Increase in Telomere Sequence-Binding Activity in Normal Human Fibroblasts in Senescence or in Cells Treated with Phorbol Ester or N-Methyl-N'-nitro-N-nitrosoguanidine

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The telomere is a specialized chromatin structure composed of unique repetitive DNA sequences and specific nuclear proteins. Telomere sequence-binding activity was measured by a mobility shift assay using nuclear extract from normal human fibroblasts. The specific binding activity of the telomere sequence increased in cells that were in a senescence state compared to that in cells at early population doublings. Treatment of cells with tumor promoting phorbol ester TPA induced an increase in the telomere sequence binding activity of nuclear extract in young cells, but the increase was marginal in senescent cells. DNA-damaging N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) also increased the telomere sequence binding activity in young cells, but not in senescent cells. As a reference, we measured the binding activity to NFκB sequence. It was activated by TPA or okadaic acid, but was not affected by MNNG or in senescence. The increase in telomere sequence-binding activity seemed to depend on activation of tyrosine phosphorylation, since an inhibitor of Tyr-kinase abolished the increase in telomere-binding activity. The molecular weight of the major binding factor in the normal human fibroblasts was approximately 32 kDa which is different from that of the telomere-associated protein, TRF-1.

Key words telomere; senescence; binding factor; alkylating agent

Telomere is a specialized chromatin domain that is essential for maintenance of eukaryotic chromosome integrity. It consists of repeated DNA sequences of TTAGGG in mammalian cells that range from about 2 to 150 kb, and is integrated into a unique nucleoprotein complex. Telomeres have unusual structure as revealed by digestion with micrococcal nuclease of nuclei of human cells, and are heterochromatic in S. cerevisiae or Tetrahymena due to packaging into condensed nucleoprotein.

RAP1 is a protein that recognizes and binds the telomere sequence specifically in S. cerevisiae, and lures several components such as Sir2, Sir3, and Sir4 to form a complex at the end of each chromosome. Protein components of the telomere in mammalian cells have recently been identified from HeLa cells, and named TRF1. TRF1 has sequence-specific telomere repeat binding activity, but has no apparent similarity to RAPI. The conserved Myb domain is suggested to participate in the binding in both cases. In RAP1 proteins are also reported to bind the telomere repeat in a sequence-specific manner, but there seems no evidence that they participate in telomere complexes of chromatin.

During cellular senescence of normal human fibroblasts, telomere length gradually decreases, possibly due to the absence of telomerase activity. The telomerase is suggested to be a molecular clock for determination of limited division potential in human fibroblasts, but molecular events that occur in the telomere complex during senescence are largely unknown at present. Cellular senescence is accompanied by alterations in the expression of several genes. For example, the inducibility of c-fos or HSP70 is decreased in senescent cells, due to a change in activity of transcription factors, and alterations in signaling molecules have also been reported. In the present report, we examined the activity of the nuclear factors that bind the telomere sequences during cellular senescence and treatment with a DNA damaging agent.

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MATERIALS AND METHODS

Cells Normal human diploid fibroblastic cells, TIG-3, were cultured in Eagle’s minimum essential medium (MEM) supplemented with 10% fetal bovine serum in a CO2 incubator. Cells were passaged at 1:4 split every week, and cells became senescent after 60 population doublings. Cells after 4 d following medium renewal were used for the experiments.

Preparation of Nuclear Extracts Cells were rinsed with phosphate buffered saline (PBS), and collected with a rubber policeman. Nuclear extracts were prepared following the procedures of Dignam et al. as described previously. A telomere DNA probe for gel shift assay was synthetic oligonucleotides annealed and subcloned into pUC19; 5'-AGCTTAGGTTAGGGTTAGGGTTAGGGTTAGGG and 5'-GATCCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCT

RESULTS AND DISCUSSION

Under our culture conditions, TIG-3 cells entered senescent stage after 59–60 population doublings. Nuclear extracts prepared from cells at different population doublings of TIG-3, and their activity to bind the telomere probe was determined by gel shift assay. Figure 1 shows that nuclear ex-
tractions from human fibroblasts at early stages of growth (population doublings of 38) had essentially no telomere probe-binding activity, but those from untreated senescence cells showed much stronger binding activity. Growth status of cells was resting in both stages, since cells were harvested 4 days after medium renewal. TRF-1 is the first mammalian telomere sequence-binding factor so far identified, and its binding activity was not altered during cell cycle.71 The binding activity described in Fig. 1 was also unaffected in growing and resting young cells (data not shown), but treatment of young cells with the phorbol ester TPA dramatically increased the activity, while the increase was marginal in senescent cells. Okadaic acid, an inhibitor of protein phosphatase, did not affect the telomere-sequence binding activity significantly.

It is still a controversial issue how cells senesce, but one of the inducing factors seems genotoxicity, since cells with hereditary disorders in DNA repair have shorter life span.18 Treatment with an alkylating agent, N-methyl-2-nitro-N-nitrosoguanidine (MNG), also increased the binding activity in young cells, but it rather decreased the binding activity in senescent cells. H2O2 which also damages DNA did not affect the telomere-binding activity. As a reference, the NFκB-binding activity was compared using the same nuclear extracts. TPA- or okadaic acid-treatment increased the NFκB-binding activity both in young and senescent cells as reported,194 but MNG-treatment did not affect the activity. H2O2 induced a slight increase in NFκB-binding activity.

From cross-linking experiments, a protein with a molecular weight of 32 kDa was a major factor that interacted with the telomere sequence probe (Fig. 2). A faint band with a molecular weight of about 70 kDa was also detected. Both bands competed with unlabeled telomere probe. Recently cloned TRF1 encodes a protein with a molecular weight of 60 kDa,80 and the factors detected in Fig. 2 seem to be distinct from TRF1 from their molecular weight. Other nuclear proteins that bind the telomere sequence are heterogeneous nuclear ribonucleoproteins (hnRNP) A1, A2/B1, D, and E.9,10 Addition of antibody against hnRNPs (a kind gift of Dr. Dreyfuss) in the mixtures of gel shift assay did not affect the binding activity (data not shown), and thus the 32 kDa factor shown in Fig. 2 may not be a component of hnRNPs.

To get some insight in the signaling pathways that activate telomere-binding, the effects of protein kinase inhibitors were tested. The results of Fig. 3 indicate that an inhibitor of protein tyrosine kinase, genistein, inhibited the activation of telomere-binding activity caused by MNG. In Fig. 3, the shifted band with telomere probe was mainly fast migrating, but the appearance of the slow migrating band shown in Fig. 1(a) varied depending on the preparations of nuclear extracts. Staurosporin, an inhibitor for Ser/Thr kinase, did not affect the activation (data not shown).

An interesting observation was the increase in telomere-binding activity in senescent cells and in young cells treated with MNG. Senescent cells are known to contain a higher level of damaged DNA,20 and genotoxic drugs activate the c-
Fig. 3. Effect of Kinase Inhibitor on the Increase in Telomere Sequence-Binding Activity

TIG-3 cells at population doublings 48 were untreated (1), treated with 5 μg/ml MNNG (2) or 5 μg/ml MNNG + 1 μmol genistein (3), and nuclear extracts were prepared. Binding activity to telomere (a) or NFκB (b) probe was examined by gel shift assay.

Abl protein tyrosine kinase. Such a kinase may participate in the activation of telomere-binding factor in MNNG-treated or senescent cells. We have been trying to isolate cDNA for the 32 kDa binding factor by the Far-western method, but have been so far unsuccessful. Identification of such a factor will facilitate the understanding of mechanisms underlying cellular senescence.

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REFERENCES