Constitutive Induction of p-Erk1/2 Accompanied by Reduced Activities of Protein Phosphatases 1 and 2A and MKP3 Due to Reactive Oxygen Species during Cellular Senescence*

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The mechanism of senescence-associated cytoplasmic induction of p-Erk1/2 (SA-p-Erk1/2) proteins in human diploid fibroblasts was investigated. p-Erk1/2 proteins were efficiently dephosphorylated in vitro by protein phosphatases 1 and 2A (PP1/2A) and MAPK phosphatase 3 (MKP3). Specific activity of PP1/2A and MKP3 activity significantly decreased during cellular senescence, whereas their protein expression levels did not. To investigate possible mechanism of phosphatase inactivation, we measured reactive oxygen species (ROS) generation by fluorescence-activated cell sorting analysis and found it was much higher in mid-old cells than the young cells. Treating the young cells once with 1 mM H2O2 remarkably induced p-Erk1/2 expression; however, it was transient unless repeatedly treated until 72 h. Multiple treatment of the cells with 0.2 mM H2O2 significantly duplicated inactivation of PP1/2A; however, thiol-specific reagents could reverse the PP1/2A activities, suggesting the oxidation of cysteine molecule in PP1/2A by the increased ROS. When the cells were pre-treated with 10 mM N-acetyl-L-cysteine for 1 h, Erk1/2 activation was completely blocked. To elucidate which cysteine residue and/or metal ion in PP1/2A was modified by H2O2, electrospray ionization-tandem mass spectrometry analyses were performed with purified PP1C-α and found Cys62SO3H and Cys105SO3H implicating the tertiary structure perturbation. H2O2 inhibited purified PP1C-α activity by both oxidation of Cys residues and metal ion(s), evidenced by dithiothreitol and ascorbate-restoration assay. In summary, SA-p-Erk1/2 was most likely due to the exposure of PP1/2A, which resulted from the continuous exposure of the cells to vast amounts of ROS generated during cellular senescence by oxidation of Cys62 and Cys105 in PP1C-α and metal ion(s).

Decreased growth rate, limited cell division, flat and large cell shapes, and tight binding of the cells to culture dished (1, 2) are well known characteristics of cells entering into replicative senescence (3). Primary mouse embryo fibroblasts exposed to oncogenic Ras overexpression undergo premature senescence in response to constitutive MAPK kinase/MAPK mitogenic signaling (4, 5), whereas established variants lacking p53 or p19ARF are efficiently transformed (6). Ha-Ras mutants that interact preferentially with specific Ras effector proteins are known as V12S35, V12C40, and V12G37 (7, 8); V12S35 binds preferentially to Raf-1 and activates MAPK without any effect on membrane ruffling (7), and the V12C40 associates with phosphatidylinositol 3-kinase (7). A distinctive feature of the cellular senescence induced by overexpression of the Ha-ras mutants as well as the replicative senescence in normal human diploid fibroblasts (HDF) is the markedly increased phosphorylation of extracellular signal-regulated protein kinase (p-Erk1/2) without nuclear translocation (10). However, its biochemical mechanism has not yet been clarified.

MAPK activity is tightly regulated by phosphorylation and dephosphorylation. The activation of the MAPK activity requires the dual phosphorylation of the Ser/Thr and Tyr residues in the TXY kinase activation motif (11–13), and deactivation occurs through the action of either Ser/Thr protein phosphatase (14), protein-tyrosine phosphatase (PTP) (14, 15), or dual specificity phosphatases (16, 17). The dual phosphatases are capable of catalyzing the removal of the phosphoryl group from Tyr(P) as well as Ser(P)/Thr(P) residues. The dual specificity phosphatases that specifically dephosphorylate and inactivate the p-Erk1/2 are called MAPK phosphatases (MKPs), and at least 9 mammalian MKPs have been identified so far (18, 19). It has been reported that MKP3 (20), also termed Pyst1 (21) or VH6 (22), is predominantly localized in the cytoplasm, is highly specific for Erk1/2 inactivation, and is not inducible by either growth factor or stress (20–23). An elegant series of studies have shown that the N-terminal domain of MKP3 can physically associate with Erk1/2 (24), and this association can activate phosphatase activity of MKP3 present in the C-terminal domain (25). Moreover, the C-terminal domain of MKP3 is significantly homologous to VHR, one of the MKPs (26), and Erk1/2 proteins are efficient substrates for VHR. MKP3 dual specificity phosphatase is not related to the Ser/Thr protein phosphatases but belongs to the PTP superfamily.

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‡ The abbreviations used are: MAPK, mitogen-activated protein kinase; PP1, phosphatase 1; and PP2A phosphatase 2A; p-Erk1/2, extracellular signal-regulated protein kinase 1/2; SA-p-Erk1/2, senescence-associated persistent induction of p-Erk1/2; HDF, human diploid fibroblasts; PTP, protein-tyrosine phosphatase; MKPs, MAPK phosphatases; VHR, vacinia H1-related; rVHR, recombinant VHR; ROS, reactive oxygen species; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; pNPP, p-nitrophenyl phosphate; IP, immunoprecipitation; FACS, fluorescence-activated cell sorting; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; NAC, N-acetyl-L-cysteine; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectroscopy; EGF, epidermal growth factor; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; TOF, time-of-flight; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-[hydroxymethyl]propane-1,3-diol.
Constitutive Induction of p-Erk1/2 with Decreased PP1/2A

