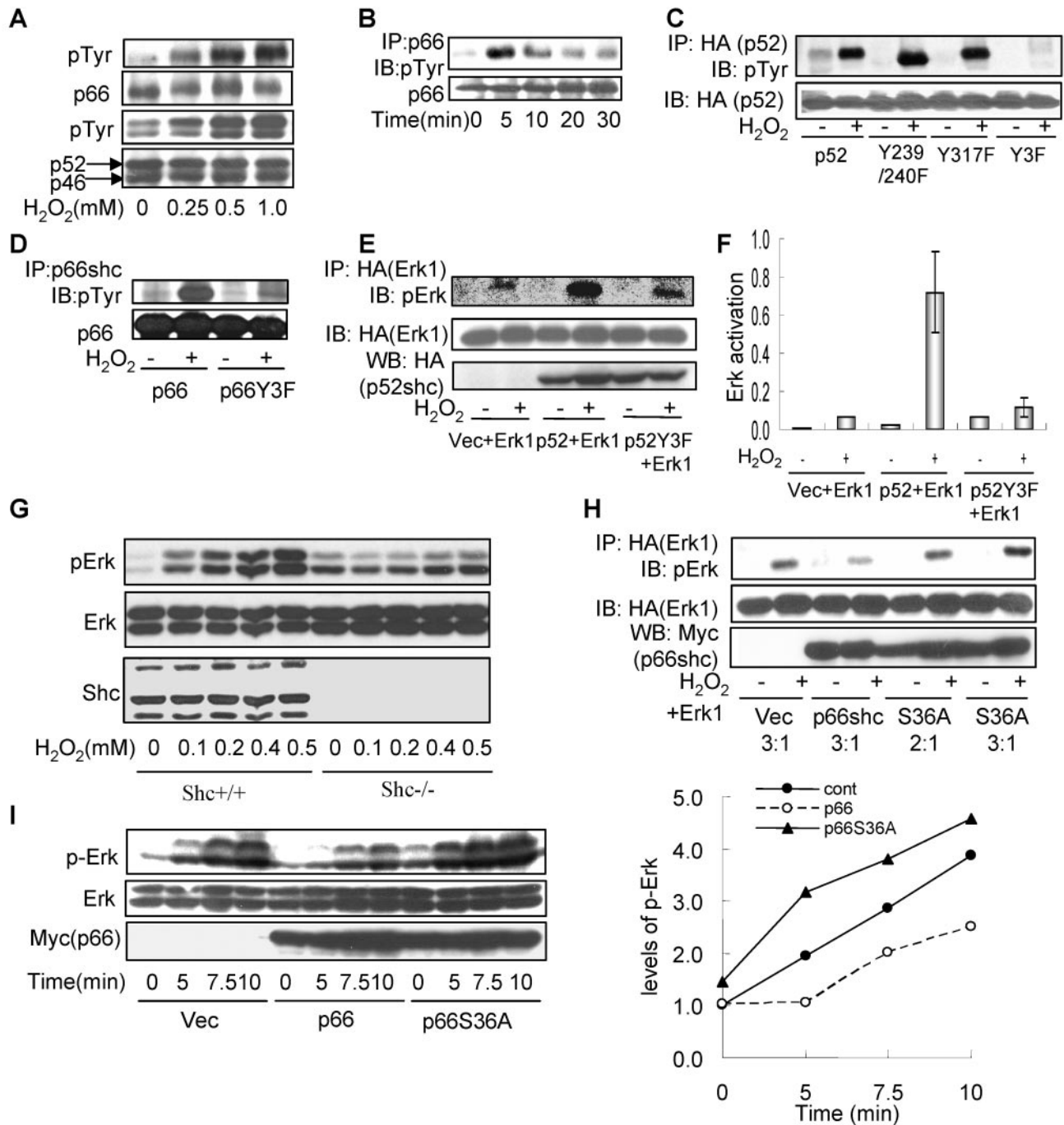


**Figure 5.** H<sub>2</sub>O<sub>2</sub> treatment down-regulated expression of p27 and its role in oxidative stress response. (A) H<sub>2</sub>O<sub>2</sub> treatment down-regulated expression of p27 without affecting MnSOD. Cells were treated with 0.1–0.5 mM of H<sub>2</sub>O<sub>2</sub> for 20 h. The levels of p27 were determined by Western blot. Inhibition of ERKs was found to rescue down-regulation of p27. (B) RT-PCR experiments showed that H<sub>2</sub>O<sub>2</sub> treatment down-regulated expression of p27 at mRNA levels. Notice that the p27 is a negative image of the gel. The experiments were done as in A. Total RNA was isolated from the cells and RT-PCR was performed using a commercial kit. (C) *ShcA*<sup>-/-</sup> MEFs showed no down-regulation of p27 in response to H<sub>2</sub>O<sub>2</sub>. *ShcA*<sup>-/-</sup> and control MEFs were treated with different concentrations of H<sub>2</sub>O<sub>2</sub> and the levels of p27 were determined by Western blot analysis. Bottom panel: quantitation data. (D) Ser36 of p66shcA played an important role in down-regulation of p27. *ShcA*<sup>-/-</sup> MEFs expressing p66shcA, p66shcA(S36A), or empty vector (top panel) were treated with different concentrations of H<sub>2</sub>O<sub>2</sub> and the levels of p27 were determined by Western blot analysis. Bottom panel: quantitation data. (E) p27-deficient MEFs showed an improved survival than control wild-type cells. Primary mutant and wild-type MEFs were challenged with different concentrations of H<sub>2</sub>O<sub>2</sub> for 5 h, and the cell survival rates were determined as described in *Materials and Methods*.

phosphorylated residues are the same as those induced by growth factors. Because the levels of phosphorylation of ShcA isoforms are proportional to their protein levels, we

assume that the tyrosine residue(s) might be shared by three ShcA isoforms and we used the p52shcA to identify the tyrosine residues. NIH3T3 cells were transfected with con-





