Significance of Nuclear Relocalization of ERK1/2 in Reactivation of c-fos Transcription and DNA Synthesis in Senescent Fibroblasts*

Two of mitogen-activated protein kinases (MAPK), p44mapk/p42mapk extracellular signal-regulated kinases (ERK1/2), translocate into nuclei following activation and play critical roles in connecting the signal to gene expression and allowing cell-cycle entry. Here we found that the nuclear translocation of ERK1/2 in response to growth stimuli was significantly inhibited in senescent cells that were irreversibly growth arrested, compared with presenescent cells. The activation step of these enzymes was not impaired, since ERK1/2 were phosphorylated and activated in senescent cells as efficiently as in presenescent cells. By elaborately localizing ERK2 in the nuclei of senescent cells, we could restore c-fos transcriptional activity upon growth stimuli, which was repressed in senescent cells. Furthermore, the nuclear localization of ERK1/2 has been suggested to potentiate the proliferative activity of the senescent cells in collaboration with adenovirus E1A protein. More importantly, SV40 large T antigen, the strong inducer of DNA synthesis, had the inherent ability to restore nuclear relocalization of active ERK1/2 in senescent cells, which was essentially required for the reinitiation of DNA synthesis. Thus, manipulating the relocalization of ERK1/2 into nuclei was expected to open the way to overcome some of the senescent phenotypes.

Cellular senescence is a postmitotic state which normal cells reach after a finite number of cell divisions (1). Attention has recently focused on the important roles of the INK4a locus (2–4) and telomere shortening in this process (5, 6). Concerning the mechanisms of the irreversible growth arrest of senescent cells, p21 and p16 are believed to play critical roles in the persistent hypophosphorylation of Rb and thereby inhibit cell-cycle entry of senescent cells (7, 8). However, much remains to be resolved for the comprehension of senescence. For example, the upstream signaling that accounts for up-regulation of p21 and p16 has been less understood.

Mitogen-activated protein kinases (MAPK) are serine/threonine kinases that play a central role in a wide variety of signaling pathways in eukaryotic organisms. They are activated at most downstream in the signaling cascade, which are initiated by various extracellular stimuli, including growth factors, hormones, and stresses. Subsequently, they convert those stimuli to intracellular signals that control gene expression, eventually leading to cell proliferation, differentiation, and programmed cell death (9, 10). In mammalian cells, MAP kinases have been classified into three subfamilies: extracellular signal-regulated kinase (ERK1 and 2, JNK, and p38 kinase. The best studied MAP kinases, ERK1/2, have been shown to play a pivotal role in processes such as re-entry of fibroblasts into the cell cycle (11). These enzymes are catalytically activated by MAPK kinase (MAPKK) through dual phosphorylation at two key regulatory threonine and tyrosine residues (12). The substrates of ERK1/2 distribute throughout cells from plasma membrane to the nucleus. Thus, subcellular localization of ERK1/2 is an important determinant of their functions. In response to extracellular stimuli, ERK1/2 are activated and translocate into the nucleus, while they are largely cytoplasmic in unstimulated cells (13–15).

In this study, we investigated activation and localization of ERK1/2 in replicatively senescent human fibroblasts and tried to elucidate their involvement in cellular senescence. Given the positive role of ERK1/2 in cell cycle entry, it was suspected that the signaling pathway controlled by ERK1/2 should be down-regulated somehow in the senescent cells that withdraw from cell cycle irreversibly. We show evidence that nuclear localization but not activation of ERK1/2 is impaired in the senescent cells and that relocalization of ERK1/2 in the nuclei of the senescent cells is sufficient or required for overcoming some of the senescent phenotypes.

EXPERIMENTAL PROCEDURES

Cell Culture—TIG-3 and TIG-7, the human cell lines of normal diploid fibroblasts, provided by the Japanese Cancer Resources Bank were grown in a Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS) and kanamycin (50 μg/ml). The cultures were divided 1:4; 1 passage was comparable to 2 PD (population doublings). We considered the cell population around 60 PD as the senescent stage when less than 6% of the cells incorporated BrdUrd after 72 h and more than 90% were senescence-associated β-galactosidase positive, which is a biomarker of cellular senescence (16).

