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A NOVEL GENE ENCODING A SMOOTH MUSCLE PROTEIN IS OVEREXPRESSED IN SENESCENT HUMAN FIBROBLASTS

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SUMMARY: In order to identify genes that may be causally involved in replicative senescence, we have isolated several gene sequences that are overexpressed in senescent human fibroblasts by differential screening of a cDNA library derived from mRNA of a subject with Werner syndrome of premature aging (Murano, S., et al., Molec. Cell. Biol., 3905-3914, 1991). Herein, we describe the sequence and expression of one of these genes, WS3-10, which encodes a novel human cytoplasmic protein of 22.5 kilodaltons. The steady-state mRNA levels of WS3-10 mRNA were higher in WS and late-passage normal cells compared to early-passage normal cells following serum depletion and subsequent repletion. Computer analysis showed similarities between WS3-10 and certain proteins in other species, indicating that WS3-10 represents the human homolog of a smooth muscle protein involved in calcium interactions that may contribute to replicative senescence.

Human diploid fibroblasts (HDF) have a finite replicative lifespan measured by the number of mean population doublings (MPD) accruing before senescence (1). Although the precise mechanisms underlying this process remain enigmatic (2), the maximum number of MPD attained by HDF cultures is inversely proportional to donor age (3). Moreover, HDF derived from individuals with genetic disorders of premature aging, such as Werner syndrome (WS), show premature replicative senescence (4). WS is characterized by the premature appearance of several features of aging, including the loss and graying of hair, cataracts, osteoporosis, atherosclerosis, neoplasia, type II diabetes mellitus, skin ulceration, and early death (5). In cell culture, WS HDF, as compared to normal HDF, undergo premature replicative senescence preceded by slow growth, cellular enlargement, irregular shape and polarity, and the

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Abbreviations: HDF, human diploid fibroblasts; MPD, mean population doublings; WS, Werner syndrome; FBS, fetal bovine serum; kDa, kilodalton.

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accumulation of intracellular inclusions and extracellular debris (6). Moreover, senescent HDF, defined either as WS cells or late-passage normal cells, exert a dominant suppressive effect on DNA synthesis in somatic cell hybrids with early-passage HDF capable of vigorous proliferation (7). In order to identify genes that may be causally involved in replicative senescence, we recently constructed a cDNA library from WS mRNA and isolated 18 overexpressed gene sequences; nine of these represented novel sequences that are overexpressed in WS and old normal HDF (8). One of the hitherto unknown cDNAs, WS3-10, which encodes a novel human cytoplasmic protein, is described here in greater detail.

MATERIALS AND METHODS

Cell Culture—WS8 fibroblasts, derived from the skin of a 47 year old male subject with classical WS, and HDF strain J065, derived from a normal 56 year old male, were used in these studies. Young J065 cells were in the first half of their replicative lifespan ([^{3}H]thymidine labeling index [TLI] $\geq$ 90%), old J065 cells were in the last 10% of their lifespan (TLI $\leq$ 13%, maximum replicative lifespan 50 MPD), and WS cells were about halfway through their lifespan (TLI $<$ 10%, maximum replicative lifespan 18 MPD).

Northern Analysis—Cells were exposed to serum depleted medium for five days (Eagle's minimum essential medium plus 0.5% FBS), refed with regular growth medium (Eagle's medium containing 15% FBS), and total RNA was isolated at the various time points indicated. The RNA was fractionated on agarose-formaldehyde gels, transferred to a nylon filter and probed with ^{32}P-labeled WS3-10 cDNA. The filter was stripped of this probe and reprobed with ^{32}P-labeled 28S rDNA to assess and correct for minor differences in loading and transfer of RNA in each lane (8).

Sequence Analysis—DNA sequencing was carried out by the dideoxy chain termination method (9) using the Sequenase kit (US Biochemical Co.). Primers consisted of the sense-strand of the Okayama-Berg vector immediately 5′ to the G-tail region (5′-TCTAGGCCTGTACGGAA-3′) (8), a universal primer for the 3′end region consisting of a mixture of (5′-3′) T_{20}A, T_{20}C, and T_{20}G (10), and the SP6 and T7 promoter primers used to sequence interior segments of WS3-10 subcloned into the Gemini 2 vector (Promega Co.). These subclones included a PstI fragment beginning immediately 5′ to the G-tail in the vector and continuing to the PstI site at position 581, an EcoRI-BamHI fragment from position 559 to the BamHI site 3′ to the polyA tail, a BamHI-HindIII fragment beginning at the BamHI site within the vector 100 nucleotides upstream of the G-tail and continuing until the HindIII site at position 788, and a HindIII-BamHI fragment extending from position 788 to the BamHI site downstream of the polyA tail. The open reading frame was established with the PC Gene program (Intelligenetics, Inc., Mountain View, CA).