There is increasing evidence that relatively few Ser- or Thr-specific protein phosphatases with pleiotropic action participate in cellular regulation. Four major classes of protein phosphatase, type 1 (PP1), type 2A (PP2A), type 2B (PP2B or calcineurin), and type 2C (PP2C), have been identified in mammalian cells (27, 28). PP1 occurs in three isoforms (α, β, and γ) and contains a catalytic subunit (PP1C), regulatory subunit (inhibitor-2), α G subunit, and an M subunit. PP1C binds to two cytosolic thermostable proteins, termed inhibitor-1 (I-1) and inhibitor-2 (I-2), which completely abolish its activity at nanomolar concentrations (29). The I-1 tightly binds to PP1 both in vitro and in vivo and requires phosphorylation by cAMP-dependent protein kinase to be inhibitory of a number of protein phosphatases (30–32), whereas phosphorylation of I-2 at Thr^{72} by glycogen synthase kinase leads to activation of the enzyme (33, 34). PP2A is a heterotrimeric enzyme consisting of a 36-kDa catalytic subunit (C), 65-kDa structural subunit (A), and a variable regulatory subunit (B). PP2A is unaffected by the above inhibitors but highly effectively inhibited by low concentrations of okadaic acid (27, 35, 36). PP2A is active toward most substrates in the absence of divalent cation, whereas PP2B and PP2C have absolute requirements for Ca^{2+} and Mg^{2+}, respectively (27).

There are numerous reports that cellular redox status plays an important role in the mechanisms to regulate the function of growth factors and tyrosine phosphorylation-dependent signal transduction pathways (37–42). Furthermore, ROS such as H_{2}O_{2} have been shown to be involved in growth factor signaling pathways (43, 44), perhaps as a second messenger. Therefore, PTP appears to be a logical target of H_{2}O_{2}, leading to transient and reversible inactivation of phosphatase activity by oxidizing catalytic cysteine residue to sulfenic acid (45). It has been reported that local levels of H_{2}O_{2} (200 μM) robustly activate Erk1/2 (26, 46–49) but only slightly activate pS8 MAPK and c-Jun N-terminal kinase/stress-activated protein kinase in COS1 cells by inactivating endogenous VHR to dephosphorylate Erk.

Very recently, a paper (50) with great relevance to our work has suggested that the PP1 is a molecular constraint on learning and memory and a potential mediator of cognitive decline during aging. It has been shown with tet-O-inhibitor-1 transgenic mice model that the transgene was expressed in the hippocampus and cortex of the mice brain. Because these tissues have been implicated in the memory process, the above observation offers a great credence to the physiological relevance of the PP1-dependent mechanism of forgetting, particularly in aging-related memory decline (51). Therefore, in order to investigate the mechanism of SA-p-Erk1/2, enzymatic activities of PP1/2A and MKP3 together with their protein expression levels were evaluated during cellular senescence. Furthermore, the difference of ROS generation between the young and old cells and the effects of ROS on PP1/2A and MKP3 were also elucidated using primary culture of the young, mid-old, and old HDF cells.

**EXPERIMENTAL PROCEDURES**

**HDF Cell Cultures**—The primary culture of normal HDF was isolated from the foreskin of a 4-year-old boy by the method described previously (10) and Ha-ras double mutants (V12S35, V12G37, and V12C40) overexpressing HDF cell lines, and the number of population doublings as well as the doubling time were continuously counted. Young, mid-old, and old cells used in the present experiments were defined as the HDF with a doubling time of around 24 h, 10–12 days, and over 2 weeks, respectively.

**p-Erk1/2 Immunocytochemistry**—HDF cells were cultured on a cover glass and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After washing the monolayer with PBS, cells were permeabilized with 0.15% Triton X-100 in PBS for 5 min and then blocked with 2% bovine serum albumin in PBS for 1 h at room temperature. Cells were incubated overnight at 4 °C with anti-p-Erk1/2 antibody (catalog number 9102, Beverly, MA), diluted (1:200) in 2% bovine serum albumin solution, followed by washing with PBS, and fluorescein isothiocyanate-labeled anti-rabbit IgG (1:200, Jackson ImmunoResearch, West Grove, PA) was applied for 1 h at room temperature. After washing with PBS, the cover glass was mounted with Mowiol mounting medium (Hoechst Celanese, Charlotte, NC) containing antifade agent 1,4-diazabicyclo[2.2.2]octane (Aldrich, catalog number 337300). Images were obtained with an Axioskop microscope (Zeiss, Jena, Germany) equipped with a cooled digital camera (Sony, Princeton Instruments). For quantification, Image J software was used.

**PP2A Immunoblot Analyses of Mono- and Di-phosphorylated Erk1/2 Proteins**—Young and old HDF cells were solubilized with RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, 50 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 1 μg/ml leupeptin), and 40 μg of cell lysates were resolved on 10% SDS-PAGE in 25 mM Tris-glycine buffer. After transferring protein bands to polyvinylidene difluoride membrane (catalog number 162-0177, Bio-Rad) at 150 mA for 1 h and 40 min, anti-p-Erk1/2 antibody was applied overnight at 4 °C. For the loading control, anti-α-tubulin (Oncogene catalog number C066, Boston, anti-Erk1/2 (Cell Signaling catalog number 9102, Beverly, MA), or anti-actin (Sigma, catalog number A5390) antibodies were used. ECL kit was employed to visualize protein expression levels. In order to elucidate whether the anti-p-Erk1/2 antibody used in our experiments detects both monophosphorylated and dual phosphorylated Erk1/2 proteins or not, purified PP1 (Sigma, catalog number P7937) and PP2A (Calbiochem, catalog number 533508) enzymes were applied to the activated Erk1 (p-Erk1, Stratagene, catalog number 206110) and cell lysates, respectively, and then repeated immunoblot analyses were performed using antiphospho-Thr^{202} and antiphospho-Thr^{204} antibodies (Upstate Biotechnology, Inc., catalog number 05-321) as well as anti-p-Erk1/2 and anti-Erk1/2 antibodies.

