

Induction of Extracellular Matrix-Remodeling Genes by the Senescence-Associated Protein APA-1

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Human fibroblasts undergo cellular senescence after a finite number of divisions, in response to the erosion of telomeres. In addition to being terminally arrested in the cell cycle, senescent fibroblasts express genes that are normally induced upon wounding, including genes that remodel the extracellular matrix. We have identified the novel zinc finger protein APA-1, whose expression increased in senescent human fibroblasts independent of telomere shortening. Extended passage, telomerase-immortalized fibroblasts had increased levels of APA-1 as well as the cyclin-dependent kinase inhibitor p16. In fibroblasts, APA-1 was modified by the ubiquitin-like protein SUMO-1, which increased APA-1 half-life, possibly by blocking ubiquitin-mediated degradation. Overexpression of APA-1 did not cause cell cycle arrest; but, it induced transcription of the extracellular matrix-remodeling genes *MMP1* and *PAI2*, which are associated with fibroblast senescence. *MMP1* and *PAI2* transcript levels also increased in telomerase-immortalized fibroblasts that had high levels of APA-1, demonstrating that the matrix-remodeling phenotype of senescent fibroblasts was not induced by telomere attrition alone. APA-1 was able to transactivate and bind to the *MMP1* promoter, suggesting that APA-1 is a transcription factor that regulates expression of matrix-remodeling genes during fibroblast senescence.

One defining characteristic of tumor cells is that they proliferate indefinitely when grown in culture. In contrast, most normal mammalian cells have a limited life span and undergo cellular senescence, an irreversible cell cycle arrest, after a defined number of population doublings. This terminal arrest is one mechanism of tumor suppression that cells must overcome during the transformation process (6).

Cellular senescence is initiated in different ways, depending on the cell type and growth conditions. In human cells, telomere length is a critical determinant of cellular life span (38). With each division, telomeres at the ends of chromosomes get incrementally shorter, eventually sending a DNA damage signal that initiates cell cycle arrest. Human fibroblasts will divide 70 to 90 times in culture until their telomeres reach a critically short length. Fibroblasts can be immortalized if telomeres are lengthened through expression of the enzyme telomerase (2).

Human epithelial cells reach an additional block to immortalization before telomeres become critically short. Both keratinocytes and mammary epithelial cells arrest after fewer than 30 population doublings due to elevated levels of the cyclin-dependent kinase inhibitor p16. If epithelial cells repress transcription of p16 through methylation of the p16 promoter or express the human papillomavirus oncogene E7, which disrupts the retinoblastoma pathway, they can bypass this early

arrest and continue dividing until their telomeres reach a critical length (21). Induction of p16 can also be delayed if epithelial cells are grown on feeder layers, leaving telomere length as the only barrier to immortalization (35).

Telomere length is not a factor in senescence of all cell types. Mouse embryo fibroblasts (MEFs), which arrest after very few passages in culture, have extremely long telomeres that do not shorten significantly before the cells reach senescence (40). Instead, mouse fibroblasts accumulate cell cycle inhibitors as they are passaged and arrest due to induction of the ARF-p53 pathway. Cells from *ARF*^{-/-} and *p53*^{-/-} mice as well as cells that acquire mutations and lose function of either gene can bypass senescence and divide continuously (20). The signals that induce ARF and p53 in mouse fibroblasts and p16 in human epithelial cells are not known but may result from the accumulated stress from growth in culture. Primary cells can also undergo senescence in response to activation of oncogenes, such as *RAS* (38). Broadly defined, cellular senescence can be triggered by both internal signals, such as telomere attrition and oncogene activation, and external signals, such as growth conditions. Senescence limits the number of divisions a cell can undergo and therefore acts as a block to transformation.

In addition to being arrested in the cell cycle, senescent cells show altered differentiation functions (5). In the case of human fibroblasts, cells can remain metabolically active for extended periods of time, but they show an altered pattern of gene expression. Senescent fibroblasts express genes consistent with an activated, or wound-healing, function; they express growth

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factors, cytokines, and enzymes that remodel the extracellular matrix (7). Experiments with cDNA microarrays have confirmed this relationship between fibroblast senescence and wound healing, as there is considerable overlap between the transcriptional profiles of senescent cells and cells stimulated with serum (16, 39). The factors that induce transcription of wound-healing genes during senescence are not known, but senescent cells have an altered complement of transcription factors that may contribute to gene expression changes (13).

These phenotypic changes in senescent fibroblasts are an important component of cellular senescence, even though they have not been linked to telomere erosion or cell cycle arrest. Recent studies have demonstrated that senescent but not presenescent fibroblasts can stimulate the proliferation of nearby, initiated epithelial cells, perhaps through expression of secreted proteins (22). This secretory phenotype has also been described in fibroblasts isolated adjacent to tumors in vivo (33), suggesting that senescent cells may stimulate tumorigenesis in vivo through the misexpression of wound-healing genes. Although this seems to contradict the model in which senescence acts as a tumor suppression mechanism, some evidence argues that limiting cellular life span may act both to prevent cancer formation early in the life of an organism and to promote tumorigenesis later in life (6).

A great deal is known about which genes change expression upon fibroblast senescence, but the regulatory molecules that translate the number of cell divisions into an altered phenotype remain to be discovered. It also remains to be determined if telomere attrition and cell cycle arrest are necessary for induction of wound-healing genes upon senescence. While searching for proteins that interact with the tumor suppressor p14^{ARF}, we discovered an uncharacterized zinc finger protein whose expression increased in senescent human fibroblasts. This zinc finger protein, called APA-1 (another partner for ARF), resembled a transcription factor but had no characterized homologs. APA-1 levels increased in both senescent fibroblasts and telomerase (hTert)-immortalized fibroblasts, arguing that its upregulation was independent of telomere length. APA-1 was regulated posttranscriptionally in fibroblasts through modification by the ubiquitin-like protein SUMO-1, which increased APA-1 half-life. Overexpression of APA-1 did not affect the cell cycle, but it did induce transcription of the matrix-remodeling genes *MMP1* and *PAI2*, which are associated with fibroblast senescence. APA-1 both transactivated and bound to the promoter of *MMP1*, suggesting that it is a transcription factor that acts directly on these promoters. We also found that extended-passage, hTert-immortalized fibroblasts expressing high levels of APA-1 had elevated levels of *MMP1* and *PAI2* transcripts, suggesting that APA-1 transcriptionally regulates a senescent phenotype in fibroblasts through a telomere-independent pathway.

MATERIALS AND METHODS

Plasmids. The APA-1 cDNA clone (clone 23667 or GenBank accession number U90919), originally described by Soares et al. (42) and obtained from Wei Yu (Baylor College of Medicine), was subcloned into pGEMT-Easy (Promega) for in vitro translation, pCDNA3 (with and without a hemagglutinin [HA] tag) for transfection, and LXSXN for retroviral transduction. The mouse APA-1 cDNA (GenBank accession number AF295806) was obtained from the American Type Culture Collection (ATCC) (IMAGE clone 1884982).

Human p14^{ARF} was cloned by reverse transcription (RT)-PCR from human

mammary epithelial cells and subcloned into LXSXN for retroviral transduction. The p16 cDNA (originally from Yue Xiong, University of North Carolina) was also subcloned into LXSXN. Plasmids for producing retroviruses (pJK3, pCMV/tat, and pL-VSV-G) were provided by the laboratory of Michael Emerman (Fred Hutchinson Cancer Research Center) (1). HA-tagged ubiquitin, pHA-ubi, was provided by James Roberts (Fred Hutchinson Cancer Research Center). The *MMP1* promoter fragments (GenBank accession number AF023338) *MMP1*-1606 and *MMP1*-624 were cloned by PCR from human fibroblast genomic DNA and subcloned into pGL3-Basic (Promega) for luciferase assays. Quick-change mutagenesis (Stratagene) was used to introduce mutations in the APA-1 binding site within pGL3-MMP1-624, generating pGL3-MMP1-624m.