Homology Analysis—Sequence data were compared with data bases in GenBank (release 71), SWISS-PROT (release 21) and EMBL (release 21) following standard procedures (11).

RESULTS AND DISCUSSION

Levels of WS3-10 mRNA expression were determined in cells after five days of serum depletion (time 0) and at several intervals following serum repletion. Steady-state levels of WS3-10 mRNA were elevated in WS and old normal cells compared to young normal cells at all time points examined from 0 to 48 hours (FIG 1). Levels of peak overexpression, which

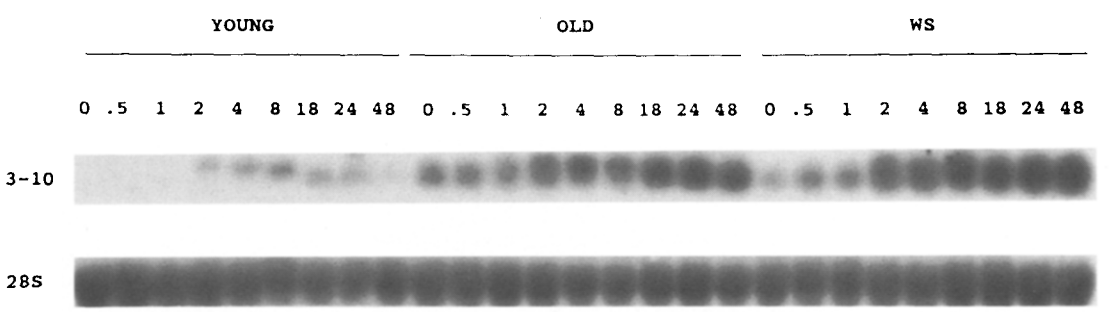

FIG.1. Northern analysis of WS3-10 mRNA in WS and normal young and old fibroblasts. Cells were exposed to serum-depleted medium for 5 days, repleted with medium containing 15% FBS and total RNA was isolated at the various time points indicated (in hours). Blots were probed sequentially with ^{32}P-labeled WS3-10 cDNA and ^{32}P-labeled 28S rDNA.

were evident between the time of serum repletion and one hour, were similar in WS and old cells, while WS3-10 mRNA was undetectable in young cells during this interval.

The sequence of WS3-10 cDNA was determined (FIG 2), and found to consist of 1083 nucleotides from the 5′-end to the poly A tail. The sequence contains a 5′-untranslated region

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ATCCTGTCTGTCCGAACCCAGACACAAGTCTTCACTCCTTCCTGCGAGCCCTGAGGAAGC 0060
CTTCTTTCCCCAGACATGGCCAACAAGGGTCCTTCCTATGGCATGAGCCGCGAAGTGCAG 0120
                MetAlaAsnLysGlyProSerTyrGlyMetSerArgGluValGln  015

TCCAAAATCGAGAAGAAGTATGACGAGGAGCTGGAGGAGCGGCTGGTGGAGTGGATCATA 0180
SerLysIleGluLysLysTyrAspGluGluLeuGluGluArgLeuValGluTrpIleIle  035

GTGCAGTGTGGCCCTGATGTGGGCCGCCCAGACCGTGGGCCCTTGGGCTTCCAGGTGTGG 0240
ValGlnCysGlyProAspValGlyArgProAspArgGlyProLeuGlyPheGlnValTrp  055

CTGAAGAATGGCGTGATTCTGAGCAAGCTGGTGAACAGCCTGTACCCTGATGGCTCCAAG 0300
LeuLysAsnGlyValIleLeuSerLysLeuValAsnSerLeuTyrProAspGlySerLys  075

CCGGTGAAGGTGCCCGAGAACCCACCCTCCATGGTCTTCAAGCAGATGGAGCAGGTGGCT 0360
ProValLysValProGluAsnProProSerMetValPheLysGlnMetGluGlnValAla  095

CAGTTCCTGAAGGCGGCTGAGGACTCTGGGGTCATCAAGACTGACATGTTCCAGACTGTT 0420
GlnPheLeuLysAlaAlaGluAspTyrGlyValIleLysThrAspMetPheGlnThrVal  115

GACCTCTTTGAAGGCAAAGACATGGCAGCAGTGCAGAGGACCCTGATGGCTTTGGGCAGC 0480
AspLeuPheGluGlyLysAspMetAlaAlaValGlnArgThrLeuMetAlaLeuGlySer  135

