Cloning and Expression of SAG: A Novel Marker of Cellular Senescence

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Unlike immortalized cell lines, normal human fibroblasts in culture undergo replicative senescence in which the number of population doublings is limited. While fibroblasts display a variety of changes as they senesce in vitro, little is known about how gene expression varies as a function of population doubling level. We have used differential hybridization screening to identify human genes that are preferentially expressed in senescent cells. While we found several isolates that were up-regulated in late-passage cells, all appeared to be variants of the same cDNA, which we named senescence-associated gene (SAG). Our data show that SAG expression is threefold higher in senescent fibroblasts and closely parallels the progressive slowdown in growth potential, but is not cell-cycle regulated. Thus, SAG serves as an accurate marker for fibroblast growth potential during replicative senescence. Further studies demonstrated that SAG is a novel gene active in nearly all tissue types tested and that it is conserved through evolution. DNA sequencing data indicate that SAG contains a potential DNA-binding domain, suggesting that SAG may function as a regulatory protein.

INTRODUCTION

Diploid human fibroblast cells have a finite replicative life span in culture of 50 to 70 doublings and have been used as a model to study senescence at the cellular level [1-5]. While a number of morphological and physiological changes accompany replicative senescence, the principle observable changes are the increases in cell size and generation time [3-6]. At the end of their life spans, senescent cells become postmitotic, but still retain viability for many months [6].

Several lines of evidence indicate that senescent cells express a dominant inhibitor of cell proliferation that may be part of the mechanism controlling the senes-
genes are involved in the control of cellular senescence. For example, some of the genes, such as statin [32, 35], are expressed at similar levels in both young and senescent cells made quiescent by growth in low serum media and thus may be regulated more by the cell cycle than by the stage of replicative senescence.

In this manuscript we describe the use of differential screening of a senescent fibroblast cDNA library with 32P-labeled cDNA from quiescent early- and late-passage cells to identify genes whose expression increases in late-passage fibroblasts. We report the cloning and characterization of a novel senescence-specific cDNA whose mRNA level increases with cellular senescence. We further show that SAG expression is independent of the growth state in early-passage cells. These properties make SAG a good marker of replicative senescence and a potential regulatory site for the growth-inhibitory mechanism.

MATERIALS AND METHODS

Cell culture. Human diploid fibroblast MRC-5 cells were obtained from the NIH Aging Cell Repository at a population doubling level (PDL) of 15. The cells were cultured in a 1:1 mixture of Dulbecco modified Eagle medium and Ham nutrient F-12 medium (DME/F12, Sigma, 8900) supplemented with 10% calf serum (Hazelton), 100 units/ml penicillin, 50 µg/ml streptomycin, and 50 µg/ml neomycin. To subculture cells, confluent fibroblasts were washed in BSS buffer (15 mM Hepes, 130 mM NaCl, 5 mM KCl, 1 mM Na2PO4, 0.3% glucose) and then treated with BSS buffer containing 0.25% trypsin/EDTA (Sigma). The trypsin was siphoned off after 1 min and the cells were incubated for 2 more min at room temperature. The cells were then resuspended in DME/F12 medium and aliquots were counted in a hemocytometer before splitting them 1:4 into new flasks or plates. Cells were kept in an incubator humidified in the presence of 3.5% CO2. All other cell lines were grown under similar conditions. However, subculture ratios varied from 1:4 to 1:10, depending on the growth rate of the cells. To harvest the cells, they were washed with BSS buffer, scraped from the plates, and collected by centrifugation at 1300g for 5 min.

DNA isolation, blotting, and hybridization. DNA was purified from nuclei as previously described [36]. The DNA was digested with EcoRI restriction endonuclease at 37°C for 2 h and size fractionated on 1.2% agarose gel. The cDNA in-
FIG. 1. Identification of senescence-specific genes by differential screening of a λ Zap cDNA library. Two nitrocellulose plaque lifts were taken from the same plate containing 400 plaques and were hybridized to labeled cDNA from early- or late-passage MRC-5 cells. A, B, C, and D label four of the down-regulated isolates. The downward arrow points to an up-regulated SAG isolate. The rightward arrow in the PDL 69 blot points to a false positive.

from late-passage senescent MRC-5 cells (PDL 69). Since we were most interested in isolating genes that were not modulated with the cell cycle or the growth state of the cells, mRNA was prepared from cells which were quiescent following 7 days of confluency.

