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Upregulation of the Gene Encoding a Cytoplasmic Dynein Intermediate Chain in Senescent Human Cells

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Abstract Normal human somatic cells, unlike cancer cells, stop dividing after a limited number of cell divisions through the process termed cellular senescence or replicative senescence, which functions as a tumor-suppressive mechanism and may be related to organismal aging. By means of the cDNA subtractive hybridization, we identified eight genes upregulated during normal chromosome 3-induced cellular senescence in a human renal cell carcinoma cell line. Among them is the *DNCI1* gene encoding an intermediate chain 1 of the cytoplasmic dynein, a microtubule motor that plays a role in chromosome movement and organelle transport. The DNCI1 mRNA was also upregulated during in vitro aging of primary human fibroblasts. In contrast, other components of cytoplasmic dynein showed no significant change in mRNA expression during cellular aging. Cell growth arrest by serum starvation, contact inhibition, or γ-irradiation did not induce the DNCI1 mRNA, suggesting its specific role in cellular senescence. The *DNCI1* gene is on the long arm of chromosome 7 where tumor suppressor genes and a senescence-inducing gene for a group of immortal cell lines (complementation group D) are mapped. This is the first report that links a component of molecular motor complex to cellular senescence, providing a new insight into molecular mechanisms of cellular senescence. J. Cell. Biochem. 82: 415–421, 2001.

Key words: cDNA subtraction; cellular senescence; cytoplasmic dynein; molecular motor; chromosome 7q

Normal human somatic cells stop dividing after a finite number of cell divisions. This process is termed cellular senescence or replicative senescence. In contrast, most cancer cells have an infinite growth potential, indicating that escape from cellular senescence, or immortalization, is an important step in human carcinogenesis [Chiu and Harley, 1997; Wynford-Thomas, 1999; Horikawa et al., 2000]. Cellular senescence is a complex cellular process regulated by multiple genetic pathways, which reflect various intracellular and extracellular signals [Horikawa et al., 2000]. Cell hybrid and chromosome transfer experiments have suggested that immortalization results from functional inactivation of senescence genes [Pereira-Smith and Smith, 1983, 1988]. Although a class of senescence genes is suggested to function through the repression of telomerase activity [Horikawa et al., 1998, 2000; Tanaka et al., 1998; Cuthbert et al., 1999], molecular mechanisms of cellular senescence are still largely elusive. An approach to better understanding of cellular senescence is to investigate senescence-associated changes in gene expression. Such investigations have found a number of senescence-associated genes, such as cell cycle regulatory genes, growth regulatory genes, and extracellular matrix-related genes [Cristofalo et al., 1998; Jansen-Dürr, 1998].

We previously reported that microcell-mediated transfer of a normal human chromosome 3 into a renal cell carcinoma cell line

Abbreviations used: DNCI1, cytoplasmic dynein intermediate chain 1; SSH, suppression subtractive hybridization; PDs, population doublings; NHF, normal human fibroblasts; RT-PCR, reverse transcription-PCR.

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(RCC23) induced cellular senescence through the repression of telomerase activity and the progressive shortening of telomeric DNA [Horikawa et al., 1998]. By means of an efficient cDNA subtraction method [suppression subtractive hybridization (SSH)] [Diatchenko et al., 1996], we here identify the genes whose expression is upregulated during the chromosome 3-induced cellular senescence. One of those genes encodes a subunit of a microtubule-associated molecular motor complex, providing a novel insight into the molecular mechanism of cellular senescence.

MATERIALS AND METHODS

Cells

A renal cell carcinoma cell line, RCC23, was previously established from a non-papillary renal cell carcinoma and expresses telomerase activity [Horikawa et al., 1998]. Microcell-mediated transfer of a single copy of normal human chromosome 3 into RCC23 cells produced a telomerase-negative microcell hybrid (RCC23+3), which showed progressive shortening of telomeres as a function of cell division numbers [Horikawa et al., 1998]. RCC23+3 cells used in this study [clone #3-C in Horikawa et al., 1998] stopped growing at 41 population doublings (PDs) with an expression of the senescence-associated β-galactosidase [Dimri et al., 1995]. A revertant clone (REV) emerged from the senescent RCC23+3 cells with a loss of the 3p12–p22 region of the transferred chromosome. REV cells showed telomerase activity comparable to parental RCC23 cells, and continued to grow over 100 PDs without any sign of senescence [Horikawa et al., 1998].