TTGGCAGTGACCAAGAATGATGGGCACTACCGTGGAGATCCCAACTGGTTTATGAAGAAA 0540
LeuAlaValThrLysAsnAspGlyHisTyrArgGlyAspProAsnTrpPheMetLysLys  155

GCGCAGGAGCATAAGAGGGAATTCACAGAGAGCCAGCTGCAGGAGGGAAAGCATGTCATT 0600
AlaGlnGluHisLysArgGluPheThrGluSerGlnLeuGlnGluGlyLysHisValIle  175

GGCCTTCAGATGGGCAGCAACAGAGGGGCCTCCCAGGCCGGCATGACAGGCTACGGACGA 0660
GlyLeuGlnMetGlySerAsnArgGlyAlaSerGlnAlaGlyMetThrGlyTyrGlyArg  195

CCTCGGCAGATCATCAGTTAGAGCGGAGAGGGCTAGCCCTGAGCCCGGCGCTCCCCCAGC 0720
ProArgGlnIleIleSer---                                         201

TCCTTGGCTGCAGCCATCCCGCTTAGCCTGCCTCACCCACACCCGTGTGGTACCTTCAGC 0780
CCTGGCCAAGCTTTGAGGCTCTGTCACTGAGCAATGGTAACTGCACCTGGGCAGCTCCTC 0840
CCTGTGCCCCCAGCCTCAGCCCAACTTCTTACCCGAAAGCATCACTGCCTTGGCCCCTCC 0900
CTCCCGGCGGCCCCCATCACCTCTACTGTCTCCTCCCTGGGCTAAGCAGGGGAGAAGCGG 0960
GCTGGGGGTAGCCTGGATGTGGGCGAAGTCCACTGTCCTCCTTGGCGGCAAAAGCCCATT 1020
GAAGAAGAACCAGCCCAGCCTGCCCCCTATCTTGTACCTGGAATATTTTTGGGGTTGGAA 1080
CTCAAAAAA
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FIG.2. Nucleotide and deduced amino acid sequence of WS3-10 cDNA. The sequence, consisting of 1083 nucleotides before the start of the polyA tail, contains an open reading frame extending from the first ATG at position 76 to a stop codon at position 679, thus encoding a protein of 201 amino acids. Three potential phosphorylation sites occur at SER-16, THR-139, and SER-181. The polyadenylation signal AATA at position 1062 is highlighted.

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DMP20   -------MSLERA*RA**AS*RNP*MDKEAQ***EAIIAEKFPA*QSYEDV*K  046
TCALP   -M*R**A**L*A**KN*LAQ***PQT*RQ*RV**EGAT*RRI--*DN------  043
CSM22   ******A*****D**********D***D******VA***SS*--******R*-  050
WS310   MANKGPSYGMSREVQSKIEKKYDEELEERLVEWIIVQCGPDV--GRPDRGPL-  050

DMP20   DG**LC*LINV**PNAVPK***SG---------------GQ**F**NINN*Q*  084
TCALP   -*XDG**D****ME*----I*K*Q*GSVQK*ND*V----QNWHKL*NIGN**R  087
CSM22   *********IV**Q*----*************I*DS**T********I*****  099
WS310   GFQVWLKNGVILSKL----VNSLYPDGSKPVKVPENPPSMVFKQMEQVAQFLK  099

DMP20   *LKE***PDI*V******Y*K**I*N*TN*IF***-R*TY*HADFKGPFLG--  134
TCALP   *IKH***KPH*I*EAN****NTNHTQ**S**I**A*Q*K**GNNVGL*V----  136
CSM22   *******V***********LA**********V**************H******  152
WS310   AAEDYGVIKTDMFQTVDLFEGKDMAAVQRTLMALGSLAVTKNDGHYRGDPNWF  152

DMP20   P*P*D*C**D***E**KA*QTIV***A***K**T***QNL-*AG*K*LLGK    184
TCALP   -*Y*EKQQ*R*QPEK*R**RNI******T*KF***Q***A*              176
CSM22   **********S****K***NI******T*K*******S-*******        198
WS310   MKKAQEHKREFTESQLQEGKHVIGLQMGSNRGASQAGMTGYGRPRQIIS     201
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FIG. 3. Alignment of amino acid sequences encoding human WS3-10, chicken SM22, drosophila MP20, and the first 176 amino acids of turkey calponin, representing 73% of its N-terminal portion. Since the turkey and chicken calponin sequences are approximately 98% homologous in this region, only the turkey calponin sequence is shown. Identical residues are denoted by stars; dashes represent gaps introduced into the protein sequence in order to obtain the best fit. The two underlined regions in DMP20 represent proposed calcium binding regions (14).

of 75 nucleotides, an open reading frame extending from the first ATG at position 76 through a stop codon at position 679, and a 3′-untranslated region of over 400 bases. The context of this ATG codon, GACATGG, accords well with the consensus sequence found to favor efficient initiation of translation (12). The deduced amino acid sequence contains 201 amino acids, coding for a protein of 22.5 kDa, with three potential sites for phosphorylation at SER-16, THR-139, and SER-181.