**Figure 6.** H<sub>2</sub>O<sub>2</sub>-induced Y239/240/317 phosphorylation of p46/52shcA facilitated ERK activation. (A) Dosage studies of tyrosine phosphorylation of all three ShcA isoforms in response to H<sub>2</sub>O<sub>2</sub> treatment. Cells were treated as described in Figure 1C. p66shcA and p52/46shcA were precipitated with anti-p66shcA and anti-ShcA, respectively, and tyrosine phosphorylation was detected by Western blot using anti-phospho-tyrosine antibodies. (B) Cells were treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for different periods of time; all ShcA proteins were immunoprecipitated using anti-ShcA antibodies and analyzed by Western blot using anti-phospho-tyrosine antibodies. (C) Point mutation analysis to identify the tyrosine residues that was phosphorylated in the presence of H<sub>2</sub>O<sub>2</sub>. p52shcA carrying mutations of Y239/240F, Y317F, or Y239/240/317F (Y3F) were expressed in NIH3T3 cells (with or without H<sub>2</sub>O<sub>2</sub> treatment), and tyrosine phosphorylation was determined as described in A. (D) p66shcA shared the phosphorylated tyrosine residues with p46shcA. NIH3T3 cells were transfected with p66shcA or p66shcA (Y3F) for 24 h, serum-starved for overnight, and stimulated with H<sub>2</sub>O<sub>2</sub> for 10 min. p66shcA proteins were immunoprecipitated and the tyrosine phosphorylation was detected using anti-phospho-tyrosine antibodies. (E) ERK activation by H<sub>2</sub>O<sub>2</sub> required tyrosine phosphorylation of p52/46shcA. NIH3T3 cells were transfected with HA-tagged ERK1 and p52shcA, p52shcA(Y3F), or vector, serum-starved for 20 h, stimulated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 10 min. ERK1 was immunoprecipitated and its activation was determined by Western blot using antibodies that specifically recognized activated ERK. The blot was stripped and reblotted with anti-HA antibodies. Western blot was carried out to check the expression of ShcA and activation of ERK. (F) Quantitation data from three repeated experiments. (G) Activation of ERKs by H<sub>2</sub>O<sub>2</sub> was diminished in ShcA<sup>-/-</sup> MEFs. Mutant and control MEFs were treated H<sub>2</sub>O<sub>2</sub> with for different periods of time and activation of ERKs was determined by Western blot analysis. (H) Coexpression of p66shcA slightly inhibited ERK activation in a Ser36-dependent manner. (I) Time-course phosphorylation of p66shcA and ERK.