When investigating the fraction levels of p-Erk1/2 regulated by PP2A in young and old HDF cells, which were maintained in the serum-free media for 24 h, were treated with 40 μM okadaic acid, a specific inhibitor for PP2A, and the cells were harvested every 4 h until 12 h for immunoblot analysis with anti-p-Erk1/2 antibody.

**Alkaline Phosphatase Assay—pNPP tablet set (N-1891, Sigma FASTT) purchased from Sigma was used for alkaline phosphatase assay. Each pNP and Tris Buffer tablets were dissolved in 5 ml of distilled water and used as substrate. Three hundred and fifty μl of a reaction mixture containing 200 μl of the substrate and 150 μl of cell lysate (100 μg), which was prepared in 1% Nonidet P-40 in PBS, 1 mM PMSF, and 1 μg/ml leupeptin, was incubated in the dark for 2 h at room temperature, and the reaction was stopped by cooling down in ice. After washing at 400 μl was wound up, and phosphatase activity was calculated from the standard curve prepared with p-nitrophenol.

**Protein Phosphatases 1 and 2A Assay**—To determine the activity of PP1/2A, cells were washed twice with phosphate-free medium and lysed with a buffer solution, containing 100 mM Tris-HCl (pH 7.4), 1 mM PMSF, 1 μg/ml leupeptin, by sonication (Upstate Biotechnology, Inc., catalog number 05-321) as well as anti-phospho-Thr^{202} and anti-phospho-Thr^{204} antibodies. When investigating the fraction levels of p-Erk1/2 regulated by PP2A in young and old HDF cells, which were maintained in the serum-free media for 24 h, were treated with 40 μM okadaic acid, a specific inhibitor for PP2A, and the cells were harvested every 4 h until 12 h for immunoblot analysis with anti-p-Erk1/2 antibody.

**Immunoblot Analyses of Mono- and Di-phosphorylated Erk1/2 Proteins**—Young and old HDF cells were solubilized with RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, 50 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 1 μg/ml leupeptin), and 40 μg of cell lysates were resolved on 10% SDS-PAGE in 25 mM Tris-glycine buffer. After transferring protein bands to polyvinylidene difluoride membrane (catalog number 100–0177, Bio-Rad) at 150 mA for 1 h and 40 min, anti-p-Erk1/2 antibody was applied overnight at 4 °C. For the loading control, anti-α-tubulin (Oncogene catalog number C066, Boston, anti-Erk1/2 (Cell Signaling catalog number 9102, Beverly, MA), or anti-actin (Sigma, catalog number A5390) antibodies were used. ECL kit was employed to visualize protein expression levels. In order to elucidate whether the anti-p-Erk1/2 antibody used in our experiments detects both monophosphorylated and dual phosphorylated Erk1/2 proteins or not, purified PP1 (Sigma, catalog number P7937) and PP2A (Calbiochem, catalog number 533508) enzymes were applied to the activated Erk1 (p-Erk1, Stratagene, catalog number 206110) and cell lysates, respectively, and then repeated immunoblot analyses were performed using antiphospho-Thr^{202} and antiphospho-Thr^{204} antibodies (Upstate Biotechnology, Inc., catalog number 05-321) as well as anti-p-Erk1/2 and anti-Erk1/2 antibodies.

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whether the H2O2 oxidized the active site cysteine residue in PP1/2A or then prepared according to the method described above. To examine with phosphate-free medium within 10 min, and the cell lysates were mutants, such as V12C40, V12G37, and V12S35, induced premature senescent HDF cells. Overexpression of Ha-Ras double mutants, such as V12C40, V12G37, and V12S35, induced premature senescence in HDF cells. Cells lysates were prepared in RIPA buffer by sonication, and immunoblot analyses were performed with the cell lysates (40 µg/lane). The rest of the experimental procedures were the same as described under Fig. 1. Y and O indicate young and old cells, respectively.

Restoration of Protein Phosphatases 1 and 2A Activity by Thiolspecific Reagents—To investigate the role of ROS played in a significantly reduced PP1/2A activity during cellular senescence, young HDF cells were pretreated once with 1 mM H2O2; since the PP1/2A activity in the mid-old cells had been reduced, most likely due to accumulated H2O2, these cells were not pretreated with H2O2. The cells were washed with phosphate-free medium within 10 min, and the cell lysates were then prepared according to the method described above. To examine whether the H2O2 oxidized the active site cysteine residue in PP1/2A or not, DTT (1 mM), β-mercaptoethanol (0.1%), or NAC (10 mM) was individually added to the young and mid-old cell lysates before the PP1/2A assay. All of the experiments with young and mid-old HDF cells were repeated 3 times. All the thiol reagents were purchased from Sigma.

Preparation of MKP3 Antibody—Recombinant MKP3 protein was expressed in the BL21 cells using pGEX4T3-GST-MKP3 cDNA (originally isolated by Muda et al. [20] and kindly donated by Dr. Montserrat Camps (Serono Pharmaceutical Research Institute, Geneva, Switzerland)). MKP3 protein (500 µg) mixed with an equal volume of complete Freund’s adjuvant (F-5506, Sigma) was injected subcutaneously into back, footpad, and thigh of New Zealand White rabbits. In 3 weeks, the first booster injection was done with MKP3 protein (500 µg) mixed with equal volume of incomplete Freund’s adjuvant (F-5506, Sigma), and the rabbits were sacrificed 1 week after the second booster injected similarly to the first. Whole serum was then obtained, and IgG was purified by incubating the serum with protein A-agarose beads (Invitrogen, catalog number 15920-010).