Cell culture. Human fibroblasts (HFFs) and keratinocytes (HKFs) were derived from neonatal foreskins. LXSXN- and LXSXN/hTert-expressing HFFs were described previously (21). Human fibroblasts and their simian virus 40 (SV40) derivatives (31), HFF, ARF^{-/-} MEF, 293T, and HeLa cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 10% fetal bovine serum and penicillin-streptomycin. HKFs were grown in keratinocyte serum-free medium with supplied supplements (Gibco-BRL). U2OS cells (ATCC) were grown in McCoy's 5a medium with penicillin-streptomycin and 10% fetal bovine serum.

Western blotting. Lysates were prepared for Western blotting by trypsinizing cells, washing with phosphate-buffered saline, and resuspending in WE16th lysis buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 20% glycerol, complete protease inhibitor tablet [Roche]). Lysates were then sonicated and clarified by centrifugation.

Protein concentrations were determined by using the DC protein assay (Bio-Rad). Protein lysates were electrophoresed on SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). Western blots were performed with mouse anti-human p16 (Pharmingen), goat antiactin (Santa Cruz Biotechnology, I-19), mouse anti-p21 (Oncogene Science, WAF1 Ab1), mouse anti-HA (BabCo, 16B12), mouse anti-GMP-1 (SUMO-1, Zymed), mouse anti-p53 (Oncogene Science, Ab6), and goat anti-p14^{ARF} (Santa Cruz Biotechnology, C-18). APA-1 antiserum was generated in rabbits by injection of recombinant, His-tagged APA-1 protein.

Northern blotting. Total cellular RNA was prepared with Qiagen's RNeasy kit. From 20 to 60 µg of total RNA was electrophoresed on 1% agarose-formaldehyde gels, transferred to Hybond-N membranes (Amersham), and hybridized to ³²P-labeled probes. Probes for APA-1 were generated by digesting and gel purifying the 5' half of the APA-1 cDNA and labeling by primer extension with a single antisense primer. All other probes were labeled by random hexamer priming of cDNAs (Roche). The 36B4 loading control probe has been described before (21). IMAGE clones were obtained from the ATCC for the *MMP1* (clone 589115), *PAI2* (clone 70692), *MMP12* (clone 196612), and *MMP2* (clone 1474174) probes. Signals were quantified by phosphorimaging.

Immunoprecipitations. Denaturing immunoprecipitations were used to examine sumoylated APA-1 and HA-ubiquitin-conjugated proteins. Cells were trypsinized, rinsed in phosphate-buffered saline, resuspended in 2% SDS-Tris-buffered saline (TBS), and boiled for 10 min. After boiling, lysates were cooled on ice, and 8 volumes of TBS were added. Lysates were then sonicated on ice and precleared by the addition of 50 µl of protein A/G-agarose (Roche) and rotating at 4°C for 30 min. After clarifying by centrifugation, immunoprecipitations were done overnight at 4°C and then purified by adding protein A/G-agarose for 1 h. Immunocomplexes were washed once with 0.5 M LiCl-TBS and twice with TBS and then eluted into sample buffer. Elutions were electrophoresed on SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotted as described above. In HA-ubiquitin immunoprecipitation experiments, U2OS cells were plated in 15-cm plates and transfected with 10 µg of pCDNA/APA-1 and 10 µg of pHA-ubi (or empty vector control) with the Fugene6 transfection reagent (Roche). After 20 h, 25 µM MG132 (Calbiochem) was added to cells. Lysates were collected 24 h after transfection.

Cycloheximide and proteasome inhibitor treatment. For half-life analysis, HFFs were treated with 25 µM cycloheximide (Calbiochem) and harvested in WE16th lysis buffer at the indicated time points. Protein levels were then examined by Western blotting for APA-1 as described above. In proteasome inhibitor experiments, cells were treated with 25 µM MG132 (Calbiochem), *N*-acetyl-Leu-Leu-Norleu-al (ALLN; Sigma), *n*-acetyl-Leu-Leu-Met-al (ALLM; Sigma), or an equal volume of dimethyl sulfoxide (solvent control) for 4 h.

Retroviral infections. Retroviruses expressing APA-1, p16, p14^{ARF}, or LXSXN were produced and concentrated as previously described (1). Concentrated retroviruses were then used to infect HFFs. At 24 h after infection, cells were expanded 1:2 into complete medium containing 1 mg of G418 per ml. At 10 to 11 days after infection, when selection was complete, cells were harvested for analysis.