Normal human fibroblasts (NHF) were derived from neonatal foreskin. They were passaged at a 1:4 split ratio until they senesced at passage number 31. Young NHF cells (at passage number 18) were growth-arrested by serum starvation (0.5% fetal calf serum), contact inhibition (confluent), or γ-irradiation (3 Gy) as previously described [Burkhart et al., 1999].

cDNA Subtraction by SSH

Cytoplasmic RNA was isolated from RCC23 and RCC23+3 cells (at 30 PDs) as previously described [Sambrook et al., 1989] and treated with DNase I (Amplification Grade, Life Technologies, Inc., Gaithersburg, MD). Poly(A)$^+$ RNA was purified from the cytoplasmic RNA by using the Oligotex Direct mRNA Kit (Qiagen, Inc., Chatsworth, CA). To isolate genes which are preferentially or exclusively expressed in RCC23+3 cells, SSH was performed using the PCR-Select cDNA subtraction kit (Clontech Labs, Inc., Palo Alto, CA) as previously described [Diatchenko et al., 1996]. Starting with 2 μg of poly(A)$^+$ RNA from RCC23+3 as a tester and RCC23 as a driver, first- and second-strand cDNA synthesis, Rsa I digestion of cDNA, ligation of the adapters to the tester cDNA, two steps of subtractive hybridization, and PCR amplification of the subtracted cDNA were carried out according to the manufacturer's instruction. The final PCR products were cloned in pCR2.1 vector (Invitrogen, Corp., San Diego, CA). Differential screening was performed using the ^{32}P-labeled, subtracted cDNA described above and the ^{32}P-labeled, reverse-subtracted (RCC23 minus RCC23+3) cDNA similarly obtained as the probes. Plasmid DNA was purified from bacterial colonies which showed preferential hybridization signals with the former probe.

DNA Sequencing and Homology Search

Double-stranded plasmid DNA was sequenced with M13-forward and reverse primers and, if necessary, gene-specific internal primers using the dRhodamine terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA). DNA homology search was performed at the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST). Chromosomal localization of the genes was based on the Online Mendelian Inheritance in Man (OMIM; http://www3.ncbi.nlm.nih.gov/Omim/searchmap.html) and/or the GeneMap98 (http://www.ncbi.nlm.nih.gov/genemap98).

Northern Blot and Reverse Transcription (RT)-PCR Analyses

Total cellular RNA was isolated by the RNeasy Mini kit (Qiagen, Inc.) and electrophoresed through a 1.2% agarose–formaldehyde gel. Northern blotting and hybridization were carried out as previously described [Horikawa et al., 1998].

For reverse transcription-PCR (RT-PCR) analyses, 2 μg of total cellular RNA was reverse-transcribed with oligo(dT) primer by

using the SuperScript preamplification system for first strand cDNA synthesis (Life Technologies, Inc.). A part of the reaction (0.5 μl) was used as a template for a 10-μl PCR amplification by the Advantage cDNA PCR kit (Clontech Labs, Inc.). Primer sequences and size of the amplified region are as follows: for cytoplasmic dynein intermediate chain 1 (DNCI1) mRNA, 5′-CTC AAC AAT GAC ACC GAG GT-3′ and 5′-CAC CCA ATC ATA TCT GGC TTC-3′ (632 bps); for cytoplasmic dynein light chain 1 mRNA, 5′-CAT GTG CGA CCG AAA GGC-3′ and 5′-CAG TCC TTG TTT CTG GAT GG-3′ (321 bps); for cytoplasmic dynein intermediate chain 2 mRNA, 5′-CCA GAG TAT GTG TTT CAC TGC-3′ and 5′-TCC AAC AGG GAA GGA CAT AG-3′ (354 bps); for cytoplasmic dynein heavy chain 1 mRNA, 5′-TGC AGA ACA TCT CAC TGG CA-3′ and 5′-TTC CTC CAA CCT CAG ACC AA-3′ (596 bps); and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, 5′-CCA TCT TCC AGG AGC GAG A-3′ and 5′-TGT CAT ACC AGG AAA TGA GC-3′ (723 bps). PCR reactions were processed through 18 temperature cycles for GAPDH mRNA or through 28 temperature cycles for the others.