MKP3 Immunoprecipitation—Phosphatase (IP-phosphatase) Assay—IP with anti-MKP3 IgG was performed with young and old HDF cell lysates (300 µg) in a solution containing 20 mM Tris-HCl (pH 7.0), 1% Triton X-100, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 0.1% β-mercaptoethanol, 1 mM PMSF, and 1 µg/ml leupeptin by the standard method. MKP3-IP-phosphatase assay mixture (200 µl) containing the above MKP3 immunoprecipitates and 50 mM pnP in 50 mM succinate buffer (pH 7.0) was incubated at 37 °C for 1 h, and the phosphate released was measured with enzyme-linked immunosorbent assay reader (Envision, Molecular Devices, Sunnyvale, CA) at 405 nm. As a control blank, heat-inactivated cell lysate and reagent were also run for each assay. When comparing the amount of p-Erk1/2 proteins sequestered by MKP3 in young and old HDF cells, whole cell lysates, MKP3 immunoprecipitates, and the IP supernatants after MKP3-IP were separated on 10% SDS-PAGE, and immunoblot analysis was performed against p-Erk1/2 antibody.

Measurement of ROS by FACS Analysis—H2O2 generation during cellular senescence was measured by FACS analysis using 50 µM 2′,7’-dichlorodihydrofluorescein diacetate (H2-DCFDA, D-399, Molecular Probes, Eugene, OR) in cell culture system. Young and mid-old HDF cells (old cells were too large to analyze by FACS) were plated in a 60-mm culture dish with 25 and 70% confluence, respectively. The next day, media were changed, and the cells were incubated in complete medium for another 24 h. The cells were then pretreated with 10 mM NAC (Sigma, catalog number A-7250) for 1 h, and H2-DCFDA was added 10 min prior to cell harvest. ROS generation was determined with FACScan (BD Biosciences) by measuring the fluorescence ofDCF arising from the oxidation of H2-DCFDA.

ESI-MS/MS Analysis—Nano-ESI on a Q-TOF2 mass spectrometer (Micromass, Manchester, UK) was used for sequence analysis of Cys62 and Cys105 containing peptides after in-gel trypsin digestion (overnight at 37 °C). Data were collected for positive ions at m/z 400–2000. For sequence analysis of peptide, once the parent ions were identified, the mass spectrometer was set up to obtain collision-induced dissociation
MS/MS spectra of the parent ions. The quadrupole analyzer was used to select parent ions for fragmentation in the hexapole collision cell. The collision gas was argon at a pressure of 6–7 \times 10^{-5} \text{ mbar}, and the collision energy was 20–30 V. A potential of 1 kV was applied to the precoated borosilicate nanoelectrospray needles in the ion source combined with a nitrogen back pressure of 0–5 pounds/square inch to produce a stable flow rate (10–30 nL/min). A new needle was used for each analysis to eliminate the risk of cross-contamination between different peptide digests. Product ions were analyzed using an orthogonal time-of-flight (TOF) analyzer, fitted with a reflector, a microchannel plate detector, and a time-to-digital converter. The data were processed using a Mass Lynx Windows NT PC system.

RESULTS
Constitutive Induction of p-Erk1/2 with Decreased PP1/2A
time of over 2 weeks, p-Erk1/2 levels were measured by immuno blot analysis. Expression of p-Erk1/2 was significantly higher in the old cells (Fig. 1D) as compared with the young cells. Moreover, immunocytochemical analysis revealed no response to EGF treatment in the old cells, significant induction of p-Erk1/2 level in the young cells (Fig. 1, B and C), and no change in the old cells (Fig. 1, E and F). It should also be noted that the basal level of p-Erk1/2 was markedly different between these cells (compare Fig. 1, B versus E). To investigate whether the marked induction of p-Erk1/2 was limited only to replicative senescence HDF cells, Ha-ras double mutant-induced premature senescence cells were also employed, and immunoblot analysis was carried out. As shown in Fig. 2, the constitutive induction of p-Erk1/2 was apparent in both the replicative senescence (HDF control) and oncogenic Ras-induced premature senescence cells (V12C40, V12G37, and V12S35). Therefore, we denote this phenomenon as “senescence-associated persistent induction of p-Erk1/2” or “SA-p-Erk1/2.”

**Which Phosphatase Can Possibly Be Involved in Dephosphorylation of p-Erk1/2 Proteins?**—To discern phosphatases possi-
Constitutive Induction of p-Erk1/2 with Decreased PP1/2A

Table I

| Comparison of protein phosphatase 1 and 2A expressed in young and old HDF cells |
|--------------------------------------------------|
| Number of cells used for comparison | Young cells | Old cells |
| Total activity of PP1 and PP2A (pmol phosphate/min/cell) | 56.3 ± 3.0 (1) | 142.6 ± 1.1 (2.5) |
| Specific activity of PP1 and PP2A (pmol phosphate/min/μg protein) | 28.4 ± 1.5 (1) | 17.4 ± 0.7 (0.6) |
| Amount of protein (μg)/cell | 121 (1) | 495 (4.1) |

Numbers in parentheses indicate relative values between the young and old cells. The old cells used in these experiments could divide once in over 2 weeks.