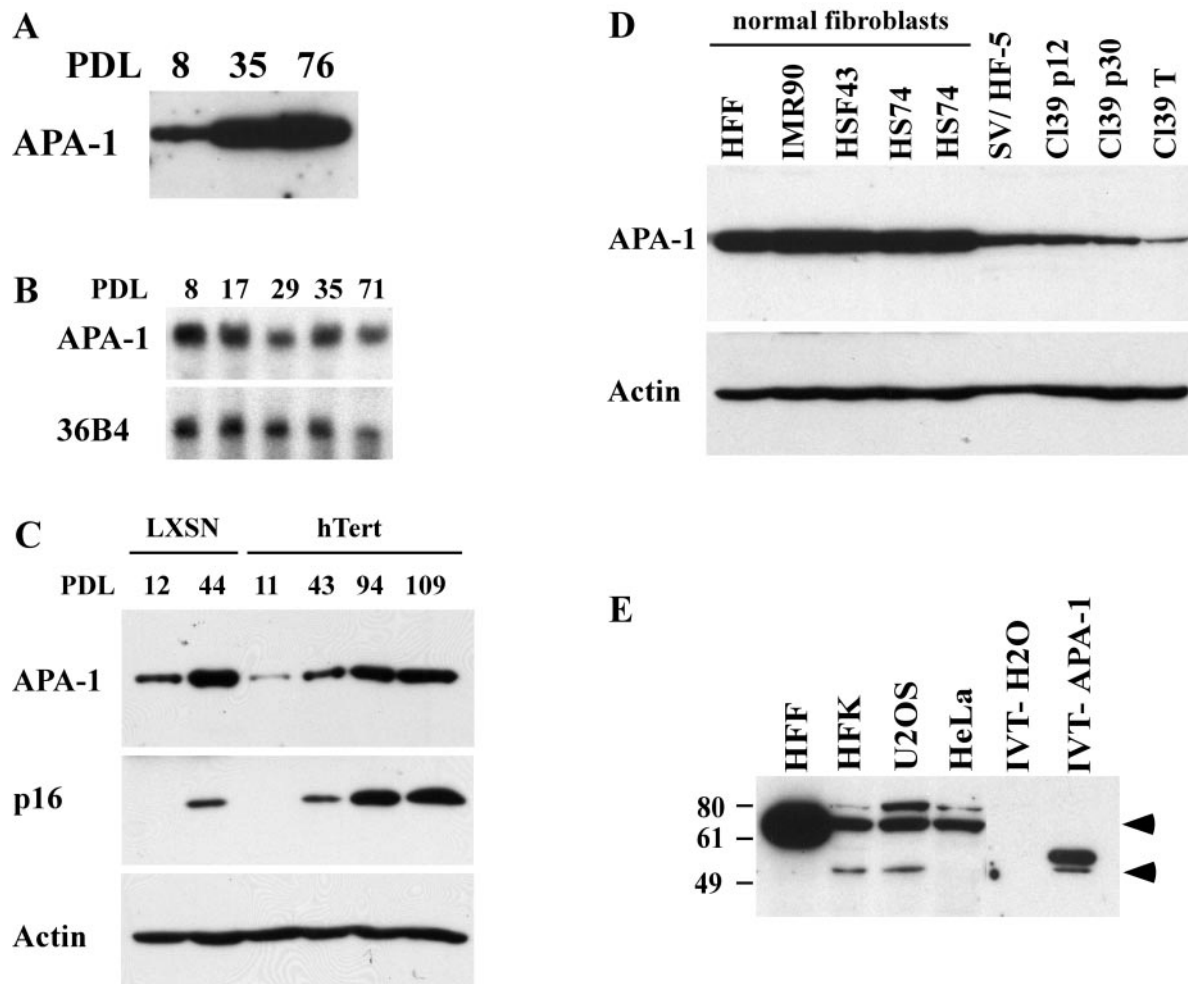


FIG. 2. APA-1 expression in human fibroblasts. (A) Increased APA-1 protein in senescent HFFs. Western blot of APA-1 protein in HFFs at increasing population doubling levels (PDLs). Cells were senescent at PDL 76. Protein concentration of each lysate was measured, and 40 μ g of total protein was analyzed by Western blotting for APA-1. (B) Constant APA-1 mRNA in HFFs throughout their life span. Total RNA (20 μ g) from HFFs at the indicated PDLs were analyzed by Northern blot with probes to APA-1 and 36B4 (loading control). (C) APA-1 and p16 increase in hTert-immortalized fibroblasts. Western blots of 40 μ g of total protein in control (LXSN) and hTert-transduced HFFs. Cells were transduced mid-life span. PDL represent population doublings after selection. LXSN cells reached senescence at PDL 44, and hTert-immortalized cells continued proliferating past PDL 109. Lysates were examined for expression of APA-1, p16, and actin. (D) Decreased APA-1 expression in SV40-transformed fibroblasts. Lysates were collected from several normal human fibroblast types (HFF, IMR90, HSF43, and HS74) as well as cells containing SV40 (SV/HF-5, CI39 p12 and p30, and CI39T). Total protein (40 μ g) was analyzed by Western blot for APA-1 and actin. (E) APA-1 expression in various cell types. Western blot of 40 μ g of protein lysate from HFF, HFK, U2OS, and HeLa cells as well as in vitro-translated APA-1 (IVT-APA-1) and a negative control (IVT-H₂O, rabbit reticulocyte lysate alone). The two predominant forms of APA-1 protein run at approximately 67 and 49 kDa and are indicated with arrowheads

script (Fig. 2B). APA-1 mRNA remained relatively constant at each PDL, demonstrating that increased protein levels in late-passage cells were due to posttranscriptional regulation.

The expression pattern of APA-1 suggested that it may be involved in cellular senescence, so APA-1 expression was analyzed in fibroblasts immortalized by expression of the catalytic subunit of telomerase, hTert. Fibroblasts immortalized by hTert expression bypass senescence but do not adopt a transformed phenotype (2, 18, 29). Telomerase-expressing fibroblasts were established previously and shown to bypass senescence as well as extend the length of telomeres past that of control cells (21). LXSN control cells reached senescence at

PDL 44 after selection, while hTert-expressing cells continued dividing past 109 population doublings.

Surprisingly, APA-1 levels increased with population doubling level in hTert-immortalized cells (Fig. 2C). APA-1 levels were lower in hTert-immortalized cells than LXSN cells at comparable passages, and levels in senescent LXSN cells (PDL 44) were equivalent to those in extended-passage hTert-expressing cells (PDL 109). The cyclin-dependent kinase inhibitor p16 was also found to increase in hTert-immortalized fibroblasts (Fig. 2C). In contrast to APA-1, p16 levels increased at similar rates in LXSN and hTert-immortalized cells, with even higher levels of p16 in extended-passage hTert-immortal-

ized cells (PDL 109) than senescent controls (LXSN PDL 44). The fact that APA-1 increased in both normal and hTert-immortalized fibroblasts suggested that increased APA-1 protein levels were not due to progressive telomere shortening. Instead, a different mechanism is likely to regulate p16 and APA-1 levels in fibroblasts.

In order to clarify the relationship between APA-1 expression and senescence or immortality, we examined APA-1 levels in a variety of primary and transformed cell types. As shown in Fig. 2D, APA-1 was expressed at high levels in several types of presenescent human fibroblasts, including IMR90 fetal lung fibroblasts, a second foreskin fibroblast line (HSF43), and normal bone marrow stromal fibroblasts (HS74). In contrast (Fig. 2E and data not shown), APA-1 expression was reduced in primary human epithelial cells (HFKs, human mammary epithelial cells). We also examined a previously described line of SV40-transformed primary fibroblasts and several other immortal cell lines. SV40-transformed fibroblasts were generated by expression of an origin-deficient mutant of SV40 in normal HS74 fibroblasts (31), resulting in cells with an extended life span (SV/HF-5). These cells subsequently underwent crisis, and rare immortal clones were isolated. One clone, CI39, divided slowly at early passages, but the doubling time decreased at later passages. At passage 30, subclones were isolated, one of which was designated CI39T. The transformants had numerous genetic alterations (31), a telomerase-independent mechanism of stabilizing their telomeres (41), and an altered morphology with some epithelium-like features. Reduced APA-1 levels were seen in SV40 transformants, with the largest decrease in the CI39T subclone (Fig. 2D). APA-1 levels were also significantly reduced in other transformed cell lines tested, including U2OS, HeLa, C33A, SiHa, MDA-MB-231, ZR75-1, HCT116, 293, and 293T (Fig. 2E and data not shown). Taken together, these data suggest that APA-1 may be related to fibroblast-specific functions and that expression increases with prolonged passage in culture.

APA-1 is modified by the ubiquitin-like protein SUMO-1. In addition to our observation that transformed cell lines and primary epithelial cells had reduced levels of the 67-kDa APA-1 protein, we noticed that some cell types (HFKs and U2OS) expressed a smaller form of the protein, of approximately 49 kDa. Also, a smaller form of APA-1 was expressed following transduction with an APA-1-expressing retrovirus (Fig. 4A, 5B, and 6A). This smaller form of APA-1 ran closer in size to the predicted size of 52 kDa and *in vitro*-translated APA-1, although an intermediate band was detected in the *in vitro* translation reaction (Fig. 2E). This suggested that APA-1 was undergoing a large modification in cells and a different, smaller modification in the rabbit reticulocyte lysate.

To rule out the possibility that the large form of APA-1 detected in cells was a cross-reacting protein and confirm that the 67-kDa band was in fact APA-1, a stable line of U2OS cells expressing HA-tagged APA-1 was generated. An APA-1 Western blot revealed that the stable transfectant was in fact overexpressing both forms of APA-1 that had been detected in cells, although the molecular sizes were slightly different due to the HA tag (Fig. 3A). Occasionally, a band of greater than 67 kDa was detected by the APA-1 antibody, but this band varied between experiments and the connection to APA-1 remains unknown. An anti-HA Western blot confirmed that two over-

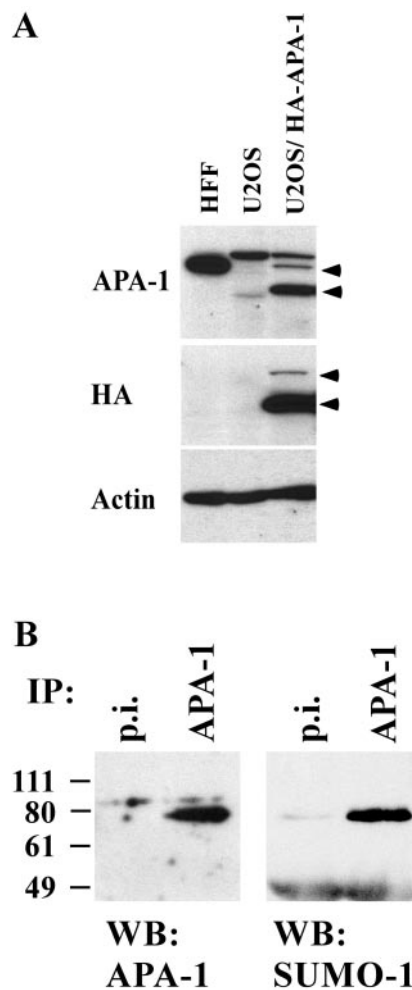


FIG. 3. APA-1 is modified by SUMO-1 *in vivo*. (A) U2OS transfectants express two forms of APA-1. U2OS cells were transfected with HA-tagged APA-1. After selection, the pool of cells was compared to untransfected U2OS cells and HFFs for expression of APA-1. Two forms of APA-1 are overexpressed in the HA-APA-1 cells (arrowheads), one of which runs close to the 67-kDa form seen in fibroblasts. These were observed by Western blot with both anti-APA-1 and anti-HA antibodies. (B) APA-1 in HFFs is modified by SUMO-1. HFFs were lysed by denaturing lysis in SDS, diluted, and immunoprecipitated (IP) with anti-APA-1 antiserum or preimmune serum (p.i.). Immunoprecipitates were eluted in sample buffer, split in half, and loaded onto two gels. These were Western blotted for APA-1 or SUMO-1. The 67-kDa form of APA-1 was immunoprecipitated and was recognized by both antibodies.

expressed forms of APA-1 detected with APA-1 antibody resulted from the exogenous HA-APA-1 cDNA and that APA-1 was undergoing a modification of approximately 18 kDa in cells.