RESULTS

Successful Application of SSH to the Identification of Differentially Expressed Genes

When ~500 bacterial colonies were screened with the subtracted and reverse-subtracted probes (see Materials and Methods), ~100 (20%) colonies showed exclusive or preferential hybridization to the former probe. Ten colonies with relatively strong hybridization signal were grown, sequenced, and found to represent eight different genes (Table I). Northern blot analysis confirmed that all of these eight genes were expressed more abundantly in RCC23+3 cells than in parental RCC23 cells (data not shown; Fig. 1A). Four genes (plasminogen activator inhibitor-1, extracellular protein S1-5, fibronectin, and 22-kDa smooth muscle protein) were previously reported to be upregulated during cellular senescence [Thweatt et al., 1992; Kumazaki et al., 1993; Lecka-Czernik et al., 1996; West et al., 1996]. Two genes (growth arrest specific protein-6 and follistatin-related protein precursor) were previously linked to cell growth arrest [Schneider et al., 1988; Shibanuma et al., 1993], consistent with their upregulation during cellular senescence. Successful identification of these genes suggests that SSH method worked well in this study and that RCC23+3 cells are a good cell system to identify senescence-associated genes. One clone showed identity to two sequences of unknown function (GenBank accession numbers: AB033025 and AK000465) that seemed to represent partial cDNA clones. Isolation and detailed characterization of the full-length cDNA will be reported elsewhere. We here direct our attention to the *DNCI1* gene encoding an intermediate chain 1 of the cytoplasmic dynein, a microtubule-associated molecular motor complex that plays a role in chromosome movement and intracellular transport [Vallee and Sheetz, 1996; Endow, 1999; Karki and Holzbaur, 1999] but has not been linked to cellular senescence.

TABLE I. List of Genes Identified in this Study

Gene	Chromosomal location[a]
Plasminogen activator inhibitor-1	7q21.3–q22
Extracellular protein S1-5	2p16
Growth arrest specific protein-6	13q34
Fibronectin	2q34
22-kDa smooth muscle protein	11q23.2
Follistatin-related protein precursor	3q
Cytoplasmic dynein intermediate chain 1	7q21.3–q22.1
AB033025/AK000465 (unknown function)	15q

[a]Chromosomal bands are from the cytogenetic map at the Online Mendelian Inheritance in Man (http://www3.ncbi.nlm.nih.gov/Omim/searchmap.html). In case cytogenetic mapping data is not available, chromosomal arms from the radiation hybrid mapping (http://www.ncbi.nlm.nih.gov/genemap98) are shown.

DNCI1 mRNA Expression Increases as Cells Age

A 740-bp RsaI–RsaI fragment from 3′-region of the DNCI1 cDNA (nucleotides 1792–2531 of GenBank AF063228) [Crackower et al., 1999] was initially isolated by SSH. 5′-RACE (rapid amplification of cDNA end) using the primers within this 740-bp region resulted in ~2.0-kb fragment containing the entire coding region, which matched the reported DNCI1 sequence. Northern blot analysis (Fig. 1A) showed that RCC23+3 cells expressed higher levels of DNCI1 mRNA than parental RCC23. The DNCI1 mRNA expression in RCC23+3 cells increased as a function of PDs (compare lanes 2–4 in Fig. 1A), with a maximum expression at

senescent cells. In the revertant REV cells, upon loss of the chromosomal region responsible for the induction of cellular senescence (3p14.2–p21.1; see Materials and Methods and Tanaka et al., 1998), the amount of DNCI1 mRNA decreased back to an undetectable level (lane 5 in Fig. 1A). RT-PCR analysis also confirmed a marked increase in DNCI1 mRNA in RCC23+3 cells (Fig. 1B). These results suggest that induction of the DNCI1 expression is associated with cellular senescence induced by chromosome 3 in RCC23 cells. Cellular aging-associated DNCI1 upregulation was also observed in NHF that were primary cultures from neonatal foreskin successively passaged until they became senescent (Fig. 2). Expression of DNCI1 mRNA was hardly detected in young fibroblasts (passage 13 in lane 1), became detectable in middle-aged ones (passage 21 in lane 3), and reached a maximum level in senescent ones (passage 31 in lane 5).