Is MKP3 Responsible for Cytoplasmic Retention of p-Erk1/2 Proteins?—Because p-Erk1/2 has been shown to strongly bind to the N-terminal domain of MKP3, allosterically activates MKP3 C-terminal phosphatase domain (52), and is exclusively located in the cytoplasm of sympathetic neurons (20), the possibility of MKP3 as a potential candidate to sequester SA-p-Erk1/2 in the cytoplasm was first investigated by determining the amounts of p-Erk1/2 bound to MKP3 in the IP-supernatant, and the whole homogenates by immunoblot against p-Erk1/2 antibody. As seen in Fig. 6A, a large amount of p-Erk1/2 protein was bound to MKP3 in the old cells, whereas there was hardly any p-Erk1/2 bound to MKP3 in the young cells. Next, we measured the MKP3 protein expression in both the young and old cells with IP-immunoblot analysis (Fig. 6B). However, no significant change was observed during HDF senescence. On the other hand, when its activity was measured by IP-phosphatase assay, the activity in the old cells (21.2 ± 4.2 pmol/min/mg protein) was significantly lower than in the young cells (34.5 ± 2.7 pmol/min/mg protein) (Fig. 6C).

What Is the Mechanism of Reduction of PP1/2A Proteins during Cellular Senescence?—In order to investigate further the mechanism underlying the significant reduction of PP1/2A and MKP3 activities and elevated p-Erk1/2 level dur-
Constitutive Induction of p-Erk1/2 with Decreased PP1/2A

Fig. 6. Sequestration of p-Erk1/2 by MKP3 in the old HDF cells. A, to investigate whether the p-Erk1/2 was bound to MKP3 or not and also to compare the bound p-Erk1/2 between the young and old cells, 300 μg of each cell lysate in immunoprecipitation buffer (1% Triton X-100, 250 mM sucrose, 20 mM Tris-HCl (pH 7.0), 1 mM EGTA, 1 mM EDTA, 0.1% β-mercaptoethanol, 1 mM PMSF, 2 μg/ml leupeptin) were incubated with 1 μg of anti-MKP3 antibody. The immunoprecipitate was incubated with 50% protein G-agarose beads at 4 °C overnight and then washed 3 times with the same buffer. The IP-supernatant and the washing solution were collected, and they were concentrated by Centricon-10 (catalog number 4206, Millipore, Bedford, MA). The whole cell lysates equivalent to the amount of the supernatants were also concentrated with Centricon. The whole cell lysate, IP-supernatant, and the washing solution were collected, and they were concentrated by Centricon-10 (catalog number 4206, Millipore, Bedford, MA). The whole cell lysates equivalent to the amount of the supernatants were also concentrated with Centricon. The whole cell lysate, IP-supernatant, and the MKP3-IP were resolved on 10% SDS-PAGE and then immunoblotted with anti-p-Erk1/2 antibody. B, MKP3 protein expression in the young (1st lane) and old (2nd lane) cells, with 40 μg of whole cell lysate applied per each lane. α-Tubulin bands represent loading control between the young and old cells. C, MKP3-IP phosphatase activity. Five hundred μg of the young and old cell lysates were applied for IP with anti-MKP3 antibody and then washed 3 times with IP-buffer. Phosphatase assay was carried out with 50 mM pNPP in 50 mM succinate buffer (pH 7.0). Assays were repeated 3 times with duplicates in each case, and the results of 34.5 ± 2.7 and 21.2 ± 4.2 pmol/min/mg protein indicate the mean ± S.D. in the young and old cells, respectively. Upper panel reveals the MKP3 IP-immunoblot findings used for the MKP3 IP-phosphatase assays.

Fig. D. Sequestration of p-Erk1/2 by MKP3 in the old HDF cells. A, to investigate whether the p-Erk1/2 was bound to MKP3 or not and also to compare the bound p-Erk1/2 between the young and old cells, 300 μg of each cell lysate in immunoprecipitation buffer (1% Triton X-100, 250 mM sucrose, 20 mM Tris-HCl (pH 7.0), 1 mM EGTA, 1 mM EDTA, 0.1% β-mercaptoethanol, 1 mM PMSF, 2 μg/ml leupeptin) were incubated with 1 μg of anti-MKP3 antibody. The immunoprecipitate was incubated with 50% protein G-agarose beads at 4 °C overnight and then washed 3 times with the same buffer. The IP-supernatant and the washing solution were collected, and they were concentrated by Centricon-10 (catalog number 4206, Millipore, Bedford, MA). The whole cell lysates equivalent to the amount of the supernatants were also concentrated with Centricon. The whole cell lysate, IP-supernatant, and the MKP3-IP were resolved on 10% SDS-PAGE and then immunoblotted with anti-p-Erk1/2 antibody. Lanes 1–3 and lanes 4–6 indicate the whole cell lysates, IP-supernatants, and MKP3-precipitate of the young and old cells, respectively. Note the p-Erk1/2 bound to MKP3 only in the old cells but not in the young cells. B, MKP3 protein expression in the young (1st lane) and old (2nd lane) cells, with 40 μg of whole cell lysate applied per each lane. α-Tubulin bands represent loading control between the young and old cells. C, MKP3-IP phosphatase activity. Five hundred μg of the young and old cell lysates were applied for IP with anti-MKP3 antibody and then washed 3 times with IP-buffer. Phosphatase assay was carried out with 50 mM pNPP in 50 mM succinate buffer (pH 7.0). Assays were repeated 3 times with duplicates in each case, and the results of 34.5 ± 2.7 and 21.2 ± 4.2 pmol/min/mg protein indicate the mean ± S.D. in the young and old cells, respectively. Upper panel reveals the MKP3 IP-immunoblot findings used for the MKP3 IP-phosphatase assays.
FIG. 7. Reactive oxygen species generated during cellular senescence-regulated PP1/2A activities and p-Erk1/2 levels. A, marked difference of ROS level between young and mid-old HDF cells, measured by FACS analysis. The young and mid-old cells were seeded into culture dishes up to 25 and 70%, respectively. After 24 h, the monolayer was re-fed with complete medium and then treated with H$_2$DCF-DA 10 min prior to harvesting. The cells were then stained with DCFDA and analyzed by flow cytometry.

| Cells            | Treatment | M2   | Fl (mean) |
|------------------|-----------|------|-----------|
| Young (DT=1d)    | Cont.     | 4.25 | 131       |
|                  | NAC       | 2.08 | 105       |
| Mid-old (DT=10d) | Cont.     | 79.82| 415       |
|                  | NAC       | 49.56| 293       |

* p<0.05 vs. 0h

B, constitutive induction of p-Erk1/2 with decreased PP1/2A activity. Western blot analysis of p-Erk1/2 and α-tubulin expression levels over time after treatment with H$_2$O$_2$.