Several types of protein modifications result in large molecular weight shifts of target proteins. One of these is modification by the ubiquitin-like protein SUMO-1. SUMO-1 is an 11-kDa protein, but when added to targets, it can result in a shift of approximately 20 kDa in apparent molecular mass during electrophoresis (27). To test whether APA-1 was modified by SUMO-1 in HFFs, denaturing immunoprecipitations were carried out with APA-1 antibody. APA-1 immunoprecipi-

tates and preimmune controls were then analyzed by Western blotting with an antibody specific to SUMO-1. As shown in Fig. 3B, the large form of APA-1 was recognized by a SUMO-1 antibody, demonstrating that APA-1 is modified by SUMO-1 in vivo.

Sumoylation increases APA-1 half-life. SUMO-1 modification affects the function of target proteins in several ways, depending on the target. In the case of Mdm2 and NF- κ B, SUMO-1 conjugation blocks the addition of polyubiquitin chains and increases the half-life of the proteins (3, 12). To test whether sumoylation affects the stability of APA-1, the half-life of sumoylated APA-1 was compared to that of unmodified APA-1. HFFs expressed high levels of sumoylated APA-1 and almost undetectable levels of unmodified APA-1, but introduction of the APA-1 cDNA into HFFs resulted in overexpression of the unmodified form (Fig. 4A, 0 h). To determine relative half-life, time course experiments were carried out in cycloheximide with control (LXSN) and APA-1-overexpressing HFFs. As shown in Fig. 4A, sumoylated APA-1 (APA-1-S) remained stable and highly expressed in both cell populations throughout the experiment. In contrast, unmodified APA-1 had a half-life of approximately 2 h. The data suggest that sumoylation increases the half-life of APA-1.

One mechanism by which sumoylation could increase APA-1 half-life is that it may block ubiquitin-mediated degradation of APA-1. In order to determine if APA-1 is normally degraded by the ubiquitin-proteasome pathway, HFFs were treated with the proteasome inhibitors MG132 and ALLN. In addition to acting on the proteasome, both of these inhibitors can also block the activity of cysteine proteases and calpains. For this reason, the cysteine protease/calpain-specific inhibitor ALLM was also tested, in order to demonstrate proteasome-specific effects (24). As shown in Fig. 4B, when HFFs were treated with MG132 and ALLN, there was an increase in the level of unmodified APA-1 protein compared to the solvent control. In addition, there was no effect upon treatment by ALLM, demonstrating that increased APA-1 stability resulted specifically from inhibition of the proteasome. As expected, levels of sumoylated APA-1 were not affected by proteasome inhibitor treatment. p53 is known to be degraded by the ubiquitin-proteasome pathway (9, 25) and was also increased in MG132- and ALLN-treated cells.

In most cases, proteins targeted for degradation in the proteasome are first tagged by the addition of polyubiquitin chains (10). We tested whether APA-1 could be polyubiquitinated in vivo by transfecting cells with a plasmid encoding HA-tagged ubiquitin, followed by immunoprecipitation and Western blotting (43). As shown in Fig. 4C, when HA immunoprecipitates were analyzed with APA-1 antibody, a ladder of bands larger than unmodified APA-1 was evident. This modification could also be demonstrated by immunoprecipitating APA-1 and Western blotting for HA-ubiquitin. As previously reported for the c-Jun and cyclin E proteins (11, 43), this assay results in different patterns of high-molecular-weight species depending on the antibody used in the Western blot. This is presumed to occur because the anti-HA antibody will preferentially detect higher-molecular-weight forms that contain greater numbers of attached HA epitopes.

APA-1 overexpression does not induce premature senes-

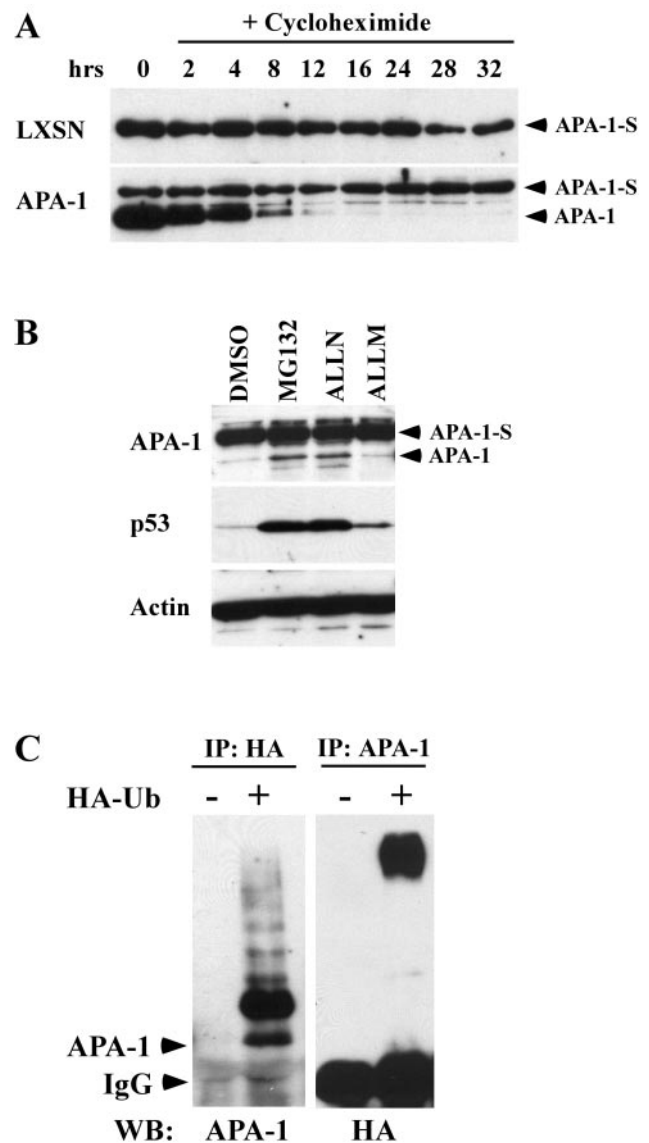


FIG. 4. Sumoylation increases the half-life of APA-1. (A) Cycloheximide time course comparing the stability of unmodified and sumoylated APA-1. HFFs were transfected with either empty vector (LXSN) or APA-1. After selection, cells were plated in 10-cm dishes and treated with 25 μ M cycloheximide to block protein synthesis. Lysates were collected at the indicated time points between 0 and 32 h. Protein concentrations were determined, and 40 μ g of each lysate was analyzed by Western blotting for APA-1. Arrowheads represent sumoylated (APA-1-S) and unmodified (APA-1) APA-1 proteins. Approximate protein levels were quantitated with a Fluor-S-Multiimager (Bio-Rad), and the half-life of unmodified APA-1 was determined to be approximately 2 h. (B) Proteasome inhibitors increase levels of unmodified APA-1 in HFFs. HFFs were treated with 25 μ M each of the proteasome inhibitors MG132 and ALLN, the calpain inhibitor ALLM, or an equivalent volume of dimethyl sulfoxide (DMSO) for 4 h, and lysates were collected. Then 40 μ g of total protein was analyzed for levels of APA-1, p53, and actin. (C) APA-1 is polyubiquitinated in vivo. U2OS cells were transfected with APA-1 and HA-tagged ubiquitin (HA-Ub) or an empty vector control. Denaturing lysates were made, diluted, and immunoprecipitated (IP) with antibodies against HA or APA-1, as indicated. HA immunoprecipitates were analyzed by Western blotting (WB) for APA-1, and APA-1 immunoprecipitates were analyzed by Western blotting for HA. The positions of unmodified APA-1 and immunoglobulin G heavy chain (IgG) are indicated by arrowheads.