Figure 1 shows an example of such a primary differential screen. Even though the λ library was made from mRNA of senescent cells, we consistently found some 10 times more clones that appeared to be down-regulated with senescence than those that appeared up-regulated (such as the weakly labeled colonies A to D in the senescent-probed blot of Fig. 1). In our initial screening of 4000 plaques, we isolated 32 clones that hybridized strongly to senescent cDNA. These clones were amplified and the DNA dot blotted for hybridization to early- and late-passage cDNAs. Eight of these clones proved positive again (data not shown). DNA was purified from each of the positive isolates. Restriction analysis showed that four of the clones had a 600-bp insert, while a few had larger inserts up to 1.6 kb in length (data not shown). The 1.6-kb insert was found to strongly cross hybridize to all of the other up-regulated isolates (data not shown), indicating that the isolates were copies of the same or similar mRNA species. Supporting this interpretation, the 1.6-kb insert readily deleted to 600 bp if amplified in the λ vector. The clone bearing the 1.6-kb insert was designated SAG for senescence-associated gene and was used for all of the subsequent analysis.

SAG expression during replicative senescence. To characterize the changes in SAG mRNA levels during senescence, equal amounts of total RNA were electrophoretically fractionated from both early (PDL 27) and late (PDL 69) passage fibroblasts and hybridized to labeled SAG cDNA after Northern blotting. Figure 2 demonstrates that SAG hybridizes to a single 2.8-kb band. The level of SAG mRNA was threefold higher in late relative to early passage MRC-5 cells. As a control for RNA loading or degradation artifacts, the Northern blot probed with SAG (left side) was washed at 70°C for 1 h in deionized water to remove all traces of radioactivity and reprobed with a 32P-labeled DNA fragment from an exon of the retinoblastoma gene (right half of Fig. 2). The level of retinoblastoma mRNA has been reported to remain nearly constant during the in vitro senescence of human fibroblasts [27]. Figure 2 indicates that the level of SAG mRNA increases relative to retinoblastoma with population doubling. To determine the reproducibility of the threefold increase in SAG mRNA levels, seven separate Northern blot experiments were carried out using total RNA from early- and late-passage cells. The level of SAG mRNA was greater in the late-passage senescent cells by a factor of 3.0 ± 0.3 for the seven experiments.

To determine the developmental progression of SAG mRNA during replicative senescence, total RNA was prepared from MRC-5 cells at various population doubling levels and was subjected to Northern blot analysis as above. In one such experiment, four identical Northern blots were hybridized to retinoblastoma, α1-collagen, β-actin, or SAG (Fig. 3). Compared to the near constant expression of the retinoblastoma and β-actin genes, expression of the collagen α1 chain mRNA decreased 10-fold during replicative senescence. In contrast, SAG expression increased with senescence, but mainly during the last 20% of the replicative life span.

To compare the changes in SAG expression to the rate of cell proliferation, the Northern blot data from three separate experiments were quantitated by densitometry and the values plotted as a function of the per-
percentage of the life span completed. We then plotted the time for each population doubling versus the percentage of life span completed. Figure 4 demonstrates that changes in SAG mRNA closely parallel the rate of cell doubling. These results indicate that SAG is an accurate positive marker for the decline in fibroblast doubling potential. The α1-collagen gene is also a good marker, although its mRNA expression declines rather than increases with replicative senescence.

SAG expression remains unchanged during the cell cycle. It has been proposed that postmitotic senescent fibroblasts reside in a different part of the cell cycle (the G1/S boundary) than do quiescent early passage cells [5, 45]. To determine whether the levels of SAG mRNA reflect cell-cycle changes rather than true senescence, RNA was isolated from early-passage cells at different points in the cell cycle. MRC-5 cells (PDL 30) were first cultured for 72 h in medium containing 0.5% serum to induce quiescence. The fibroblasts were then harvested at regular intervals after growth stimulation with media containing 10% serum. RNA purified from those cells was subjected to Northern blotting and then hybridized to probes for SAG, retinoblastoma, β-actin, and α1-collagen. The top two panels in Fig. 5 show that retinoblastoma and α1-collagen mRNA levels change little during the cell cycle. In contrast, β-actin expression is strongly stimulated by serum, as has been shown previously [46]. The last two panels show that the level of SAG mRNA does not change significantly in response to serum. Moreover, no increase in SAG message is seen in these early-passage cells as they traverse the G1/S boundary of the cell cycle (Fig. 5, bottom panel, which occurs some 10 to 18 h after serum stimulation [34, 47–48]). We conclude that the changes in SAG mRNA levels that occur during in vitro culture of MRC-5 cells are not due to cell-cycle differences in early- versus late-passage cells. These data also indicate that the SAG gene is not serum responsive, as are many of the genes involved in growth regulation.