structs expressing various fragments of p46shcA (HA tagged) and stressed with H<sub>2</sub>O<sub>2</sub> for 10 min. ShcA fragments were immunoprecipitated with anti-HA antibodies and analyzed by Western blot using anti-phospho-tyrosine antibodies. We found that tyrosine phosphorylation only occurred to CH1 (unpublished data), the domain for tyrosine phosphorylation upon a variety of stimuli, e.g., platelet-derived growth factor receptor (Y239/240) and Src kinase (Y317; van der Geer *et al.*, 1996; Blake *et al.*, 2000). We then made point mutations at Y239/240/F, Y317/F, and Y239/240/317F by site-directed mutagenesis in p52shcA. NIH3T3 cells were transfected with DNA expressing the mutant p52shcA and subsequently treated with H<sub>2</sub>O<sub>2</sub> for 10 min. Tyrosine phosphorylation of these proteins was analyzed by Western blot using anti-phospho-tyrosine antibodies (Figure 6C). Neither Y239/240/F nor Y317/F mutations significantly affected the phosphorylation, whereas triple mutations completely abolished tyrosine phosphorylation, indicating that all three sites were phosphorylated upon H<sub>2</sub>O<sub>2</sub> treatment. We then swapped the triple mutations into p66shcA and found that they were the only tyrosine residues phosphorylated by H<sub>2</sub>O<sub>2</sub> treatment (Figure 6D). We showed, for the first time, that H<sub>2</sub>O<sub>2</sub> treatment led to phosphorylation of all three tyrosine residues in all three ShcA isoforms. Although Src kinase and PDGFR could phosphorylate these residues, we found that pretreated NIH3T3 with inhibitors for Src, PDGFR, or a combination of the two did not significantly affect H<sub>2</sub>O<sub>2</sub>-induced tyrosine phosphorylation of ShcA proteins (unpublished data). Furthermore, Src<sup>-/-</sup> MEF cells showed normal tyrosine phosphorylation of all three ShcA isoforms even in the presence of PDGFR inhibitor (unpublished data), suggesting that neither Src nor PDGFR is required for ShcA tyrosine phosphorylation or that there exist functionally redundant tyrosine kinases in the cell.

Tyrosine phosphorylation has been involved in growth factor induced ERK activation. Like growth factors, H<sub>2</sub>O<sub>2</sub> can activate ERK1/2 in a dosage-dependent manner (Rao, 1996). Similarly, we found that in NIH3T3 cells, 0.25 mM of H<sub>2</sub>O<sub>2</sub> could significantly activate ERK1/2 and this was accompanied by increased complex formation between ShcA and Grb2 (unpublished data). It has been reported that Src plays important roles in H<sub>2</sub>O<sub>2</sub>-induced ERK activation (Aikawa *et al.*, 1997). We tested this conclusion using the Src<sup>-/-</sup> and control MEFs and found that Src was not required for ERK activation (unpublished data), suggesting that redundant kinases may indeed exist in the cell. The results are in agreement with our finding that Src deficiency did not alter ShcA tyrosine phosphorylation.

To determine whether tyrosine phosphorylation of ShcA plays a role in ERK activation, NIH3T3 cells were transfected with HA-tagged ERK1, in combination with p52shcA or p52shcA (Y239/240/317F), serum-starved for 20 h, and treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 10 min. ERK1 was immunoprecipitated using polyclonal anti-HA antibodies, and activated ERK1 was detected with anti-phosphorylated ERK antibodies by Western blot. Expression of normal p52shcA was able to enhance activation of ERK1 in response to H<sub>2</sub>O<sub>2</sub>

(Figure 6, E and F). On the other hand, expression of the mutant p52shcA (Y239/240/317F) failed to do so (Figure 6, E and F), suggesting that tyrosine phosphorylation of ShcA is necessary for activation of ERKs in response to H<sub>2</sub>O<sub>2</sub>. The triple mutations (Y239/240/317F) should not affect the folding of ShcA proteins, because p66shcA carrying the triple mutations was similarly phosphorylated at Ser36 upon H<sub>2</sub>O<sub>2</sub> treatment and that p66shcA carrying these mutations still possessed its ability to physically interact with its partner p52shcA (Supplementary Data S3 and S4). To further confirm that ShcA proteins are involved in ERK activation in response to H<sub>2</sub>O<sub>2</sub>, ShcA<sup>-/-</sup> fibroblasts were challenged with different concentrations of H<sub>2</sub>O<sub>2</sub>, and ERK activation was assessed by Western blot analysis. It was found that ShcA<sup>-/-</sup> fibroblasts showed compromised ERK activation (Figure 6G), although the basal level of activated ERKs was slightly higher in the ShcA-deficient cells. A similar defect in ERK activation in response to low concentrations of growth factors has been reported in ShcA<sup>-/-</sup> fibroblasts (Lai and Pawson, 2000). Taken together, our data indicate that H<sub>2</sub>O<sub>2</sub> treatment results in tyrosine phosphorylation of p52/46shcA, which in turn facilitates H<sub>2</sub>O<sub>2</sub>-induced ERK activation.