Antibodies—Monoclonal antibodies used in this study are anti-HA epitope (12CA5, Roche Molecular Biochemicals), anti-SV40 large T antigen (Pub101, Santa Cruz Biotechnology, Inc.), anti-pan ERK1/2 (ERK1 + ERK2, Zymed Laboratories Inc.), anti-activated MAP kinase (Sigma), and anti-Myc tag (Invitrogen). Polyclonal antibodies are anti-Sp-1 (sp-1 (pogp2)-G, Santa Cruz Biotechnology, Inc.), anti-active ERK1/2 (the dual-phosphorylated p44/42MAPK, Promega), anti-phospho-Erk-1 (Ser383) (New England Biolabs), anti-HA (Zymed Laboratories Inc.), and anti-luciferase (Transformation Research, Inc.). Secondary antibodies, fluorescein isothiocyanate-conjugated goat anti-mouse modeoxyuridine; HA, hemagglutinin; NLS, nuclear localization signal; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole.
FIG. 1. Activation of ERK1/2 in the pre- and senescent cells after growth stimuli. A, pre-(39 or 31 PD) and senescent (60 or 57 PD) cells of TIG-3 or -7 were stimulated with 10% FCS for 0 (−) or 1 h (+). Total proteins were analyzed by Western blotting with the indicated antibody. In C, TIG-7 cells of the indicated PD were treated as in A and fractionated as described under "Experimental Procedures." Triton-soluble and -insoluble fractions were designated as S and P, respectively. The upper and lower bands corresponded to ERK1 and -2, respectively. Almost equal amounts of total ERK2 detected with anti-pan ERK2/2 were present in each set of extracts. B, TIG-7 cells of the indicated PD that were serum starved for 48 h were stimulated with 10% FCS. After the indicated times, cell lysate was prepared and ERK1/2 kinase activity was determined in vitro according to the manufacturer's instructions. First, active ERK1/2 kinase were selectively immunoprecipitated by a phospho-antibody to the kinase and then, the resulting immunoprecipitate was incubated with a Elk-1 fusion protein as a substrate in the presence of ATP. The amount of phosphorylated Elk-1 fusion protein was determined by Western blotting with a phospho-Elk-1 antibody followed by quantification with Lightcapture (ATTO Co., Tokyo, Japan). The values were shown as a ratio to the control (time 0 of 38 PD presenescent cells) after normalization with the total amounts of ERK1/2 in each cell lysate that were estimated by Western blotting with anti-pan ERK1/2.

IgG antibody and tetramethylrhodamine B isothiocyanate-conjugated goat anti-rabbit IgG antibody were purchased from DAKO.

Western Blot and Kinase Activity Assay—Cells were solubilized in lysis buffer (50 mM Heps, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 2 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) or disrupted with Dounce homogenizer and fractionated into Triton X-100-soluble and -resistant fractions in the buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 2 mM MgCl2, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin). The proteins were quantified using Bio-Rad Protein Assay (Bio-Rad), and equal amounts were loaded in each lane. After SDS-polyacrylamide gel electrophoresis, resolved proteins were electrophoretically transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech). The membranes were then probed with a primary antibody followed by a secondary one. The immune complexes were detected using enhanced chemiluminescence, according to the manufacturer’s protocol (NEN Life Science Products).

ERK kinase activity was measured with p44/42 MAP Kinase Assay Kit (New England Biolabs) according to the manufacturer’s instructions. Briefly, immune complex precipitated by anti-active ERK1/2 antibody were incubated with a Elk-1 fusion protein in the presence of ATP, and phosphorylation of Elk-1 at Ser383, which was a major phosphorylation site by ERK1/2, was evaluated by Western blotting using a phospho-Elk-1 antibody. The bands derived from phospho-Elk-1 was quantified by Lightcapture (ATTO, Co., Tokyo) and normalized by total amounts of total ERK/2 detected with anti-pan ERK/2 were present in each set of extracts.