WS3-10 was determined to represent the human gene sequence equivalent to chicken SM22 cDNA (13) after a search of GenBank and EMBL DNA databases (11). We also found homologies of amino acid sequence to four proteins present in different organisms (FIG 3): 34% homology to MP20, a drosophila muscle protein (14), 38% homology to the 22 kDa amino terminal ends of turkey gizzard calponin (15) and chicken gizzard calponin (16), and 84% homology to SM22, a smooth muscle protein first isolated from chicken gizzard but also detected in a variety of smooth muscle-containing tissues including intestine, uterus, oesophagus, and aorta (17,18). If conservative residues are considered, the homology between WS3-10 and SM22 increases from 84% to 91%. The only difference between the amino acid sequence of SM22 deduced from the cDNA (13) versus the direct determination from the protein (17) is the presence of two extra amino acids (isoleucine and serine) at the C-terminus of the open reading frame. That these two residues are also encoded in WS3-10 cDNA suggests that a postsynthetic cleavage occurs in vivo.

MP20 represents a cytoplasmic protein confined to synchronous muscles in drosophila and is proposed to have two calcium binding sites that are presumably involved in pumping calcium into and out of the sarcoplasmic reticulum (14). While the first calcium binding site near the amino terminus of WS3-10 has only 25% identity to MP20, the second site is conserved at 75%, making it more likely to be a calcium binding region. The complete turkey calponin protein is 34 kDa and known to bind to calcium and calmodulin (15), as does the nearly identical chicken calponin protein (16). There exists a 58% conservative sequence similarity between residues 18 to 42 of chicken calponin with residues 24 to 50 of the ras GTP-binding protein, p21 (16,19), which constitutes the site of interaction between p21 and the GTPase activating protein (20). On comparing p21 to residues 14 to 38 of WS3-10, the homology was found to be 41%. The significance of these similarities is unknown at this time.

SM22 is thought to be involved in the contractile apparatus of cells, but has not been shown to bind calcium, actin, or calmodulin (18,21). Although SM22 can be phosphorylated on serine, the physiological significance of this phosphorylation is in question because it occurs at a ratio of only 0.5 moles/mole of protein (21). As noted, WS3-10 has three potential sites for phosphorylation, two of which occur on serine residues. In comparison with SM22, MP20, and calponin, it appears that WS3-10 encodes a smooth muscle protein involved in interactions with calcium, calmodulin, actin, and/or tropomyosin and is presumably involved in the cytoskeletal makeup of the cell.

The proposed interaction between the protein encoded by WS3-10 and calcium is intriguing because calcium homeostasis is involved in several cellular processes, including DNA synthesis (22). Indeed, changes in calcium homeostasis in HDF from older donors (23,24) and HDF at the end of their replicative lifespans (25) could lead to inhibition of DNA synthesis and ultimately HDF senescence. We have been unable to demonstrate inhibitory activity on DNA synthesis following transfection of WS3-10 cDNA in a eukaryotic expression vector (4,8) into young proliferatively competent HDF, or after microinjection (26) of WS3-10 mRNA transcribed in vitro (data not shown). Thus, WS3-10 may not inhibit DNA synthesis directly, or alternatively may require the concerted action of one or more of the several overexpressed gene sequences we have identified in the WS cDNA library (8).

The question arises how WS3-10 and the 17 other overexpressed genes relate to the primary defect in WS, an autosomal recessive disorder. We have proposed that the defect involves a gene locus which encodes a protein that normally represses age-dependent inhibition of DNA synthesis (27). In WS, the mutant protein would allow premature derepression of one or more of these loci, stabilize their mRNAs, or both[1], thereby suppressing cellular DNA

[1]Thweatt, R., and Goldstein, S., BioEssays, in press (1992).

synthesis. In this regard, the recent identification of a WS locus on the short arm of chromosome 8 (28) is of great interest and should soon resolve this question.

In summary, we have isolated and sequenced a novel cDNA, apparently encoding the human homolog of a previously described chicken smooth muscle protein, which is overexpressed in both WS and late-passage normal HDF, compared to early-passage normal cells. At present, it is uncertain how the product of the WS3-10 gene sequence, which is likely to be involved in calcium interactions and contractile properties of the cell, contributes to the replicative senescence of HDF, but we are currently exploring its function, alone and in concert with other overexpressed gene sequences from the WS cDNA library.

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