#### Phosphorylation of Ser36 Played a Negative Role in H<sub>2</sub>O<sub>2</sub>-induced ERK Activation

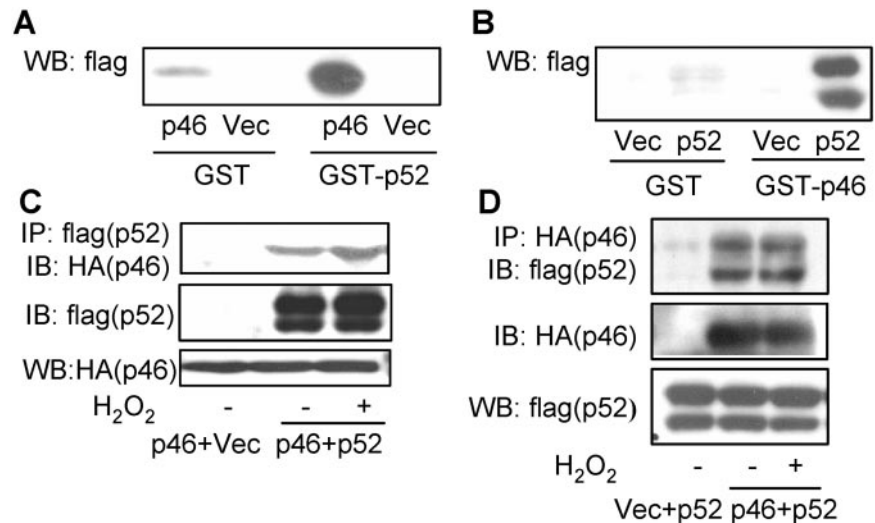
Having shown that ERK interacted with p66shcA and phosphorylated p66shcA on Ser36 and that p52/46shcA facilitated H<sub>2</sub>O<sub>2</sub>-induced ERK activation, we wanted to determine whether Ser36 phosphorylation of p66shcA played a role in H<sub>2</sub>O<sub>2</sub>-induced ERK activation. NIH3T3 cells were cotransfected with ERK1 and p66shcA or p66shcA (S36A), serum-starved for 20 h, and stimulated with H<sub>2</sub>O<sub>2</sub> for 10 min. ERK1 was precipitated with anti-HA antibodies conjugated to agarose beads, and its activation was determined by Western blot analysis. Unlike p52/46shcA, which strongly facilitate ERK activation, p66shcA was found to suppress activation of ERK (Figure 6H), consistent with the findings that p66shcA plays an inhibitory role in ERK activation stimulated by EGF (Migliaccio *et al.*, 1997; Okada *et al.*, 1997). Surprisingly, mutant p66shcA (S36A) was found to facilitate ERK activation, although only to a mild extent (Figure 6H), suggesting that the normal function of p66shcA is to negatively regulate ERK activation and that phosphorylation of Ser36 may play an important role. To confirm this conclusion, we overexpressed p66shcA, or p66shcA(S36A) in COS7 cells, which were subjected to stimulation with 0.5 mM H<sub>2</sub>O<sub>2</sub> for different periods of time and the activation of endogenous ERKs were detected by Western blot. It was found that p66shcA inhibited H<sub>2</sub>O<sub>2</sub>-induced ERK activation (Figure 6I), whereas expression of S36A mutant led to a slightly enhanced activation of ERK1/2.

#### Complex Formation among ShcA Proteins

Having shown that H<sub>2</sub>O<sub>2</sub>-induced ERK activation could be facilitated by p52/46shcA but hampered by p66shcA, we wanted to study the molecular basis for their different actions. p66shcA contains an extra 110 amino acid CH2 do-

**Figure 6 (cont).** NIH3T3 cells were transfected with HA-tagged ERK1 and p66shcA, p66shcA(S36A), or vector, serum-starved for 20 h, and stimulated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 10 min. Different amounts of DNA expressing p66shcA (S36A) was used to transfect cells to test their effects on ERK activation. ERK1 was immunoprecipitated and its activation was determined by Western blot using antibodies that only recognize activated ERK. The blot was stripped and reblotted with anti-HA antibodies. Western blot was carried out to check the expression of ShcA. (I) Time course study of inhibition of ERK activation by p66shcA. COS7 cells were transfected with empty vector or constructs expressing p66shcA or p66shcA(S36A) for 24 h, serum-starved overnight, and stimulated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for different periods of time, and ERKs activation was determined by Western blot analysis. Bottom panel: quantitation data showing the activation of ERK1 and ERK2 combined.

**Figure 7.** Interaction between p52 and p46 proteins. (A) GST-p52shcA was able to pull down p46shcA. p46shcA expressed in COS7 cells were incubated with GST-p52shcA beads overnight at 4°C. The beads were washed and p46shcA was detected on a Western blot. (B) GST-p46 was able to pull down both p52shcA and p46shcA. p52shcA and p46shcA (expressed from one mRNA) expressed in COS7 cells were incubated with GST-p46shcA beads overnight at 4°C. The beads were washed and p46shcA and p52shcA was detected by Western blot. (C) p52shcA was able to precipitate coexpressed p46shcA. COS7 cells were transfected with p52shcA (flag-tagged at C' terminus) and p46shcA (HA tagged) and treated with H<sub>2</sub>O<sub>2</sub> for 10 min. The cell lysates were incubated with anti-Flag antibodies and p46 was detected with anti-HA antibodies by Western blot. (D) p46shcA was able to precipitate the coexpressed p52shcA and p46shcA. The experiments were carried out as in C, except that the lysates were incubated with anti-HA antibodies and the Western blot was probed with anti-Flag antibodies.