Expression Plasmids—A series of HA-tagged ERK2 expression vectors, including wild-type, nuclear localization signal (NLS)-added (+NLS), and kinase-defective (kd) vectors, were constructed using a pcG-HA vector (17). The open reading frame of ERK2 was polymerase chain reaction-amplified and inserted into the vector with or without a NLS sequence. The NLS sequence used here was derived from SV40 large T antigen and expressed at the N-terminal of the resulting fusion protein with ERK2. A kinase-defective ERK2 derivative (kd-ERK2) mutated at Lys44 to Arg and Lys50 to Arg (18) was created by the Site-directed Mutagenesis System (TaKaRa Shuzoh, Co., Kyoto) using a mutagenic oligonucleotide, 5′-GAGTAGCTATCGAGAATCGCCTTTG-3′. The expression plasmids for Elk-1 (Myc tagged) (19) and constitutive active MEK1 (SDSE-MAPKK) (20) were generously provided by Dr. Nishida (Kyoto University). The luciferase reporters were based on the pGL3 basic luciferase reporter plasmid (Promega). Promoter fragments of c-fos (−2.2 or −0.74 kilobases) were subcloned into the vector to yield c-fos (−2.2) and (−0.74) luciferase reporter. The c-fos SRE sequence was mutated using the mutagenic oligonucleotide 5′-CCTCCCCCTTACAACTGATGTCCATATTAGG-3′ to yield mSRE/c-fos luciferase reporter (21). SV40 large T antigen expression plasmid, pSVb18-8-16, was obtained from DNA Bank, Tsukuba Life Science Center, RIKEN. E1A expression plasmid, pAd2-128-E.1A (22), were kindly provided by Dr. Oda (Science University of Tokyo).

Microinjection—TIG-7 cells were plated on glass coverslips. After 24 h, plasmids were microinjected into the nuclei in the culture medium buffered with 0.01 M Hepes for 0.3 s at the constant pressure of 60 hPa. Efferor and reporter plasmids were diluted with sterile phosphate-buffered saline (PBS) to final concentrations of 10 and 250 μg/ml respectively. The injection marker, mouse or rabbit normal IgG, was microinjected together with the plasmids at a concentration of 1.25 mg/ml when needed. Injections were performed using a Zeiss Axiovert 135, Eppendorf 5171 micromanipulator, and Eppendorf 5242 injector.

Immunofluorescence, Luciferase, and BrdUrd-incorporation Assay—Cells, plated on glass coverslips, were washed with PBS and fixed with 3.7% formaldehyde in PBS for 3 min at room temperature, followed by permeabilization in 0.2% Triton X-100/PBS for 2 min at room temperature. Coverslips were washed with PBS and nonspecific sites were blocked by incubation with PBS containing 3% bovine serum albumin for 30 min at room temperature. The cells on the coverslips were then incubated successively with the primary and secondary antibody diluted in PBS/bovine serum albumin for 1 h at room temperature. Each incubation was accompanied by four washes in PBS containing 0.1% Tween 20 for 15 min each. Subsequently, coverslips were incubated for 2 min in a 4,6-diamidino-2-phenylindole (DAPI) solution (0.5 μg/ml DAPI in PBS) to stain DNA, rinsed with PBS, and mounted with glycerol. Fluorescence microscopy was carried out using an Axioscope microscope (Carl Zeiss). As for the luciferase assay, the reporter and the injection marker, mouse normal IgG (mIgG), or the efferor plasmid...
were co-microinjected into the cells. The injected cells were identified by immunostaining using fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody alone or in combination with anti-HA. Luciferase positive cells were visualized by immunostaining using anti-luciferase antibody and microscopically counted. A BrdUrd incorporation assay was performed with a cell proliferation kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. The effector plasmids (10 \(\mu\)g/ml) were microinjected with the injection marker, rabbit normal IgG (10 mg/ml), into the senescent TIG-7 cells. After labeling with BrdUrd, the cells were fixed and incubated with the anti-BrdUrd monoclonal antibody, followed by a mixture of secondary antibodies to mouse IgG (fluorescein isothiocyanate-labeled) and to rabbit IgG (tetramethylrhodamine B isothiocyanate-labeled). The DNA was stained with DAPI to locate the nuclei.