cence in fibroblasts. Although the data suggested that increases in APA-1 were associated with increased passages in culture and not specifically a senescent arrest, we still thought it possible that overexpression of APA-1 in fibroblasts might induce a senescence-like cell cycle arrest, similar to what is seen when p16 is expressed at supraphysiological levels (26). In addition, we wanted to test whether induction of premature, telomere-independent senescence through overexpression of p16 or p14^{ARF} could affect APA-1 levels. To address these questions APA-1-, p16-, and p14^{ARF}-expressing retroviruses as well as an empty vector (LXSN) control virus were produced and used to infect HFFs. After selection, cells were harvested for Western blots, labeled with BrdU, and analyzed for cell cycle arrest or stained for senescence-associated β -galactosidase expression, a marker of senescent cells (15). Infections were repeated three times, and a representative experiment is shown in Fig. 5.

Overexpression of all three proteins was seen in Western blots (Fig. 5A); as noted previously, APA-1-transduced cells overexpressed primarily unmodified protein. As expected, expression of p16 and p14^{ARF} resulted in a senescence-like cell cycle arrest with increased p53 and p21 (Fig. 5A to C). In both cases, cells failed to incorporate BrdU into their DNA and stained positive for senescence-associated β -galactosidase activity. Induction of premature senescence by p16 and p14^{ARF} led to a modest increase in APA-1 protein, but the increase in protein was not as great as in late-passage HFFs. In contrast to the p16- and p14^{ARF}-expressing cells, APA-1-overexpressing cells did not arrest in the cell cycle or express detectable β -galactosidase activity despite slight increases in p53 and p21 proteins (Fig. 5A to C). These data argue that APA-1 is not involved in the cell cycle arrest of senescent cells but that it can be induced in response to induction of senescence in a telomere-independent manner.

APA-1 overexpression induces transcription of matrix-remodeling genes that are associated with fibroblast senescence. In addition to undergoing a cell cycle arrest, senescent fibroblasts are known to express genes in a pattern consistent with activation or wound repair (7). In order to determine if APA-1 overexpression altered the expression of any genes known to be associated with senescence, transcriptional profiles were analyzed with cDNA microarrays. RNAs isolated from control (LXSN) and APA-1-overexpressing HFFs were labeled and cohybridized to spotted cDNA microarrays that assay 18,000 human genes. Upon examination of genes whose expression changed more than twofold in two experiments, we identified collagenase-1 (*MMP1*) and plasminogen activator inhibitor-2 (*PAI2*), which were induced upon APA-1 overexpression (data not shown) and have been reported to be upregulated in senescent human fibroblasts (28, 39, 45, 46). The matrix-remodeling gene metalloelastase (*MMP12*) was also induced, although it had not previously been associated with fibroblast senescence.

To confirm induction of these genes by APA-1, overexpression was repeated in a second line of HFFs and mRNA levels were analyzed by Northern blotting. Levels of gelatinase-A (*MMP2*), a matrix-remodeling gene not associated with fibroblast senescence, were examined for comparison. Early-passage and senescent HFF samples were also included. First, APA-1 protein levels were examined by Western blot (Fig.

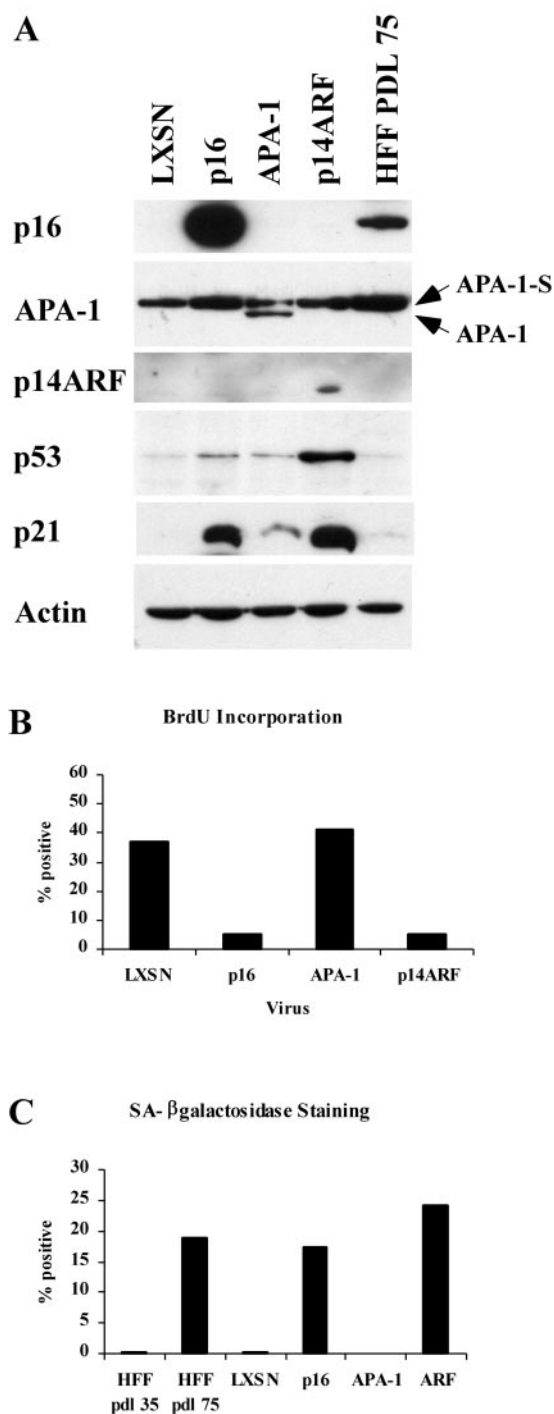


FIG. 5. APA-1 overexpression does not induce senescence. (A) Western blots of transduced HFFs. Mid-life HFFs were infected with concentrated retroviruses expressing empty vector (LXSN), p16, APA-1, or p14^{ARF}. After selection, lysates were collected and protein concentrations were measured. Then 40 μ g of total protein was analyzed by Western blot for expression of p16, APA-1, p14^{ARF}, p53, p21, and actin. Overexpression of p16, p14^{ARF}, and APA-1 was confirmed in the corresponding infections. (B) After selection, the cells from A were labeled with 10 μ M BrdU for 4 h and fixed in 70% ethanol. Nuclei were labeled with anti-BrdU-fluorescein isothiocyanate and propidium iodide and analyzed by flow cytometry. Percent BrdU-positive cells is presented. (C) The cells from A along with early-passage (HFF PDL 35) and senescent (HFF PDL 75) controls were stained for senescence-associated (SA) β -galactosidase. Percent positive cells is presented.