main compared with p52shcA and is a minor isoform in fibroblasts. We first used pulldown assays to test whether they exist as complexes in the cell. A GST-p52shcA fusion protein was made and used in pulldown experiments against p46shcA expressed in COS7 cells. Although GST-p52shcA was able to precipitate p46shcA, GST itself failed to do so (Figure 7A). Similarly, GST-p46shcA was made and used in pulldown experiments against p52shcA. COS7 cells were transfected with p52shcA, which expressed both p52shcA and p46shcA due to two different start codons being used. We found that GST-p46 was able to precipitate both p52shcA and p46shcA, suggesting that p52-p46 and p46-p46 dimers may exist (Figure 7B). Similar experiments were carried out using GST-p46shcA fusion protein to precipitate CH1, PTB, or SH2 domains alone, and it was found that PTB and CH1 domains were able to bind p46shcA (unpublished data), suggesting that those two domains are involved in complex formation between ShcA proteins. Because p66shcA contains PTB and CH1 domains, we believe that p66shcA could also form complexes with p52shcA or p46shcA.

Interaction between p46 and p52 was confirmed by co-IP experiments. We found that p52shcA was able to immunoprecipitate coexpressed p46shcA, whereas p46 could not be precipitated when p52 was not coexpressed (Figure 7C). H<sub>2</sub>O<sub>2</sub> treatment did not significantly promote p52-p46 interaction. Reciprocally, p46shcA was able to precipitate both p52shcA and p46shcA (expressed from the same construct; Figure 7D), and H<sub>2</sub>O<sub>2</sub> treatment did not significantly enhance the complex formation. These results suggest that ShcA proteins do interact *in vivo*.

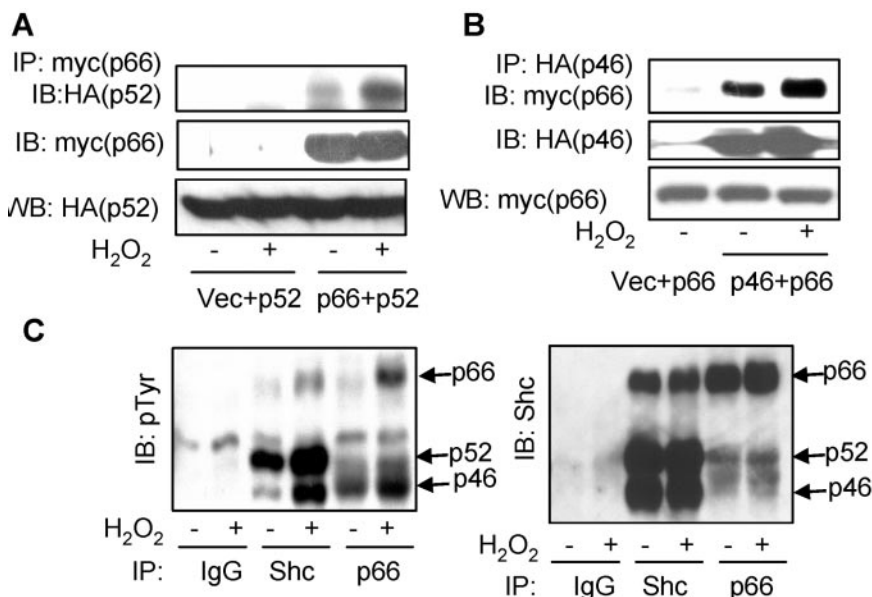
As mentioned above, p66shcA and p52/46shcA play distinct roles in ERK activation induced by growth factors or H<sub>2</sub>O<sub>2</sub> (Figure 6 and Migliaccio *et al.*, 1997). To test whether p66shcA interact with p52 or p46, we transfected COS7 cells with myc-tagged p66 and HA-tagged p52. One set was stimulated with H<sub>2</sub>O<sub>2</sub>, whereas the other set was left untreated. p66 and associated proteins were precipitated with anti-myc antibodies and fractionated onto an 8% SDS-PAGE gel. p52 was detected with anti-HA antibody on a Western blot (Figure 8A). Dimers of p66-p52 were found even when

cells were not treated, and H<sub>2</sub>O<sub>2</sub> treatment modestly enhanced this dimerization. Similar results were obtained for p66-p46 interaction (Figure 8B). Because p66 and p52/46 have different functions in ERK activation, increased dimer formation between p66shcA and p52shcA or p46shcA in response to H<sub>2</sub>O<sub>2</sub> may help the cells to attenuate signal transmission. This is consistent with our findings that tyrosine phosphorylation of p52 and p46 precedes Ser36 phosphorylation of p66.