RESULTS AND DISCUSSION

Activation of ERK1/2 in Pre- and Senescent Cells—We first examined the activation of ERK1/2 upon growth stimuli in pre- and senescent TIG-3 and -7 human normal diploid fibroblasts. The results of Western blotting using the antibody that specifically recognizes the dually phosphorylated ERK1/2 (anti-active ERK1/2) clearly showed that both ERK1/2 were phosphorylated in senescent cells in response to 10% FCS as efficiently as in presenescent cells with the same time course (Fig. 1A). To verify and compare the kinase activity of ERK1/2 quantitatively between pre- and senescent cells, \textit{in vitro} kinase assay was performed using Elk-1 as a substrate. In parallel with the increase in phosphorylation, kinase activity of ERK1/2 was remarkably elevated in the cell lysate from the senescent cells as well as that from presenescent cells in response to 10% FCS (Fig. 1B). Almost equal levels of activation were observed in pre- and senescent cells in a time- and dose-dependent manner to the stimulus. Together, the results clearly demonstrated that the signals were properly transmitted from cell surface receptors to ERK1/2 in the senescent cells.


diagram

\begin{figure}
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\caption{Relocalization of ERK1/2 in the pre- and senescent cells after growth stimuli. Pre- (A and C) and senescent (B and D) TIG-3 cells were incubated with PDGF (10 ng/ml) for 0 (–) or 30 (+) min. Immunofluorescence was performed as described under “Experimental Procedures.” The first antibodies used were against total ERK1/2 (pan-ERK1/2) (left panels of A and B) and against active ERK1/2 (anti-activated MAP kinase from Sigma) (left panels of C and D). DNA was stained with DAPI. In A and B, Sp1 was immunostained simultaneously and shown in the right panels. The arrows in D indicated the location of the same nuclei in both panels.}
\end{figure}

Inhibition of Nuclear Relocalization of Activated ERK1/2 in the Senescent Cells—Despite the proper activation accompanying the phosphorylation, which was reported to be sufficient to cause nuclear translocation of ERK1/2 (14), we found that the
phosphorylated forms of ERK1/2 in the senescent cells were distributed exclusively in the 0.1% Triton-soluble fraction and not in the insoluble fraction containing nuclei either in the presence or absence of the stimulus. In the presenescent cells, the increase in the phosphorylated forms was observed not only in the soluble but also insoluble fractions. The increase in the insoluble fraction appeared to be underestimated possibly because of redistribution of the nuclear proteins during fractionation of the cells (Fig. 1C). To investigate the localization of ERK1/2 in the cells in further detail, we conducted immunostaining. When the presenescent cells were immunostained with the antibody to ERK1/2 after treatment with PDGF, strong nuclear staining was observed with faint residual staining in the cytoplasm from 30 to 120 min after stimulation in all of the cells, as originally found by Chen et al. (23) (Fig. 2A). In the senescent cells, however, the staining signals were not concentrated in the nuclei but diffusely distributed within the cells at any time point from 1 to 120 min after the stimulation as shown in Fig. 2B. Essentially the same results were obtained using an antibody to active forms of ERK1/2 (Fig. 2C). In this case, faint nuclear staining was sometimes observed in nearly but not completely senescent cells. However, even in such cells, the nuclear staining was remarkably weak compared with that in the presenescent cells. Therefore, it was most likely that the nuclear localization of the activated forms of ERK1/2 was significantly depressed in the senescent cells. Subsequently, we confirmed the above result using HA-tagged ERK2 exogenously expressed by microinjection and visualized with the antibody to the tag. Nuclei were barely stained in the senescent cells (Fig. 3B), while strong nuclear signals were observed in the presenescent cells in response to serum stimulation (Fig. 3A). Since Sp1 transcription factor was detected in the nuclei of the senescent cells (Fig. 2B, right panels), the accessibility of antibodies to the nuclei did not appear to be generally hindered during preparation of the senescent cells.