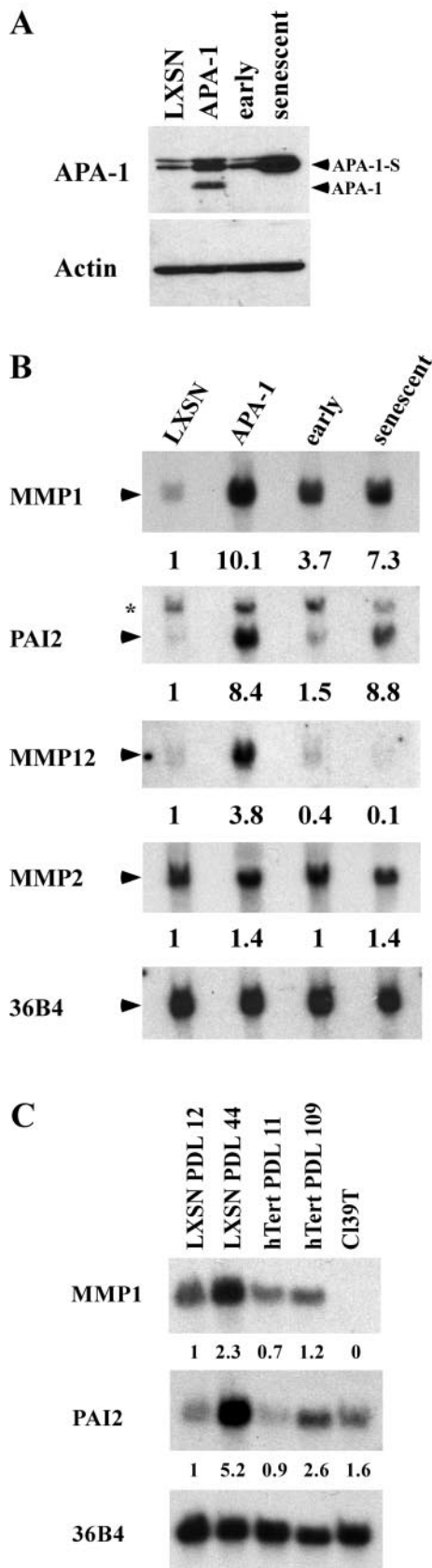


FIG. 6. APA-1 overexpression induces transcription of extracellular matrix-remodeling genes. (A) Early-passage HFFs were infected

6A). In this experiment, both the sumoylated and unmodified forms of APA-1 were increased in APA-1-overexpressing cells. Northern blotting confirmed that transcription of *MMP1*, *PAI2*, and *MMP12* was induced in cells overexpressing APA-1 (Fig. 6B). Interestingly, increases in *MMP1* and *PAI2* mRNAs resulting from APA-1 overexpression were even greater than the induction in senescent HFFs, and although *MMP12* was induced by APA-1, no senescence-associated increase in *MMP12* mRNA was seen. In contrast to the senescence-associated genes that were examined, transcription of *MMP2* was not significantly altered in APA-1-expressing cells or in senescent HFFs (Fig. 6B). These data suggest that APA-1 may transcriptionally induce genes in the wound repair pathway that is associated with fibroblast senescence.

MMP1 and PAI2 increase in hTert-immortalized fibroblasts. We would also expect that APA-1 would induce transcription of matrix-remodeling genes in extended-passage, hTert-immortalized fibroblasts, which have high levels of APA-1 protein. To test this, *MMP1* and *PAI2* mRNA levels were examined in late-passage hTert-expressing HFFs and compared to the levels in LXS control cells as well as Cl39T-transformed fibroblasts. hTert-immortalized cells were analyzed at a passage when APA-1 levels were comparable to senescent LXS cells (Fig. 2D).

For both genes, the increase in transcript level upon senescence in LXS cells was similar to what was seen in previous experiments (Fig. 6B and C). Late-passage hTert-immortalized (PDL 109) cells showed increased levels of both *MMP1* and *PAI2* mRNAs by comparison to early-passage hTert-immortalized cells (PDL 11), but the increases were not as large as in senescent cells (Fig. 6C). Compared to the corresponding early-passage time points, *MMP1* RNA increased 2.3-fold upon senescence of controls and just under 2-fold in late-passage hTert-immortalized cells. Similarly, *PAI2* RNA increased five-fold in senescent controls and threefold in late-passage hTert-immortalized cells. Cl39T cells, which have low levels of APA-1 (Fig. 2C), had intermediate levels of PAI2 and did not express any detectable MMP1.

APA-1 transactivates and binds to the *MMP1* promoter. In order to determine if APA-1 acts as a transcription factor and binds directly to promoters to activate transcription, we examined the effects of APA-1 overexpression on the *MMP1* promoter. First, the ability of APA-1 to transactivate the *MMP1*

with concentrated retrovirus expressing empty vector (LXS) or APA-1 and selected as before. APA-1 protein was analyzed by Western blot and compared to uninfected early-passage (early) and senescent HFFs. Arrowheads indicate the two forms of APA-1. (B) APA-1 induces transcription of senescence-associated matrix-remodeling genes. Total RNA was harvested from the cells described in A and analyzed by Northern blotting for *MMP1*, *PAI2*, *MMP12*, *MMP2*, and 36B4 (loading control). Specific bands are represented by arrowheads, and the asterisk (*) represents residual signal on the PAI2 blot from a previous probe. Signals were quantified by phosphorimaging, and relative signal (with respect to the 36B4 loading control) is indicated beneath each blot. (C) hTert-immortalized fibroblasts have increased levels of *MMP1* and *PAI2* mRNAs, similar to senescent controls. RNA was harvested from the cells described in Fig. 2D as well as Cl39T cells. Then 30 μ g of total RNA was analyzed by Northern blotting for *MMP1*, *PAI2*, and 36B4. Relative signals as determined by phosphorimaging are indicated beneath each blot.

promoter in reporter assays was examined. Luciferase reporter constructs containing 624 or 1,606 bp upstream of the translation start site were created. *ARF*^{-/-} mouse embryo fibroblasts were used to assay transactivation because these cells transfect easily, are immortal in culture, and are primary fibroblasts. Upon cotransfection of APA-1 and either reporter construct, luciferase expression increased 2.5- to 3-fold compared to vector controls (Fig. 7A). This suggested that APA-1 was acting on the 624-bp region of the *MMP1* promoter, just upstream of the translation start site.

Many C₂H₂ zinc finger-containing proteins are sequence-specific DNA-binding proteins (48), suggesting that APA-1 may bind directly to the promoter sequence of the *MMP1* gene. To test this the 624-bp region of the *MMP1* promoter was divided into five fragments, from 137 to 196 bp in length, and these were used as probes in gel shift assays with extracts from either control U2OS cells, expressing very low levels of endogenous APA-1, or U2OS cells transiently overexpressing HA-tagged APA-1.

APA-1 could specifically shift a probe containing the 137 bp directly upstream of the translation start site (data not shown). The 137-bp region was then narrowed down by analyzing five overlapping probes (Fig. 7B). Each probe showed several shifts that were common between control (pCDNA3) and HA-APA-1-transfected cells, but probe C showed one band that was detected only upon addition of HA-APA-1-expressing extract (Fig. 7C). This band was specific for APA-1, as it was competed by addition of either polyclonal anti-APA-1 antibody or an antibody that recognizes the HA tag. This was particularly interesting because probes B and D, which contain all of the sequence included in probe C, did not show similar shifts, suggesting that APA-1 bound to a region downstream of the PEA3 site and at the junction of the B and D fragments (Fig. 7B).

Much work has been done to investigate sequence recognition by C₂H₂ zinc finger proteins. Structural studies have shown that each zinc finger adopts a conserved ββα fold that forms a finger-like structure and that residues in the α-helix contact base pairs in the major groove of DNA (34). Mutagenesis of residues within the α-helix as well as studies of naturally occurring zinc finger proteins have led to a suggested recognition code that predicts which base pairs a particular finger may recognize (8, 48).

We examined the sequence of the zinc fingers in APA-1 and attempted to predict a DNA sequence that it might bind to. Following the suggested recognition code, the sequence X-(C/T)-X₂-(C/T)-G-X-A-X (where X is any amino acid) was predicted as a putative APA-1 binding site, assuming that three of the five zinc fingers bind to DNA in a canonical fashion.

Upon examination of gel shift probe C, we found a putative APA-1 binding site overlapping the junction of probes B and D. To test if the predicted residues were important for APA-1 binding, we made base pair substitutions in probe C, changing the sequence ATATTGGAG to AgATgaGcG (lowercase letters represent substitutions) and tested the mutated sequence in a gel shift assay. As seen previously, extract from HA-APA-1-expressing cells but not control extract resulted in a specific shift of probe C that was competed by anti-APA-1 and anti-HA antibodies, but not a preimmune antibody control (Fig. 7D). After mutation of probe C, the APA-1-specific shift was no

longer detectable upon addition of HA-APA-1 extract, indicating that the residues that APA-1 was predicted to bind to were essential for the APA-1-specific shift and further supporting the role of APA-1 as a sequence-specific DNA-binding protein.