Finally we determined whether interaction between p66shcA and p52/46shcA exists between endogenous ShcA proteins. We utilized antibodies that only recognize p66shcA but not p52/46shcA for this purpose. Theoretically, if ShcA proteins interact *in vivo*, immunoprecipitated p66shcA should be able to bring down p52/46shcA. Cells were treated with H<sub>2</sub>O<sub>2</sub> to test whether association among ShcA proteins would be enhanced under this condition. To our expectation, both p52shcA and p46shcA were found to associate with p66shcA. H<sub>2</sub>O<sub>2</sub> stimulation slightly enhanced the complex formation (Figure 8C). Because both p52shcA and p46shcA run close to the immunoglobulin heavy chain that could be recognized in a nonspecific manner by anti-ShcA antibodies (or secondary anti-rabbit antibodies), the blot was also probed with anti-phospho-tyrosine antibodies to pinpoint p52 and p46, which were tyrosine-phosphorylated in H<sub>2</sub>O<sub>2</sub>-treated cells. The results show that endogenous ShcA proteins do exist in the cells as a complex. Whether the complexes are in the form of dimer (homo or hetero) or trimer needs further investigation.

## DISCUSSION

We provide evidence to support that p66shcA is a cytoplasmic substrate for ERKs in response to H<sub>2</sub>O<sub>2</sub> or serum stimulation. Although previous studies have shown that inhibition of ERK activation inhibited the slower migration of p66shcA in response to endothelin-1 (Foschi *et al.*, 2001), our results demonstrated for the first time that p66shcA (Ser36) is a substrate of ERKs. p66shcA has a perfect MAPK recognition motif (Ser36-Pro37) in the CH2 domain. We demonstrated that p66shcA and ERK do interact *in vitro* and in



**Figure 8.** Interaction between p66shcA and p46/52shcA. (A) H<sub>2</sub>O<sub>2</sub> promoted association between p66shcA and p52shcA. Immunoprecipitation assays were done as in C. (B) H<sub>2</sub>O<sub>2</sub> promoted association between p66shcA and p46shcA. Immunoprecipitation was carried out as described in Figure 7C. (C) Endogenous p66shcA was able to precipitate endogenous p52shcA and p46shcA in a co-IP experiment. Cells were treated with H<sub>2</sub>O<sub>2</sub> for 10 min and the cell lysates were incubated with anti-p66shcA antibodies. The Western blot was probed with anti-phospho-tyrosine and anti-ShcA antibodies, respectively. Rabbit IgG was used as a control for co-IP assays.

*in vivo*. *In vitro* kinase assays confirmed that H<sub>2</sub>O<sub>2</sub>-activated ERKs phosphorylated p66shcA with Ser36 being a major site. Furthermore, ERK activation is necessary and sufficient for H<sub>2</sub>O<sub>2</sub>-induced Ser36 phosphorylation. This conclusion is further supported by the findings that inhibition of ERK activation also impeded p66shcA downstream signaling molecules such as Akt and downstream transcription factor FOXO3a in a signaling pathway triggered by H<sub>2</sub>O<sub>2</sub> (Nemoto and Finkel, 2002). Even though serum feeding also resulted in Ser36 phosphorylation in an ERK-dependent manner, inhibition of ERKs, and therefore inhibition of p66shcA phosphorylation, did not affect Akt activation or FOXO3a phosphorylation induced by serum stimulation, suggesting that the p66shcA-Akt-FOXO3a pathway is specific to H<sub>2</sub>O<sub>2</sub>, but not to growth factors. One possible explanation is that serum-induced Akt activation is mainly through RTK-PI-3 kinase pathway, whereas H<sub>2</sub>O<sub>2</sub>-induced Akt activation is mainly through p66shcA.

What cellular events does the p66shcA-Akt-FOXO pathway regulate in response to oxidative stress? Reactive oxygen species have been documented to have a proapoptotic role and under certain conditions, a mitogenic role. Meanwhile, ROS can also activate the antioxidant defense system to protect the cells. Accumulating evidence suggests that some of these effects of ROS are mediated by the FOXO transcription factors, which are known to control cell cycle, cell death, and stress detoxification by regulating transcription of different sets of genes under various conditions (Tran *et al.*, 2003). For example, H<sub>2</sub>O<sub>2</sub> has been reported to down-regulate genes involved in both H<sub>2</sub>O<sub>2</sub> scavenging and oxidative stress resistance, e.g., catalase and MnSOD, by inactivating FOXO3a through the p66shcA-Akt pathway (Nemoto and Finkel, 2002; Trinei *et al.*, 2002). In the absence of p66shcA, this pathway is disrupted and as a consequence, expression of these antioxidant proteins is maintained and p66shcA-deficient cells exhibit resistance to oxidative stress (Migliaccio *et al.*, 1999; Nemoto and Finkel, 2002; Trinei *et al.*, 2002). In this regard, the action of the p66shcA-Akt-FOXO pathway is to transduce proapoptotic signals. In contrast, FOXO proteins also control expression of genes involved in apoptotic induction such as Fas ligand and the Bcl2 family member Bim. Activation of Akt and phosphorylation of