If the above notion was correct, it was reasoned that substrates of ERK1/2 in the nuclei should remain unphosphorylated in the senescent cells even when the cells were stimulated to activate the kinase. This possibility was examined using a transcription factor, Elk-1, which was a well-established substrate of ERK1/2 in the nuclei (11, 30, 31). To this end, the expression plasmid of Myc-tagged Elk-1 was introduced into the cells by microinjection. After 24 h, the cells were stimulated with PDGF, and the phosphorylation of Elk-1 was visualized by immunostaining with the antibody to phospho-Elk-1. As predicted, the nuclei of the senescent cells were hardly immunostained with the anti-phospho-Elk antibody despite the successful expression of the Elk-1 protein, which was proven by immunostaining with anti-Myc antibody (Fig. 4A). Under the same condition, the nuclei of the presenescent cells were distinctively stained with the antibody in response to the stimulus (Fig. 4A). The fraction of the nuclei positive for phosphorylation of Elk-1 was estimated microscopically, and the abolishment of Elk-1 phosphorylation in the senescent cells was evidently shown (Fig. 4B). Similarly, the phosphorylation of Elk-1 in the nuclei was significantly inhibited in the senescent cells when the constitutive active form of MAPKK was expressed to activate ERK1/2 instead of the PDGF treatment (Fig. 4C). Only in 7.0% of the senescent cells, the phosphorylation of Elk-1 occurred, whereas 89.7% nuclei were positive in the presenescent cells. These experiments provided further support for the conclusion that nuclear localization was impaired in the senescent cells.

This phenomenon was observed not only in the senescent cells obtained from both TIG-3 and -7 but also in quasi-senescent young cells that were exposed to H2O2 and irreversibly ceased to proliferate long before replicatively senescing (data not shown). In addition, similar observations were reported in senescent and terminally differentiated melanocytes (24). In this case, however, ERK2 was retained in the cytoplasm only because it was not phosphorylated and thus inactive. These results implied that the inhibition of nuclear translocation was closely associated with the irreversible growth-arrested state and not merely a defect as a consequence of an accumulative number of cell divisions of the senescent cells. In accordance with this notion, ERK1/2 were found to be localized in the nuclei in an immortalized TIG-3 derivative that had overcome the growth-arrested state of senescence and undergone an in-
Concerning the mechanism by which ERK1/2 are transported into nuclei, several possibilities have been proposed (11, 13–15, 26); however, the details are still a matter for discussion.

Inhibition of nuclear localization in senescent cells might result from the nuclear export of ERK1/2 by a nuclear export signal-like sequence, which predominated over their import. However, this is unlikely because even in the presence of leptomycin B, which is a specific inhibitor of the nuclear export signal function (27), ERK1/2 did not accumulate in the nuclei of the senescent cells (data not shown). Another possibility was that ERK1/2 were somehow masked or degraded so promptly in the nuclei of the senescent cells that they escaped detection. This was also unlikely, because +NLS-ERK2 (ERK2 carrying the NLS sequence of SV40 large T antigen at its N-terminal) that was forced to be localized in the nuclei by its NLS was clearly detected in the nuclei of the senescent cells by a similar immunofluorescence technique (Fig. 3E).