SAG is widely expressed in tumor cell lines. To determine whether SAG is expressed in various immortalized cells from differing origins, RNA was isolated from 12 human tumor cell lines, fractionated on agarose gels,
FIG. 6. SAG expression in various cell lines. SAG expression in 12 different cell lines was analyzed by Northern blotting as before. Since no probe has been identified as a hybridization control for all of these polyploid and transformed cell types, quantitation of SAG mRNA levels was normalized to the total undegraded RNA loaded in each lane. (A) Total RNA was probed from PDL 27 MRC-5, PDL 69 MRC-5, cervical carcinoma (HeLa), bladder carcinoma (J82), and brain glioblastoma (T98G) in lanes 1-5. (B) Total RNA was probed from PDL 27 MRC-5, PDL 69 MRC-5, colon carcinoma (ATCC CCL 247), bladder carcinoma (ATCC HTB 9), lung carcinoma 1 (ATCC HTB 58), lung carcinoma 2 (ATCC HTB 53), adenovirus-transformed embryonal kidney (ATCC CRL 1573), breast duct carcinoma (T47D), nasal septum carcinoma (ATCC CCL 30), Wilm’s tumor (kidney, ATCC CRL 1441), cervical carcinoma (HeLa), and breast adenocarcinoma (ATCC HTB 132) in lanes 1-12. The positions of 28 S rRNA and SAG mRNA are shown on the right.

and Northern blotted along with RNA from early- and late-passage MRC-5 cells. The blots were then hybridized to the labeled 1.6-kb SAG cDNA. Figure 6 demonstrates that SAG is expressed in all cell lines tested with the possible exception of HeLa cells, which have little (Fig. 6B) or no (Fig. 6A) expression.

SAG contains both highly repetitive DNA and low-copy, evolutionarily-conserved elements. The requirement for stringent hybridization conditions in order to obtain clear Northern blots when probing with SAG suggested the presence of repeated elements. To test whether SAG contains highly repetitive DNA, duplicate Southern blots containing electrophoretically fractionated human DNA restricted with EcoRI were hybridized to radiolabeled SAG DNA (lane H, middle panel of Fig. 7) or to an Alu repeat (lane H, left panel of Fig. 7). The 1.6-kb SAG clone hybridizes very strongly to the full range of fractionated human DNA in the same way that the Alu-containing probe does. Since Alu is one of the most highly repeated elements in the human genome, Fig. 7 indicates that SAG contains very highly repetitive DNA.

To determine whether SAG also contains low-copy and evolutionarily-conserved elements, we included rat liver DNA on the duplicate Southern blots in Fig. 7 (lanes R). In contrast to the smeared signal from human DNA, hybridization of SAG or c-fos probes to EcoRI-restricted rat DNA gives rise to single EcoRI bands of 2.5 or 2.2 kb, respectively, indicating that the unique portions of SAG are similar to c-fos in copy number and conservation. Since the human c-fos gene is known to be a single-copy gene with high homology to rat c-fos, these data suggest that SAG contains DNA elements that are homologous to a single-copy cognate gene in rats.

To localize the SAG nonrepetitive sequences, various radiolabeled fragments of the 1.6-kb SAG clone were used to probe Southern blots containing EcoRI-restricted human DNA. A 232-bp Haelll fragment at the 5' terminus of the SAG cDNA hybridizes to a discrete 8-kb band (Fig. 7, right panel). A discrete band of the same size was also seen after hybridization to a 640-bp HindIII fragment at the 5' end of the SAG cDNA (data not shown), indicating that the first 40% of the SAG clone is nonrepetitive. As discussed below, sequencing data suggest that this nonrepetitive region coincides with the presumed SAG coding sequences. In contrast, most of the sequences 3' of the HindIII site contain repetitive DNA (data not shown).