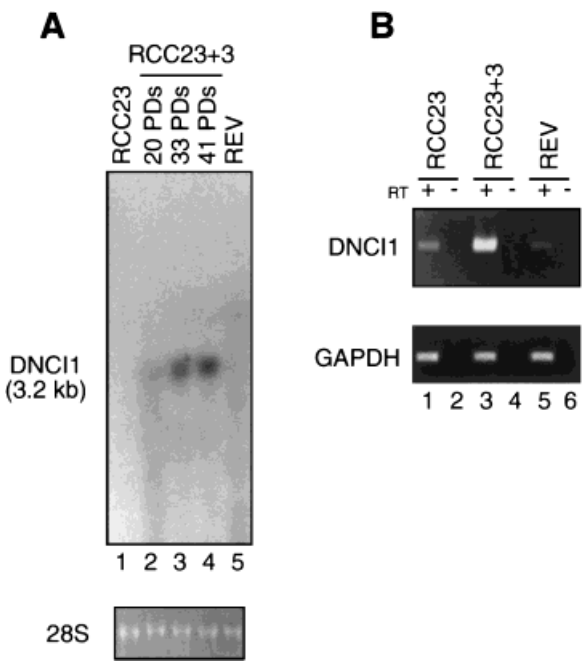

Fig. 1. Upregulation of DNCI1 mRNA during chromosome 3-induced cellular senescence in RCC23 cells. **A:** Northern blot analysis. ^{32}P-labeled DNCI1 cDNA (nucleotides 167 to 2125) was used as the hybridization probe. **Lane 1**, RCC23; **lane 2**, RCC23+3 at 20 PDs; **lane 3**, RCC23+3 at 33 PDs; **lane 4**, RCC23+3 at 41 PDs (senescence); **lane 5**, REV. Ethidium bromide staining of 28S rRNA is shown as a control for equal loading. **B:** RT-PCR analysis. Total cellular RNAs from RCC23 (**lane 1**), RCC23+3 at 33 PDs (**lane 3**) and REV (**lane 5**) were reverse-transcribed and examined for DNCI1 mRNA expression. Each sample has a negative control without the reverse transcriptase (**lanes 2, 4** and **6**). The control amplification of GAPDH is shown.

Cellular Senescence-Associated Upregulation is Unique to DNCI1 Among Multiple Components of Cytoplasmic Dynein

A cytoplasmic dynein consists of multiple components including heavy, intermediate, light intermediate, and light chains, each of which has two or more isoforms [Vallee and Sheetz, 1996; Endow, 1999; Karki and Holzbaur, 1999]. We examined by RT-PCR analyses whether the cellular senescence-associated upregulation is specific to the DNCI1 or generally observed in multiple components. As shown in Figure 3A, we did not find a significant difference in mRNA expression of the other components tested (heavy chain 1, intermediate chain 2, and light chain 1) among RCC23, RCC23+3, and REV cells. Similarly, mRNA expression of these components showed no significant change throughout the replicative life span of NHF (Fig. 3B). Thus, the cellular senescence-associated mRNA induction is likely to be unique to the DNCI1 among multiple components of cytoplasmic dynein.