Effect of Nuclear-targeted ERK2 on c-fos Transcriptional Activity in the Senescent Cells—The next question to be addressed here is as to whether relocation of ERK1/2 could restore any of the senescent phenotypes. Although several transcription factors have been known to be substrates for ERK1/2 in the nuclei (9), the biological significance of the nuclear translocation of ERK1/2 had been unclear until the recent work done by Brunet et al. (11) which substantially showed that the inhibition of this translocation repressed the gene expression as well as the re-entry of cells to the S phase following growth factor stimuli. These results, together with our above observations, suggested that inhibition of some nuclear events in the senescent cells, including gene expression and DNA synthesis, was partly due to the retention of ERK1/2 in cytoplasm under growth stimuli, thereby blocking the signals to the nuclei.

To test this hypothesis, we first focused on the c-fos gene expression in response to serum stimulation, which was repressed in the senescent cells (28). In quiescent young cells, c-fos is induced immediately after serum stimulation (29), which depends on the relocalization of ERK1/2 into the nuclei (11). The efficient transcriptional response of c-fos to serum is mediated via ternary complex formation on the serum response element (SRE) of the gene (21). As proven by Gille et al. (30, 31), ERK1/2 played essential roles in this complex formation through phosphorylation of Elk-1, one of the components of the ternary complex. In the present study, transcriptional activity of the c-fos gene was monitored using the luciferase reporter that was placed under the 2.2-kilobase upstream sequence encompassing the c-fos enhancer/promoter. Prior to the experiment, we tested whether this reporter responded to serum appropriately. We microinjected the reporter plasmid together with an injection marker, mouse IgG, into the nuclei of pre- and senescent cells. Following 48 h of incubation in a serum-starved medium, cells were stimulated with 10% FCS for 2 h and immunostained with the antibody to luciferase (Fig. 5B). In the quiescent presenescence population, the number of luciferase-positive cells increased from 10% of the basal level to 40% by serum stimulation (Fig. 5A, bar 1, pre/-FCS; 2, Pre/+FCS). In contrast, only 10% of the cells were luciferase positive even after serum stimulation of the senescent population (Fig. 5A, bar 3, Senes/-FCS; 4, Senes/+FCS). Thus, this assay was thought to reflect the transcriptional behavior of the endogenous c-fos gene. In addition, the increase in reporter activity was completely abrogated when the kinase-defective/dominant-negative ERK2 was co-microinjected with the reporter plasmid in the presenescence cells (data not shown), verifying the crucial role of ERK2 in the transcriptional response of c-fos in our system, as presented before (11, 30, 31).

Subsequently, we tried to recover c-fos inducibility in the

finite number of cell divisions (data not shown).

The above results would seem to indicate that the nuclear transport system of cellular proteins is generally impaired in the senescent cells. However, this was not the case as demonstrated in Fig. 3C. We examined the localization of SV40 large T antigen whose nuclear translocation depends on its NLS (25). SV40 large T antigen was found to be localized exclusively in the nuclei of the senescent cells as well as in normal cells, suggesting that the NLS-dependent nuclear transport system was not impaired in the senescent cells. Since nuclear translocation of ERK1/2 was independent of the NLS (13), the machinery specific to ERK1/2 transportation appeared to be inhibited.
Fig. 5. c-fos transcriptional activity was restored by the presence of +NLS-ERK2 in the nuclei of the senescent cells. A and B, pre- or senescent cells were microinjected with the c-fos (-2.2) luciferase reporter together with the marker, mouse IgG (mIgG), and incubated in a serum-starved medium for 48 h. Subsequently, the cells were treated with 10% FCS for 0 (-) or 2 h (+) and immunostained as described under “Experimental Procedures.” The injected cells and their c-fos transcriptional activity were labeled with fluorescein isothiocyanate (green, left panels of B) and tetramethylrhodamine B isothiocyanate (red, right), respectively. The luciferase positive percentages are shown in A. The numbers of cells successfully microinjected were 93, 69, 69, and 74, from bars 1 to 4, respectively. C and D, senescent cells were co-microinjected with the reporter and -NLS-wild-type or kinase dead (designated as kd) ERK2 effectors, and were cultured in a 10% serum-containing medium for 24–48 h. The percentages of luciferase positive cells (red in right panels of D) among the cells expressing the effectors (green in left panels of D) are shown in C. The results were mean ± S.D. obtained from at least three independent experiments. The numbers of cells successfully microinjected were 69, 69, 124, 151, 115, and 175, from bars 1 to 6, respectively. The basal activities were examined in pre- (bar 1, Pre mIgG/fos) and senescent cells (bar 2, mIgG/fos) in the same way as A and B. The c-fos (-2.2) and (-0.74) reporters gave substantially the same results. The basal activity of the mSRE reporter was almost the same as the parental reporter.