FOXO would lead to down-regulation of these two proteins, facilitating cell survival. Furthermore, activation of Akt, a well-studied mediator of survival, can lead to inactivation of caspase 9 and the proapoptotic Bcl2 family member Bad, in a transcription-independent manner. It appears that the Akt-FOXO pathway also transduces antiapoptotic signals. In addition, oxidative stress also activates many other signaling pathways, including the protein kinase C (PKC)  $\delta$ -Nrf2 pathway, which up-regulates the levels of antioxidant proteins such as peroxiredoxin I, promoting cell survival (Li *et al.*, 2002, 2004). Therefore, all these signals would be integrated to influence the decision making as to cell death or survival in response to oxidative stress.

We discovered that p27<sup>kip1</sup>, a well-studied target gene of FOXO3a, was down-regulated by H<sub>2</sub>O<sub>2</sub>, in correlation to inactivation of FOXO3a. Furthermore, H<sub>2</sub>O<sub>2</sub>-induced Ser36 phosphorylation, Akt activation, as well as down-regulation of p27 could be suppressed by inhibition of ERK activation. This is the first time that a connection between oxidative stress and p27 expression was established. What is the biological significance of reduced expression of p27 in response to oxidative stress? p27 is a CDK inhibitor and is a negative regulator of cell cycle progression. The levels of p27 are decreased in many human cancers and growth factor-stimulated cell proliferation is associated with down-regulation of p27. In addition, previous studies showed that ectopic expression of activated FOXO3a led to cell cycle arrest in which induction of p27 through FOXO3a appeared to play an important role (Medema *et al.*, 2000). We propose that H<sub>2</sub>O<sub>2</sub>-induced down-regulation of p27 may facilitate cell cycle entry, consistent with the finding that ROS can have a mitogenic role under certain conditions (Li *et al.*, 1997; Reth, 2002) and the recent finding that p66shcA is actually a negative regulator of cell cycle progression (Pacini *et al.*, 2004). Yet, H<sub>2</sub>O<sub>2</sub> alone was not sufficient to promote cells to enter cell cycle, because we could not detect a marked increase in proliferating cells under stimulation of a low dose of H<sub>2</sub>O<sub>2</sub>. However, we could not exclude the possibility that H<sub>2</sub>O<sub>2</sub>-induced p27 reduction may facilitate cell cycle progression under proper conditions, for example, when apoptosis is blocked or other mitogenic signals are provided. Recent studies also suggest that p27 may participate in

apoptosis. Overexpression of p27 induces apoptosis in several cell types and p27 deficiency protects cells from stress-induced apoptosis, indicating a proapoptotic role for p27 (Dijkers *et al.*, 2000). In contrast, there are also reports suggesting an antiapoptotic role for p27 (reviewed in Coqueret, 2003). It appears that p27 can have different functions in apoptosis, dependent on the cell types and stress types (Coqueret, 2003). Studies of p27<sup>-/-</sup> MEFs in comparison to wild-type control cells revealed that p27 has a proapoptotic role in response to oxidative stress, as p27<sup>-/-</sup> MEFs showed improved survival against H<sub>2</sub>O<sub>2</sub>. Therefore, H<sub>2</sub>O<sub>2</sub>-induced down-regulation of p27 may provide a protective mechanism in oxidative stress response. The modest effect of p27 supports the concept that the end result of oxidative stress is determined by a coordinated action of various pathways. Taken together, we found that H<sub>2</sub>O<sub>2</sub> led to down-regulation of p27, which may participate in cell response to oxidative stress.