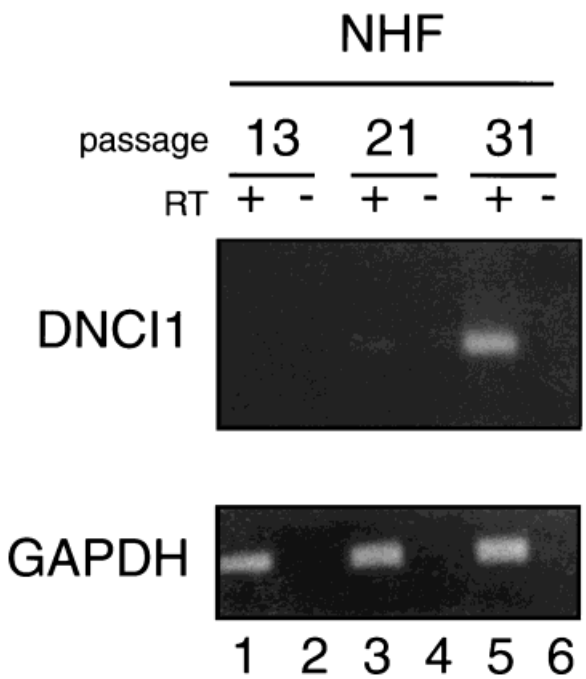

Fig. 2. RT-PCR analysis of DNCI1 mRNA expression during in vitro aging of NHF. NHF at passage 13 (**lane 1**), passage 21 (**lane 3**), and passage 31 (senescence; **lane 5**) were analyzed as in Figure 1B. **Lanes 2, 4**, and **6** are reverse transcriptase-minus controls.

Induction of DNCI1 mRNA is Specific to Cellular Senescence

To examine whether or not the upregulation of DNCI1 mRNA is associated with cell growth arrest in general, we induced growth arrest in NHF cells by serum starvation, contact inhibition or γ-irradiation. Unlike cellular senescence, none of these conditions resulted in a significant increase in DNCI1 mRNA expression (Fig. 4). This suggests that the induction of DNCI1 mRNA is characteristics of cellular senescence rather than a general feature of cell growth arrest.

DISCUSSION

In this study we identified the *DNCI1* gene, which encodes an intermediate chain 1 of the cytoplasmic dynein, as a novel senescence-associated gene. The DNCI1 upregulation during cellular aging was observed in both epithelial (RCC23+3) and fibroblastic (NHF) cells. Unlike the *DNCI1* gene, expression of genes encoding the other components of cytoplasmic dynein showed no significant change during cellular senescence. Also important is that the DNCI1 upregulation was specifically associated with cellular senescence, and not cell growth arrest in general. These findings strongly support a specific role of the *DNCI1* gene in cellular senescence. This is the first report that links a component of molecular motor complex to cellular senescence, providing a new insight into molecular mechanisms of cellular senescence.

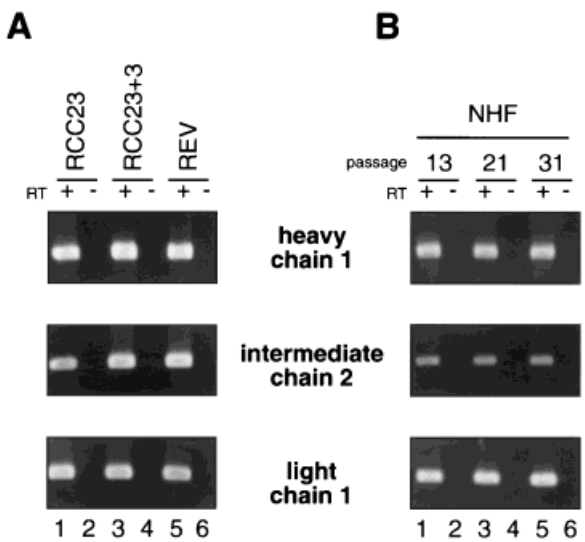

Fig. 3. RT-PCR analyses of heavy chain 1 (upper panels), intermediate chain 2 (middle panels) and light chain 1 (bottom panels) of cytoplasmic dynein. **A:** Samples are same as those in Figure 1B. **B:** Samples are same as those in Figure 2.