In order to determine if binding to the *MMP1* promoter is necessary for transactivation by APA-1, the 4-bp changes that abolished APA-1 binding in the gel shift assay were introduced into the 624-bp *MMP1* promoter-luciferase construct (MMP1-624m). These substitutions diminished transactivation of the *MMP1* promoter by APA-1 (Fig. 7E). These data indicate that APA-1 is a transcription factor that acts directly on the *MMP1* promoter to induce transcription.

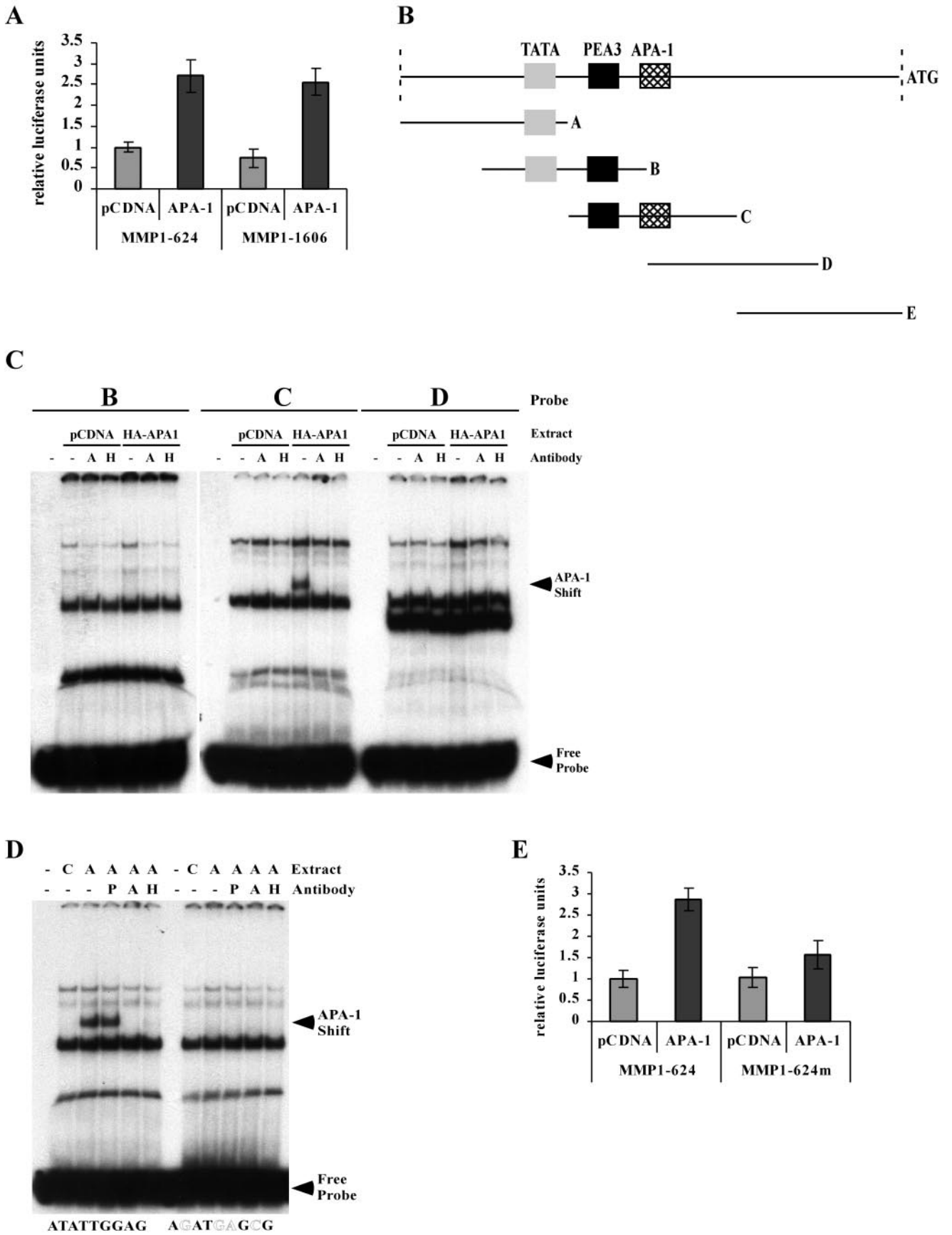
DISCUSSION

In this study, we have begun characterization of APA-1, a zinc finger protein found to be associated with fibroblast senescence. These experiments support several conclusions about the regulation and function of APA-1 during the replicative life span of human fibroblasts. First, we found that APA-1 protein increases with population doubling level in human fibroblasts and that this upregulation is independent of telomere length. Since erosion of telomeres initiates senescence-associated cell cycle arrest in fibroblasts, we analyzed expression of APA-1 in cells immortalized through expression of hTert. APA-1 increased to comparable levels in both control and hTert-immortalized cells, similar to the expression pattern of the cyclin-dependent kinase inhibitor p16. These data argue that more than one signaling mechanism is involved in the aging of human cells in culture.

p16 levels are increased in senescent cells due to altered levels of its transcriptional regulators Ets1 and Id1 (32). Although the factors that alter Ets1/Id1 levels are not known, some studies suggest that p16 increases when cells are exposed to a stress imposed by growth in cell culture. Fibroblasts can be induced to arrest after fewer divisions, with elevated p16 levels, if they are grown in chemically defined medium with low serum levels (35). These findings argue that telomeres progressively shorten until they signal a cell cycle arrest and an additional signal, possibly an accumulation of stress, leads to increases in p16 and APA-1.

The finding that APA-1 induces transcription of several matrix-remodeling genes suggested that some of the phenotypes associated with senescent fibroblasts may be the result of this second signaling mechanism and not due to telomere shortening (Fig. 8B). In fact, we found that expression of the matrix-remodeling genes *MMP1* and *PAI2* was increased in hTert-immortalized cells, which have high levels of APA-1. Increases in *MMP1* and *PAI2* were not as great in late-passage hTert-immortalized cells as in senescent controls, and therefore it is likely that other factors are involved in their transcriptional regulation as well. These data are consistent with a model in which APA-1 is one factor regulating transcription of these genes in late-passage fibroblasts. In addition, these data demonstrate that telomerase-immortalized cells may have important differences from their presenescent counterparts.

The connection between APA-1 and senescence is further strengthened by observations that APA-1 is induced in situations in which fibroblasts undergo telomere-independent se-



nescence. A senescence program can be induced in fibroblasts in response to overexpression of oncogenes and induction of the p16 and ARF tumor suppressor pathways (14, 26, 44). We found that induction of premature senescence due to the overexpression of p16 or ARF led to increased levels of APA-1 protein. In addition, the murine APA-1 protein increased upon senescence of mouse embryo fibroblasts (data not shown), a process known to be independent of telomere shortening. These data further support the model that telomere-independent factors lead to senescence-associated changes in the cell.

We have also shown that APA-1 levels are much lower in primary epithelial cells, transformed cell lines, and fibroblasts transformed by the DNA tumor virus SV40. These findings support the model that APA-1 is a transcription factor that regulates genes important for fibroblast-specific functions, such as extracellular matrix remodeling. Epithelial cells and transformed cell lines do not carry out these fibroblast-specific functions and do not express high levels of APA-1.

Third, we have demonstrated that APA-1 is modified and regulated by the ubiquitin-like protein SUMO-1. Although two predominant forms of APA-1 protein were detected in cells, all fibroblasts examined expressed exclusively the larger form of the protein. This form was modified by SUMO-1 in vivo (Fig. 4C), and recombinant APA-1 can be modified by SUMO-1 in vitro (data not shown). Addition of SUMO-1 to target proteins can have a range of effects, including altering subcellular localization, enhancing transcriptional activation, and increasing protein half-life by blocking ubiquitin-mediated degradation (30). Both forms of APA-1 are found in the cytoplasm and the nucleus, suggesting that sumoylation does not alter APA-1 localization (data not shown). Sumoylation of APA-1 increased the half-life of the protein substantially. In addition, unmodified APA-1 was degraded by the proteasome and was polyubiquitinated in vivo. These data argue that sumoylation of APA-1 may act to block ubiquitination and increase protein stability, although alternative explanations cannot yet be eliminated.