C, specific activity (pmoles/min/μg) of p-Erk1/2 over time after treatment with H$_2$O$_2$. Significant differences are indicated by * p<0.05 vs. 0h.

D, time course of p-Erk1/2 expression levels after treatment with H$_2$O$_2$. Significant differences are indicated by * p<0.05 vs. 0h.
Fig. 8. Restoration of protein phosphatase 1 and 2A activities by thiol-specific reducing agents in mid-old HDF cells. In the case of the young HDF cells, the cells were pretreated with 1 mM H$_2$O$_2$. However, the mid-old cells were not pretreated, because the PP1/2A activity was already reduced, most likely due to accumulated H$_2$O$_2$ during senescence. The cell lysates were prepared within 10 min in the phosphate-free condition. By using the phosphopeptide as a substrate, PP1/2A activity was measured by the described method with the control (C) and H$_2$O$_2$-treated cell lysates. The assay was also performed either with DTT (1 mM), β-mercaptoethanol (2ME, 0.1%), or NAC (10 mM) addition to the cell lysates of the H$_2$O$_2$-treated cell lysates. Recovery of the senescent-induced PP1/2A activity was also evaluated with the mid-old cell lysates by addition of 0.1% β-mercaptoethanol to the control cell lysate. All the samples were triplicated, and the assays were performed more than three times.

Addition of DTT, β-mercaptoethanol, and NAC, respectively. All three reagents significantly reactivated PP1/2A activity as compared with that of the H$_2$O$_2$ alone treatment (*, p < 0.002, and **, p < 0.02). However, only DTT, but not β-mercaptoethanol and NAC, significantly increased PP1/2A activity of the young cells more than the control (p < 0.005). Interestingly, when the assay was carried out with mid-old cell lysates, whose PP1/2A activity had been already reduced, most likely due to accumulated H$_2$O$_2$ during senescence, β-mercaptoethanol could significantly increase PP1/2A activity as compared with the control (22.04 ± 0.80 versus 18.71 ± 0.76 pmol/min/μg protein, p < 0.005, right panel in Fig. 8), thus strongly suggesting that the high level of ROS found in senescent cells oxidized thiol residues, which are important for phosphatase activity.

To investigate whether elimination of ROS generated during cellular senescence could revert the morphological changes characteristic to senescent cells or not, the mid-old cells were treated with NAC twice a day for 2 and 4 days, and their morphology was examined. As seen in Fig. 9, the flat and large cell characteristic to the old indeed reverted to the morphology characteristic to young cells: small, slender, and cylindrical fibroblast. In order to investigate whether the morphological changes of the young-like cells were accompanied by any biochemical changes specific to cellular senescence, the expression of senescence-associated β-galactosidase in the cells was measured. As shown in Table II, treatment of the cells with NAC for 2 days was sufficient to significantly reduce senescence-associated β-galactosidase expression in the mid-old cells.

Which Cysteine Residue and/or Metal Ion in PP1C-α Is Oxidized by H$_2$O$_2$?—To confirm oxidation of Cys-SH by H$_2$O$_2$ in vitro, 0.5 mM 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was incubated with purified PP1C-α (Sigma) based on Denu and Tanner (45). As shown in Fig. 10A, incubation of PP1C-α with 2.5 mM H$_2$O$_2$ for 10 min clearly oxidized Cys-SH to Cys-SOH, indicated by NBD-sulfoxide modification and the change of maximum absorbance from 420 to 347 nm. To elucidate the net effect of oxidation(s) on their activities, thiol-reducing agents and metal-reducing ascorbate were separately added to PP1C-α after incubation with 2.5 mM H$_2$O$_2$ for 10 min at 30 °C. Activity of PP1C-α could be restored not only by Cys-reducing agents but also by ascorbate (Fig. 10B). To determine the most vulnerable site of oxidation in cysteine of the purified PP1C-α, MS/MS analysis was performed using nano-electrospray ionization on a Q-TOF2 mass spectrometry. As shown in Fig. 11, A and C, two oxidized cysteine-containing peptides were detected in the m/z range of 740–860; Cys$^{42}$-containing peptide encom-
Constitutive Induction of p-Erk1/2 with Decreased PP1/2A

**DISCUSSION**

Replicative senescence of human fibroblasts has frequently been used as an aging model in vitro (53). In the present study, we have shown that a large amount of p-Erk1/2 proteins, denoted as SA-p-Erk1/2, were found in the cytoplasm of both senescent and prematurely senescent HDF cells induced by Ha-ras double mutants (Figs. 1 and 2) and that the SA-p-Erk1/2 double mutants (Figs. 1 and 2) and that the SA-p-Erk1/2 could be restored by both Cys-reducing agents and metal-reducing ascorbate (B).