senescent cells by forcing ERK to localize in the nuclei. To this end, we used the aforementioned +NLS-ERK2. The +NLS-ERK2 expression vector was co-microinjected with the c-fos reporter plasmid into the nuclei of the senescent cells; the cells were incubated for 48 h under the continuous presence of serum to ensure that the expressed glucaminase that was expressed specifically in keratinocytes (data not shown). This suggested that the substrate(s) targeted by +NLS-ERK2 in the nuclei was specific to the c-fos enhancer/promoter. Second, kinase-defective +NLS-ERK2 (kd-ERK) did not induce an increase in the c-fos transcription (Fig. 5C, bar 6, +NLS-kd-ERK/fos, and bottom of D). Finally, the effect of +NLS-ERK2 was reduced to 40% when the ternary complex formation on SRE, which was assumed to be the nuclear target of ERK1/2, was specifically impaired by point mutations (21) (Fig. 5C, bar 5, +NLS-ERK/mSRE/fos). Therefore, one of the key targets of +NLS-ERK2 in the nuclei was thought to be Elk-1 in the ternary complex, and not to be nonphysiological substrates. The residual activity of 40% was thought to be attributable to other transcriptional elements such as cAMP response element adjacent to SRE and activated indirectly by transcriptional activation of the reporter by +NLS-ERK2 was considered to be relevant based on the following reasons. First, +NLS-ERK2 did not affect the transcriptional activity of transglutaminase that was expressed specifically in keratinocytes.
ERK1/2 (32). Altogether, these findings substantially supported our hypothesis that the loss of c-fos inducibility in the senescent cells was the consequence of cytoplasmic retention of ERK1/2, and that nuclear targeting of ERK2 was sufficient for the transcriptional reactivation of c-fos.

Previously, Atadja et al. (33) reported on hyperphosphorylation of serum response factor (SRF), another component in the ternary complex, resulting in the loss of SRF binding activity and transcriptional inhibition of c-fos in senescent cells. They proposed an age-specific phosphatase activity as one of the regulators of SRF phosphorylation, although neither the phosphorylation sites nor the phosphatase have been identified. On the other hand, phosphorylation at a specific serine residue of SRF has been demonstrated to increase its binding ability to DNA (34). Among the kinases that have been shown to phosphorylate the site of SRF are pp90<sup>fas</sup> and MAPKAP2, both of which are substrates of ERKs (34). Therefore, to be compatible with the above results, it was speculated that nuclear-targeted ERK2 could improve not only Elk-1 activity directly but also the SRF activity indirectly through activation of either of the two kinases, leading to complete recovery of the ternary complex formation on SRE. The c-fos gene is the first to be activated in response to extracellular stimuli and thereafter affects the expression pattern of genes in a transcription factor network. Keeping in mind other transcription factors targeted by ERK1/2 in the nuclei, such as c-myc and NF-IL6 (9), whose activities also appeared to be repressed in the senescent cells, restoration of nuclear translocation of ERK1/2 could have profound effects on the expression pattern of a large number of genes in the senescent cells, leading to phenotypic rejuvenation of the cells.