One hurdle in studying sumoylated proteins has been that it is often difficult to overexpress sumoylated forms. It is possible that sumoylation may affect the transcriptional activity of APA-1 and that the unmodified form is more active than the sumoylated form. It is difficult to address this question without being able to overexpress sumoylated APA-1 in cells. Recently, several groups have discovered a number of SUMO-conjugating enzymes with different substrate specificities (17, 19, 37). Although it is not yet clear how many proteins can act as of

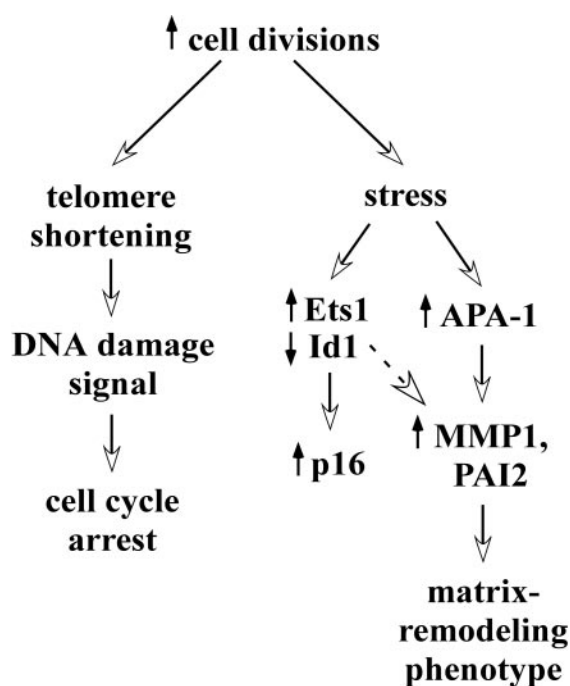


FIG. 8. Model for induction of matrix-remodeling genes during fibroblast senescence. Although telomere shortening induces the senescent cell cycle arrest in fibroblasts, an additional signal, possibly resulting from an accumulation of stress, leads to upregulation of the p16 and APA-1 proteins. If APA-1 is necessary for induction of matrix-remodeling genes in late-passage cells, this suggests that the matrix-remodeling phenotype is separable from telomere-induced cell cycle arrest during senescence.

SUMO ligases, it may be possible to find a SUMO ligase that targets APA-1, allowing us to examine the effects of sumoylation more directly.

Finally, we have shown that overexpression of APA-1 does not result in a senescence-like cell cycle arrest, but induces transcription of extracellular matrix-remodeling genes that are associated with fibroblast senescence. APA-1 was originally identified as an ARF binding protein, which suggested that its expression may affect the cell cycle. But APA-1 overexpression did not have a discernible effect on the cell cycle and did not appear to modulate any known ARF function. For these reasons, we began to examine the functions of APA-1 independent of ARF and discovered that it was capable of regulating

FIG. 7. APA-1 transactivates and binds to the *MMP1* promoter. (A) Early-passage *ARF*^{-/-} mouse embryo fibroblasts (MEFs) were transfected with the indicated reporter plasmid (MMP1-624 or MMP1-1606) and empty vector (pCDNA) or APA-1. Luciferase values were normalized to protein concentration and graphed relative to the pCDNA3 control for the MMP1-624 promoter fragment. Shown is an average of three experiments carried out in triplicate. (B) Schematic diagram of the *MMP1* promoter. Boxes indicate previously described TATA (grey) and *PEA3* (black) elements, as well as the putative APA-1 binding site (hatched). Overlapping probes (A through E) used for gel shift analysis are indicated. (C) APA-1 binds to the *MMP1* promoter. MMP1 gel shift probes B to D were used in binding reactions with control extracts (pCDNA) or extracts expressing HA-APA-1, as indicated. APA-1 antibody (A) or anti-HA antibody (H) only competed away binding of the APA-1-specific shift of probe C. (D) Four-base-pair substitutions disrupt APA-1 binding. Gel shift analysis of probe C with the wild-type sequence (ATATTGGAG) or with mutations in the putative APA-1 binding site (AGATGAGCG, with substitutions shown in outline letters) and control (C) or HA-APA-1 (A)-expressing extracts, as indicated. The APA-1-specific shift is not competed by addition of preimmune antibody (P) but is competed by addition of APA-1 (A) or anti-HA (H) antibodies. (E) APA-1 binding is necessary for transactivation of the *MMP1* promoter. *ARF*^{-/-} MEFs were transfected with MMP1-624 reporter plasmid or MMP1-624m reporter plasmid containing the same changes as in D and the vector control (pCDNA) or APA-1 expression plasmid. Luciferase values were normalized to protein concentration and graphed relative to the pCDNA3 control for the MMP1-624 reporter. Shown is an average of three experiments carried out in triplicate.

the transcription of matrix-remodeling genes that are associated with senescence. It is important to note that induction of these genes by APA-1 was even greater than what was observed in senescent cells and that in general, senescence-associated increases were not as large as have been reported by others (28, 39, 45, 46). It is likely that this is partly due to the experimental conditions. In studies where large differences have been seen, cells were grown in low-serum medium prior to comparison, while in this study cells were grown in 10% serum. Since transcription of wound-healing genes is stimulated by growth factors, it is possible that mRNA levels are artificially elevated in early-passage cells grown in 10% serum. It also remains possible that the overexpressed, unmodified APA-1 is more transcriptionally active than the endogenous, sumoylated protein, and therefore there is more APA-1 activity in cells overexpressing APA-1 than in senescent HFFs.

The amino acid sequence of APA-1 suggested that the protein may bind to DNA and directly activate transcription of target genes. We analyzed the promoter of one APA-1-inducible gene, *MMP1*, and found that APA-1 was capable of both transactivating the promoter in reporter assays and binding to the promoter in gel shift assays. With gel shift analysis, the APA-1 binding site was narrowed to a region of the promoter just downstream of the TATA box and a *PEA3* site. Furthermore, mutation of residues necessary for APA-1 binding diminished its ability to transactivate an *MMP1* reporter construct. These data demonstrate that APA-1 can act as a transcription factor and support a role for APA-1 regulation of *MMP1* during senescence.

Further mutagenesis of the *MMP1* promoter to determine the sequence elements required for APA-1 binding will allow screening of the promoters of other senescence-associated genes for potential APA-1 binding sites. Although the promoter of *MMP1* has been well characterized (36), APA-1 does not appear to bind to any previously defined sequence element. It is important to note that there are many sequence similarities in the promoters of matrix-remodeling genes that are known to be involved in coordinate transcriptional regulation in response to certain stimuli (47). Most of the *MMP* gene promoters as well as the *PAI2* promoter contain several AP-1 sites that bind to the Fos/Jun family of transcription factors (23). These sites are important for induction of the *MMP* genes during growth factor stimulation and wound healing. The promoters also contain *PEA3* elements, which bind the Ets family of transcription factors. Ets factors can function as coactivators for the AP-1 transcription complexes in *MMP* promoters (4). Expression levels of the Ets proteins Ets1, Ets2, and Id1 are also known to be altered upon senescence and to regulate p16 expression (32). In addition, the APA-1 binding site in the *MMP1* promoter is adjacent to one *PEA3* site, raising the possibility that Ets factors cooperate with APA-1 to induce transcription of matrix-remodeling genes during senescence.

In conclusion, we have shown that the zinc finger protein APA-1 is upregulated in a telomere-independent manner in senescent human fibroblasts. Although overexpression of APA-1 does not induce a senescence-like cell cycle arrest, it induces transcription of matrix-remodeling genes that are associated with fibroblast senescence. These findings are the first to suggest that senescence-associated phenotypes, such as ex-

tracellular matrix remodeling in human fibroblasts, are independent of telomere erosion in human cells.

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