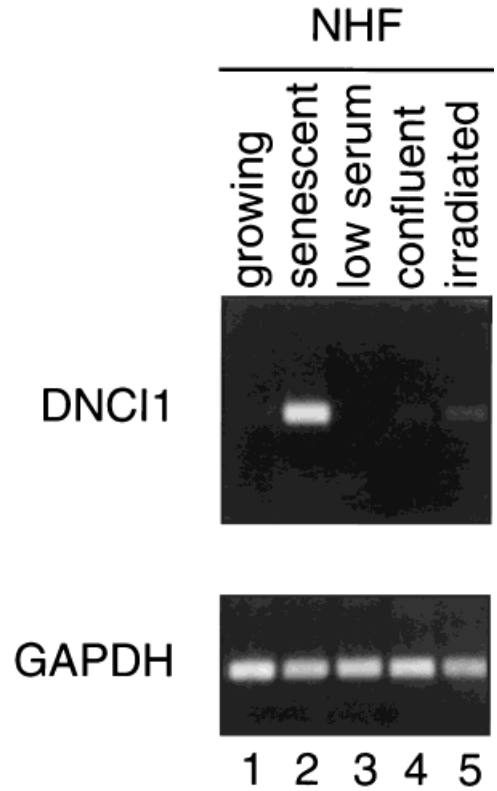

Fig. 4. DNCI1 induction is not generally associated with cell growth arrest. DNCI1 mRNA expression was examined by RT-PCR in NHF (passage 18) that were growth-arrested by serum starvation (**lane 3**), contact inhibition (**lane 4**) and γ-irradiation (**lane 5**). **Lane 1**, growing NHF at passage 18; **lane 2**, senescent NHF as a positive control.

Mouse Dnci1 mRNA is expressed most abundantly in brain [Crackower et al., 1999]. By northern blot analysis of normal human tissues (data not shown), we also observed the highest DNCI1 expression in human brain. Human heart and skeletal muscle also expressed a high level of DNCI1 mRNA. It should be noted that a low but significant level of DNCI1 expression was detected in all of the other human tissues and cells examined (i.e., pancreas, kidney, liver, lung, placenta, colon, small intestine, ovary, testis, prostate, thymus, spleen, and mammary epithelial cells). This finding suggests a physiological role of the DNCI1 in a wide range of human tissue and cell types.

A precise function of the DNCI1 protein in cellular senescence still remains to be determined. It is unlikely that the DNCI1 is related to telomerase regulation, because the progressive

increase in DNCI1 mRNA expression occurred with no change in telomerase activity (i.e., with telomerase activity kept repressed) during in vitro aging of NHF and RCC23+3 cells. Regulations of chromosome movement and organelle transport, in which the cytoplasmic dynein is suggested to play important roles [Vallee and Sheetz, 1996; Endow, 1999; Karki and Holzbaur, 1999], may be related to cellular senescence. For instance, altered control of mitotic chromosomes could contribute to permanent cell cycle arrest at cellular senescence, which may be consistent with an aging-related misregulation of mitotic cell division [Ly et al., 2000]. Production of a DNCI1-specific antibody and determination of cellular localization of the DNCI1 protein during cellular aging will facilitate better understanding of its biological significance in cellular senescence.

It should be noted that the *DNCI1* gene is located on the long arm of chromosome 7 (7q21.3–q22.1, Table I), where putative tumor suppressor genes of various human cancers (e.g., ovarian, breast, and prostate cancers) are mapped [Verma et al., 1999; Zeng et al., 1999; Neville et al., 2001]. In our preliminary examination of seven human cancer cell lines (3 breast and 4 ovarian) for DNCI1 mRNA, no or little expression was detected in a breast cancer cell line MCF-7 and an ovarian cancer cell line BG-1. The chromosome 7q is also supposed to have a senescence-inducing gene whose inactivation is critical to immortality of a group (complementation group D) of human cell lines [Ogata et al., 1995; Nakabayashi et al., 1997]. Of great interest will be whether loss or mutation of the *DNCI1* gene plays a causative role in human cell immortalization and caricinogenesis. A potential significance of alternatively spliced forms of DNCI1 [Crackower et al., 1999] also remains to be examined.

Finally, only a small number of clones were the subject of this study. The successful application of SSH shown here suggests that a large-scale study to find more differentially expressed genes may lead to the identification of a gene on chromosome 3p that triggered to induce cellular senescence in RCC23 cells.

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