Sequence analysis of the 1.6-kb SAG clone. We made three attempts to obtain a full-length cDNA clone of SAG by screening two separate cDNA libraries, but were unsuccessful. Our lack of success may have been due to the tendency for SAG sequences to be deleted from bacterial cloning vectors. Since a full-length clone was unavailable, we have sequenced the 1.6-kb SAG clone by subcloning fragments into single-stranded M13 (Fig. 8). The coding strand was determined by hybridizing Northern blots containing fractionated RNA from MRC-5 cells with 32P-labeled probes prepared from each strand of SAG cDNA in M13mp18. Only the complement to the Fig. 8 sequence showed any hybridization to SAG mRNA (data not shown), identifying the

FIG. 7. Southern blot analysis of SAG in human and rat genomic DNA. Human (lanes H) and rat (lanes R) genomic DNA was restricted with EcoRI and electrophoresed on a 1% agarose gel as duplicate sets along with HindIII-restricted λ (lane M) as a marker. The fractionated DNA was blotted to nitrocellulose and the duplicate sets were separated and hybridized to a labeled 2.1-kb PvuII-BamH1 c-fos probe [38], containing an Alu repeat and the 3' c-fos coding region (left panel), or to the labeled 1.6-kb SAG probe (middle panel). In the right panel, a Southern blot containing EcoRI-restricted human DNA was probed with a 232-bp Haelll fragment from the 5' end of SAG.
Fig. 8. Nucleotide sequence of the 1.6-kb SAG clone and the predicted C-terminal amino acid sequence of the SAG protein. Three exons with excellent protein-coding potential were predicted by GRAIL (Oak Ridge National Laboratories) and the predicted amino acid sequence is given for these exons. The single Hind111 site is underlined and labeled. Most the sequences 3' of exon 3 contain genomic repeated DNA based on hybridization data. The Alu consensus sequence starts at sequence 1305 bp and continues until the end of the sequence.

SAG has been analyzed for protein-coding potential on GRAIL (gene recognition and analysis internet link) at Oakridge National Laboratories. The program, which is optimized for human codon recognition, detected three short exons with an excellent probability of forming coding regions. These three coding regions appear to be separated by introns, suggesting that the 1.6-kb SAG fragment is actually an unprocessed mRNA precursor. Perhaps the processed SAG cDNA is spontaneously deleted out of the λ cloning vector while the rare unprocessed mRNA is more stable.

**DISCUSSION**

*SAG is a novel gene that is up-regulated with senescence.* By differentially screening a cDNA library from senescent MRC-5 fibroblast cells, we have observed an altered expression of some mRNAs during senescence, most of which appear to be down-regulated. α1-collagen is one such down-regulated gene, while many others have been reported in the literature [3, 4, 34]. In contrast, we only observed one cDNA species...
Basic Motif

\[
\text{E12: KEKKEKKKK\text{E47: KKRKKKKKK}}
\]

The antigrowth gene p53 contains a similar basic motif rich in arginine and lysine (R and K residues shown in bold) that resembles the basic DNA-binding domains found in many nuclear regulatory proteins. E12, E47, MyoD1, and c-jun are all transcription factors. The antigrowth gene p53 contains a similar basic motif at residues 273–293 [52], in which mutation of the first arginine (R273) abolishes both antigrowth and DNA-binding activity [53].

(SAG) which was up-regulated when comparing quiescent early- and late-passage MRC-5 fibroblasts. Our data show an increasing expression of SAG that directly parallels the decline in MRC-5 growth potential. Since SAG is not cell-cycle regulated, it is one of the first up-regulated markers of human fibroblast senescence that is unaffected by the cell cycle or the growth state of the cell. Blot and sequence analysis of SAG defines a novel gene which is widely expressed in many cell types and is evolutionarily conserved. However, SAG cDNA also has numerous repetitive elements that may generate instability on amplification in bacterial vectors.

**SAG may be regulatory.** While the possible functions of SAG are unknown, three lines of evidence suggest that SAG may be of regulatory significance during senescence. First, the increase in SAG expression shows an inverse correlation with the doubling rate of MRC-5 cells (Fig. 6). Moreover, two human fibroblast cell lines transformed by SV40 contained levels of SAG mRNA regulation appears to be altered in most immortal cells (Fig. 6A), suggesting a possible correlation with the gene(s) on chromosome 4.

While both increases and decreases in specific mRNA levels could contribute to replicative senescence, recent data suggest that the senescence phenotype is dominant, so that genes with increasing expression may be of greater relevance. SAG’s pattern of expression and its potential DNA-binding domain are consistent with the notion that replicative senescence is under dominant genetic control. Thus, a better understanding of SAG regulation may help elucidate the genetic mechanism of replicative senescence.

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**REFERENCES**