Further, a large amount of SA-p-Erk1/2 proteins was bound and sequestered in the cytoplasm of old cells (Fig. 6A). Erk1/2 is one of MAPK isoforms together with c-Jun N-terminal kinase/stress-activated protein kinase and p38MAPK, and the upstream components of Erk1/2 include c-Ras, c-Raf1, and MAPK kinase (54, 55). When cells are stimulated with various ligands such as growth factors, hormones, neurotransmitters, or tumor promoters, Erk1/2 is activated through dual-phosphorylation at the -pT-E-pY- motif. Subsequently, p-Erk1/2 translocates into the nucleus and phosphorlates Elk-1, thereby acting as a transcription factor for cell proliferation (56).

The phosphorylation state of any given protein in vivo depends on the balance between the activities of phosphatases and kinases, and there is increasing evidence that relatively few Ser- or Thr-specific protein phosphatases with pleiotropic action participate in cellular regulation. Therefore, the finding that a significant decrease of PP1 activities in both the cytoplasm and nuclear fractions of the senescent cells without any change in their protein expression (Fig. 5D) resulted in the induction of p-Erk1/2 levels that might possibly be explained by the existence and induction of phosphatase inhibitors such as inhibitor-1 or an unidentified inhibitor during cellular senescence. Therefore, in order to verify the presence or increase of a putative inhibitor in the old cells, we carried out two approaches as follows: detection of the known inhibitor 1 expression in the young and old cells by immunoblot analysis, and the other to measure the PP1/2A activity after mixing the young and old cell lysates. When analyzed by immunoblot analysis, the protein expression levels among the two were basically the same (data not shown). Also when increasing amounts of the old cell lysates were added to a fixed amount of young cells and the PP1/2A activity was measured, the sums of the activity measured individually were the same as those in the mixture, indicating the absence or no increase of an inhibitor in the old cell lysates (data not shown). The above two experiments verified the fact that the decreased PP1/2A activity in the old cells...
FIG. 11. Mass spectrometry analysis of oxidation state of PP1C-α. Control and H₂O₂-oxidized PP1C-α (each 5 μg/sample) were separately digested with trypsin (0.1 μg) in-gel at 37 °C for overnight and desalted using C18 nano-columns. For analysis by MS/MS, peptides were eluted with 1.5 μl of 50% methanol, 49% H₂O, 1% formic acid solution directly into a precoated borosilicate nanoelectrospray needle. A and B spectra show the oxidized Cys⁶²-containing peptide and Cys¹⁰⁵-containing peptide, respectively. C and D are tandem mass spectra of a peptide with m/z 857.4 and 771.9, respectively. The C-O₃ denotes Cys-SO₃H.
was not due to increase of a putative inhibitor in the old cells.

To investigate further the mechanistic evidence of the reduced PP1/2A activity during cellular senescence, thiol-specific reducing agents were added to the cell lysates whose cells had been pretreated with H$_2$O$_2$. As seen in Fig. 8, the reducing agents could significantly restore the H$_2$O$_2$-inhibited PP1/2A activities in the young HDF cells and increase the PP1/2A activity in mid-old cells. These data strongly indicated that the inhibition of phosphatase activity might have been due to the oxidation of reactive site cysteine residue(s) by ROS during cellular senescence. It should be noted that the catalytic domains of PP1, PP2A, and calcineurin are highly homologous, i.e. 49% identity between PP1 and PP2A and 40% identity between PP2A and calcineurin. However, their substrate specificities and interactions with regulatory molecules are very different. The crystal structure of their catalytic domains revealed similarly folded catalytic cores, each of which contains two catalytic metals (57, 58). There is very little information available about the effects of oxidants on the activity of PP1/2A. However, Sommer et al. (59) recently revealed that the activities of PP1/2A in SK-N-SH cell lysates were reversibly inhibited by H$_2$O$_2$. ROS-inhibited PP1 activity could be reversed by treating SK-N-SH cells either with thiol-reducing agent DTT or metal-reducing ascorbate. Our results together with the above observation led us to suggest that the mechanism of inhibition of PP1/2A in HDF cells was due to direct attack of H$_2$O$_2$ on cysteine residue(s) of PP1/2A but not due to a regulating factor activity.

As shown in Fig. 3 and Fig. 4, SA-p-Erk1/2 served as a substrate for PP1/2A. Because the inhibition constants ($K_i$) of PP1 and PP2A by okadaic acid are 150 nM and 32 pM, respectively (60), induction of p-Erk1/2 in the young and old HDF cells 4 h after the treatment with 40 nM okadaic acid (Fig. 5E, lanes 2–4 and 6–8) strongly indicated that the preferential inactivation of PP2A during cellular senescence could also be significantly responsible for SA-p-Erk1/2 proteins.