**Involvement of ERK in Reinitiation of DNA Synthesis in the Senescent Cells**—Next, we tested whether nuclear-targeted ERK2 counteracts cell cycle arrest during senescence. We microinjected the expression plasmids of ERK2 into senescent cells and the effect on DNA synthesis was examined. After microinjection, the cells were incubated with BrdUrd in the presence of 10% serum for 48 h and then analyzed for BrdUrd incorporation by an immunofluorescence technique. In the senescent cells, +NLS-ERK2 alone could not induce DNA synthesis (data not shown). One of the reasons was thought to be a high expression of p16<sup>INK4a</sup> that was not suppressed by +NLS-ERK2. If so, adenovirus E1A protein could aid +NLS-ERK2 in stimulating DNA synthesis in the senescent cells, since E1A is able to cancel the effect of p16<sup>INK4a</sup> through binding to Rb. Actually, +NLS-ERK2 but not -NLS-ERK2 could stimulate DNA synthesis in the presence of E1A in 25.6% of the cells, when the basal level of DNA synthesis was only 2.1% (Fig. 6A). This potentiation of DNA synthesis by +NLS-ERK2 in collaboration with E1A was reproducibly observed but the effect of +NLS-ERK2 seemed to be only modest. Based on these observations, we changed the approach and investigated the requirement of nuclear localization of ERK1/2 for reprogramming the senescent cells to proliferate. Given that the cellular senescent process comprises multiple stages and that the irreversible growth arrested state is brought about by several kinds of sequentially occurring events during the process (8), it seemed natural that nuclear relocalization of ERK was not sufficient by itself to release the senescent cells from the constraints of the growth arrest. Instead, we hypothesized that nuclear relocalization of ERK could be one of the essential requirements for reinitiation of DNA synthesis. As natural sources to impose the senescent cells to re-enter cell cycle, viral oncoproteins are famous to have such activity. Among them, SV40 large T antigen was the strongest inducer of DNA synthesis. It could induce DNA synthesis in 69.3% of the senescent cells in our system (Fig. 6D, lane −), while E1A induced DNA synthesis in only 12.8% of them (Fig. 6A, lane −). If nuclear localization of ERK1/2 is dispensable for overcoming the senescence-associated cell cycle arrest, it should accompany the effect of SV40 large T antigen in restoring DNA synthesis. Actually, nuclear localization of endogenous ERK1/2 (active forms) was detected in the senescent cells of TIG-7 when the basal level of DNA synthesis was only 2.1% (Fig. 6A). If nuclear localization of ERK1/2 is indispensable for overcoming the senescence-associated cell cycle arrest, it should accompany the effect of SV40 large T antigen in restoring DNA synthesis. Actually, nuclear localization of endogenous ERK1/2 (active forms) was detected in the senescent cells of TIG-7 when the basal level of DNA synthesis was only 2.1% (Fig. 6A). When the HA-tagged ERK2 was expressed exogenously together with SV40 large T antigen and the cells were stimulated with 10% FCS for 30 min, the

*J. K.-Kaneyama, K. Nose, and M. Shibanuma, unpublished data.*
signals visualized by either of anti-HA or anti-active ERK1/2 were concentrated in the nuclei of 58% of the senescent cells as a maximal response (Fig. 6C, lanes SV40). With or without ERK2 exogenously overexpressed, similar results were obtained. E1A could also localize ERK1/2 in the nuclei to some extent (Fig. 6C, lane E1A). However, it was less effective compared with SV40 large T antigen, which was in good agreement with the extent to which each protein could induce DNA synthesis. More importantly, DNA synthesis induced by SV40 with the extent to which each protein could induce DNA synthesis was accompanied by inhibition of the translocation of ERK1/2 in the nuclei is one of the critical requirements for overcoming senescence along with other activities such as telomerase activity.

In conclusion the growth-arrested state of senescent cells was accompanied by inhibition of the translocation of ERK1/2 into nuclei, which resulted in abrogation of nuclear activities such as gene expression and DNA synthesis in response to growth stimuli. Further investigation of the molecular mechanisms regulating this phenomenon would not only lead to a better understanding of senescence but also enable us to control the process pharmacologically or genetically.

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