Our studies provided evidence that p52/46shcA are also involved in oxidative stress response. They participated in H<sub>2</sub>O<sub>2</sub>-induced activation of ERKs. It has long been known that H<sub>2</sub>O<sub>2</sub> activates ERK but the molecular mechanisms are less clear. We found that oxidative stress led to phosphorylation of ShcA proteins on all three tyrosine residues (239/240/371), the complex formation between ShcA and Grb2, and activation of ERK. Mutant p52shcA (Y239/240/371 to Phe) inhibited H<sub>2</sub>O<sub>2</sub>-induced ERK activation, and cells deficient for ShcA showed diminished ERK activation. Thus, H<sub>2</sub>O<sub>2</sub>-induced activation of ERKs follows the same pathway triggered by growth factors. The question remains as to what are the tyrosine kinases activated by H<sub>2</sub>O<sub>2</sub> and capable of phosphorylating ShcA proteins at all three residues. Although previous studies have shown that H<sub>2</sub>O<sub>2</sub> treatment activates growth factor receptors such as EGFR and PDGFR, probably by suppressing protein tyrosine phosphatases (Knebel *et al.*, 1996; Kamata and Hirata, 1999), PDGFR was found unnecessary for ShcA phosphorylation or ERK activation. Other mechanisms have also been suggested by which H<sub>2</sub>O<sub>2</sub> activates ERK in different settings (Guyton *et al.*, 1996). Studies in cardiomyocyte showed that H<sub>2</sub>O<sub>2</sub> directly targeted G $\alpha$ <sub>i</sub> and G $\alpha$ <sub>o</sub>, which led to dissociation of G $\beta\gamma$  and subsequent activation of ERK (Nishida *et al.*, 2000). In these cells, activation of ERK required Src activation (Nishida *et al.*, 2000), but we found that deficiency of Src did not affect phosphorylation of ShcA on tyrosine residues, Akt activation, or ERK1/2 activation induced by H<sub>2</sub>O<sub>2</sub> in fibroblasts. The discrepancy could be due to the ways by which Src is inhibited. In previous studies, either an inhibitor or a dominant negative form of Src was used, whereas in our studies, Src<sup>-/-</sup> MEFs were used. It is possible that dominant negative Src may interfere with the function of Src homologues such as Yes and Lyn.

Our findings also suggest that p66shcA played a negative role in H<sub>2</sub>O<sub>2</sub>-induced ERK activation, in which Ser36 phosphorylation is critical, because mutant p66shcA (S36A) showed some stimulatory effect. A negative role for p66shcA ERK activation has been previously reported in response to growth factor (Migliaccio *et al.*, 1997; Okada *et al.*, 1997). Hence, all the three ShcA isoforms participate in p66shcA phosphorylation: p52/46shcA facilitates ERK activation; activated ERK in turn phosphorylates p66shcA, leading to FOXO3a phosphorylation. Therefore, in this event, they functioned in a cooperative way. Later, the Ser36-phosphorylated p66shcA may block ERK activation as a feedback regulation. In this regard, ShcA proteins clearly have distinct functions. What is the molecular basis for the isoform specific effects? How do ShcA isoforms achieve their functions?

We believe that our studies may provide some hints to the above questions. We found that ShcA isoforms interact with each other and may exist as dimers or multimers in the cell. H<sub>2</sub>O<sub>2</sub> treatment promotes the association between p66shcA and p52shcA or p46shcA, but not between p52 and p46. Because p52shcA and p46shcA are more abundant than p66shcA in fibroblasts, they may exist as dimers in the cell and facilitate ERK activation in response to H<sub>2</sub>O<sub>2</sub>. The switch to p66-p52 or p66-p46 may act to interfere with p52/46shcA function and thereby attenuate ERK activation. In accordance with this, it was found that ectopic expression of p66shcA in ShcA<sup>-/-</sup> MEFs did not inhibit ERK activation (Hu and Li, unpublished data), suggesting that the inhibitory effect of p66shcA on ERK activation was via p52/46shcA. Alternatively, it is possible that the interaction among ShcA isoforms may help to assemble upstream and downstream components, e.g., protein phosphatase 2A, MAPKAP kinase2, SHIP, Grb2 (all interact with ShcA), into an organized signaling complex, enhancing the efficiency of signaling propagation (Lamkin *et al.*, 1997; Ugi *et al.*, 2002; Yannoni *et al.*, 2004). In this regard, the complex may act as an anchoring scaffold to facilitate signal transmission (Burack and Shaw, 2000). Certainly, the exact function of the complex formation among ShcA proteins warrants further investigation.

The major finding of this study is that phosphorylation of p66shcA on Ser36 serves two purposes in cell response to oxidative stress: transmitting signals to modulate p27 expression to influence cell response to oxidative stress; and inhibiting ERK activation through a negative feedback mechanism. In response to H<sub>2</sub>O<sub>2</sub>, all three ShcA proteins participate in transmitting signals to inactivate transcription factor FOXO3a, in two steps: H<sub>2</sub>O<sub>2</sub> first induces phosphorylation of tyrosine residues of ShcA proteins, facilitating ERK activation; and activated ERKs in turn phosphorylate p66shcA on Ser36, leading to phosphorylation of FOXO3a by Akt and down-regulation of p27. p66shcA may inhibit ERK activation by forming complexes with p52shcA or p46shcA, the stimulatory ShcA proteins for ERK activation.

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