The reason why a huge induction of p-Erk1/2 by a single treatment with 10 mM H$_2$O$_2$ was greatly attenuated in 40 min might be found in the rapid induction of MAPK through EGF receptor (61, 62), and the significant but transient reduction of PP1 and PP2A in HDF cells, which were significantly inhibited in 10 min but recovered by 40 min and reached the control level in 8 h (Fig. 7C). It is important to note that when the cells were treated with H$_2$O$_2$ every 8 h for more than 72 h, activities of PP1/2A were persistently reduced, and the level of p-Erk1/2 was constitutively high (Fig. 7D), resulting in SA-p-Erk1/2. These findings implicate that cells in vivo might be continuously exposed to the higher level of ROS during the senescence process, thus resulting in the inhibition of PP1/2A and the constitutive increase of p-Erk1/2. This was supported by the 20 times more ROS measured in the mid-old cells than the young cells (Fig. 7A). When FACS analysis was employed to measure ROS generation, data acquisition from the old cells was impossible to obtain population-gated, because the size was too large and because of the fragility of the old cells, thus necessitating the use of the mid-old cells for the experiment. Even though the cells were stimulated with 1 mM H$_2$O$_2$ and markedly induced p-Erk1/2 levels, the huge induction could be completely prevented by NAC pretreatment (Fig. 7B). Furthermore, treatment of the young cells only once with a lower concentration of H$_2$O$_2$ (200 μM) significantly inhibited phosphatase activity along with the concomitant “increase” of p-Erk1/2 proteins 5 min after the treatment. However, when treated repeatedly with H$_2$O$_2$ at every 8 h, the PP1/2A activities remained significantly inhibited even after 72 h (Fig. 7D). The levels of p-Erk1/2 in the young cells were reciprocally regulated with the PP1/2A activities by the same treatment. Moreover, treatment of the mid-old cells with antioxidant NAC induced morphological changes of the senescent to the young cells (Fig. 9), concomitant with the reduction of senescence-associated β-galactosidase expression (Table II). H$_2$O$_2$ could directly oxidize PP1C-α, as proved by NBD-Cl modification of Cys-SOH species (Fig. 10A). However, H$_2$O$_2$ reduced PP1 activity not only by cysteine oxidation but also by oxidation of metal ions, which were located in the active site of the PP1C-α (63). This was evidenced by ascorbate (15 mM) recovery of H$_2$O$_2$-inhibited PP1 activity as much as DTT (Fig. 10B), indicating the oxidation of metal ions in the active site during cellular senescence. The concentration of ROS was 20 times higher in the mid-old cells than the young HDF; therefore, when the cysteine residue oxidized by H$_2$O$_2$ was determined by ESI-MS/MS analysis, 25 mM H$_2$O$_2$ was applied instead of 2.5 mM. H$_2$O$_2$ completely oxidized Cys$^{62}$ and Cys$^{105}$ of PP1C-α to sulfonic acid without sulfenic and sulfenic acids (Fig. 11, C and D); however, the effect of air oxidation could not be ruled out during the in-gel trypsin digestion at 37 °C overnight. Sulfonic acid is sensitive to air, as has been pointed out (64). Moreover, oxidation of Cys$^{62}$ might be very important for reducing the PP1/2A activity during cellular senescence, because of its conservation between the two enzymes. The bulky Cys-SO$_3$H mediated disruption of tertiary structure might be expected based on the stereo view of PP1C-α (Fig. 12).

These data strongly indicate that H$_2$O$_2$ generated during cellular senescence directly regulated both PP1/2A activities and p-Erk1/2 level through oxidation of cysteine residues of PP1/2A, further suggesting that H$_2$O$_2$ might be the major culprit of the significantly reduced PP1/2A activity in the senescent HDF cells. Considering that the inhibition of PP1 activity in brain cortex of the inhibitor-1 transgenic mice was significantly different depending on the training schedule and that the long-term memory was dependent on the PP1 activity in the experimental animal, which mimics the physiological aging in animal and human being (50), the significant inhibition of PP1/2A activities and accompanying phosphorylation of Erk1/2 proteins in the mid-old and old HDF cells in this study impose a great credence upon the physiological importance of PP1/2A during cellular senescence.

It has been reported recently (65) that aging compromises peroxisosomal targeting signal 1 (PTS1) protein import, with the critical antioxidant enzyme catalase; the number and appearance of peroxisomes are altered in old cells accompanied by the accumulation of Pex5p on their membranes. Concomitantly, old
cells produced increasing amounts of the toxic metabolite hydroxyl peroxide, and increased load of ROS may further exacerbate the effects of aging. It is now generally accepted that Pex5p, the receptor for most peroxisomal matrix proteins, cycles between the cytosol and the peroxisomal compartment, and insertion of Pex5p into the peroxisomal membrane is cargo protein-dependent (66). Pex5p binding to Pex13p, one of the import machinery is essential for import of catalase with PTS1-like signal (67). Therefore, failure of catalase import into peroxisomal matrix might plausibly explain the 20 times higher H₂O₂ level in the mid-old cells than that in the young cells (Fig. 7A).

MKP3 can physically associate with Erk1/2 (24), and this association can consequently activate the phosphatase activity of MKP3 (25) through conformational change of the latter. It has been proposed that MKP3 can exist in two distinct conformations, the open and closed (52). In the open conformation, the enzyme is catalytically incompetent, because the general acid (Asp262) is positioned away from the active site (His/Val)-X-Cys (293)-Arg (Ser/Thr) side chains adopted by the active site Cys293 and Arg299 residues are not optimal for catalysis. However, the binding of Erk2 to the N-terminal domain of MKP3 facilitates the repositioning of active site residues and accelerates the general acid loop closure, accompanied by the activation of MKP3 (52). The N-terminal domain of MKP3 and Erk2 revealed a high affinity for binding interaction (68). Furthermore, the p-Erk2 is a highly specific substrate for MKP3 with kcat/Km of 3.8 × 10³ mol/l that is over 6 orders of magnitude higher than Erk2-derived phosphopeptide encompassing the active site Cys293-X-Arg299-(Ser/Thr), and the side chains adopted by the active site residues of MKP3 facilitates the repositioning of active site residues and accelerates the general acid loop closure, accompanied by the activation of MKP3 (52).

In summary, SA-p-Erk1/2 proteins accumulated in the cytoplasm of old HDF cells were due to the reduced PP1/2A activities and inactivation of cytoplasmic MKP3, resulting in retention of p-Erk1/2 in the cytoplasm and prevention of their translocation to the nucleus. Failure of nuclear translocation of p-Erk1/2 might result in stoppage or slow down of mitosis in old cells. These observations are most plausibly explained by oxidation of the conserved Cys262 in PP1/2A and the molecules around the active site of MKP3 by ROS, which was immensely generated during the senescence process.

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Constitutive Induction of p-Erk1/2 with Decreased PP1/2A

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