Scanning probe microscope visualization of t-loop assembly by TRF2 in cells

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ABSTRCT

Telomeres are essential nucleoprotein structure at the ends of all eukaryotic chromosomes. Our previous work demonstrated that mammalian telomeres were shown to end in a large t-loop structure in vitro and the formation of t-loops was dependent on the presence of TRF2. In this work, the telomere DNA and its complex of TRF2 in HeLa cells has been direct observed in the nanometer resolution regime by atomic force microscopy (AFM) and scanning near-field optical microscopy (SNOM). AFM images showed that the looped structures exited in cell extract containing TRF2, but it disappeared in the protein-deleted samples. When cells were pretreated by UV light plus psoralen, the looped structure could be observed in the protein-deleted samples. SNOM images further demonstrated TRF2 and p53 proteins in cell was bound at the loop junction. Above results suggest that the telomere t-loop structure by TRF2 play a important role in cell-senescence, and might signals p53 protein directly through association with the t-loop junction in cells.

Keywords: Telomeres, scanning near-field optical microscopy, atomic force microscopy, telomere DNA, TRF2 protein, p53 protein, looped structures, t-loop assembly, fluorescence, HeLa cell

1. Introduction

Telomeres are essential nucleoprotein structure at the ends of all eukaryotic chromosomes [1]. by acting as caps, protecting chromosome ends from fusion and degradation [2,3]. The telomeric DNA usually consists of a simple repeated sequence with one G-rich strand. This G-strand extends to the 3′ end of the chromosome and protrudes to form a single-stranded overhang. In vitro, single-stranded G-rich telomere DNA can form a variety of noncanonical structures including G quartets, triple helices and G·G base pairing [1,4]. Electron microscopy (EM) showed that mammalian telomeres were shown to end in a large loop resembling a lasso, termed the telomeric t-loop [5]. Recent studies have implicated several proteins required for DNA damage recognition and repair in telomere maintenance. The telomere-specific protein TRF2 plays a central role in concealing telomere ends from ds break recognition and repair factors, suggesting that inhibition of TRF2 function at the telomeres signals p53 directly. It was demonstrated by EM the ability of p53 to hind t-loop structures [6]. However, EM couldn't quantitative the width and height of the telomeric DNA and can not resolve details of structure. On other hand, telomere DNA used in these observations was from cells with UV-psoralen treatment. The structure of the telomeric t-loop and its interaction with protein in cells is still not known.
Atomic force microscopy (AFM) has its advantages not only in observing the real morphology with high resolution but

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also in the rapid sample preparation. AFM can images individual DNA or proteins under physiological conditions [7], Scanning Near- field Optical Microscopy (SNOM) overcome the diffraction limit of light microscopic technique [8,9]. And the topographical image, light transmission (absorbency) and fluorescence images can be recorded simultaneously by SNOM. [10]. It has become possible to observe single molecule events on the telomere structure and its interaction with proteins using this technique.

Our previous work demonstrated by atomic force microscopy (AFM) that mammalian telomeres end in a large t-loop structure in Hela cells with UV-psoralen treatment [11]. In this work, the telomere DNA and its complex of human TRF2 and p53 has been observed directly in the nanometer resolution regime by AFM and SNOM. Results showed that the reversible control of the telomere t-loop structure by TRF2 protein play a important role in cell-senescence, and might signals tumor suppressor protein p53 directly through association with the t-loop junction in cells.

2. MATERIALS AND METHODS

2.1 Materials

Human HeLa cells were obtained from Beijing Medical University (Beijing, China). The cells were maintained in RMPI 1640 medium, with 10% fetal bovine serum, 100 IU/ ml penicillin and 100 IU/ ml streptomycin, and in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. TRF2 protein was a gift of Prof. Lange (Laboratory of Cell Biology and Genetics, Rockefeller University). Human p53 protein was from Neomaker co Inc. mouse anti-human TRF2 protein was from BD Co. Mouse anti-ribbit IgG, TRITC from Jingmei Biotech Co. Ltd..

2.2 Preparation of cell nuclei

All operations were carried out at 0-4 °C. Nuclei were isolated from 2-3 x10^8 HeLa cells by lysis in 40 mM Tris-Hcl, (pH 7.5), 2 mM DTT and 5 mM $MgCl_2$, homogenized with 12 strokes in a glass homogenizer and centrifugation through a glycerol cushion (25% glycerol, 10 mM Tris-Hcl, (pH 8.0), 1.5 mM $MgCl_2$). Transmission electron microscopy was used to assess the purity of the nuclear preparations and confirmed the absence of organellar contamination. Nuclei were washed gently in a buffer containing 150 mM KCI, 5 mM $MgCl_2$ and 25 mM Tris-Hcl (pH 7.5) Nuclei were washed again and suspended in a buffer containing 50 mM Tris-Hcl (pH 8.0), 0.1 mM DTT and 0.1 mM EDTA For Chromatin preparation or suspended in 3 ml of cross-linking buffer (15 mM Tris-Hcl, 15 mM NaCl, 60 mM KCl, 1mM EDTA, 0.1mM EGTA, and 0.25 M sucrose, pH7.4) for isolation and purification of Telomere DNA.

2.3 Preparation of chromatin

The preparation of chromatin from purified nuclei was carried out according to the method of Oudet et al. [12], in order to prevent DNAase action and drastic shearing as much as possible. All homogenirations were done manually. Nuclei from cells were lysed in 250 ml of a buffer containing 10 mM Tris-HCI (pH 7.9).80 mM NaCl, 20 mM EDTA and 0.01% Triton X-100 using a Teflon glass homogenizer. After 10 min of centrifugation at 12000 g, the pellet was suspended in 40 ml of a buffer containing 10 mM Tris-HCl (pH 7.9). 150 mM NaCl,20 mM EDTA and 0.01% Triton X-100 and homngenized. A 20 ml -aliquot of the suspension was then centrifuged for 3 h at 54 000 g through two layers of sucrose, the upper one (10 ml) containing 15 mM TRIS-HCI (pH 7.9), 150 mM NaCl, 20 mM EDTA and 0.01% Triton X 100 and 1.65 M sucrose, whereas the lower one (25 ml) contained 15 mM Tris-HCI (pH 7.9).150 mM NaCl, 20 mM EDTA and 0.01% Triton X-100 and-1.7 tasucrose. The pellet was then homogenized in 50 ml of a buffer containing 10 mM Tris-

HCI (pH 7.9), 15 mM NaCl and 20 mM EDTA and then centrifuged for 10 min at 12000 g. The purified chromatin Pellet was finally suspended in 5 ml of 10 mM Tris-HCI (pH 7.9), 5 mM sodium bisulphite and 0.1 mM dithiothreitol and stored at –20 ºC.

The preparation of lysine-rich histone-depleted (HI-depleted) chrornatin was carried out according to the method of Oudet et al. [12] and modified for AFM observation. Purified chromatin was diluted to 100 μg DNA ml^{-1} in a buffer containing 10 mM Tris-HCI (pH 8.0), 700 mM NaCl and 5 mM sodium bisulphite (buffer A). Chromatin solubilization. which was accompanied by a marked increase in viscosity, was achieves in 2-3 h at 4 ºC. A 1 ml align of the very viscous solution was diluted in 19 ml of tridistilled water, homogenized. Kept at 4 ºC for 1 h and then centrifuged for 20 mim at low speed. The supernatant containing lysine-rich histone-depleted (HI-depleted) chromatin was taken out for AFM imaging.

24 Isolation and purification of telomere DNA [5,11]

Psoralen (10 mg/ml stock dissolved in DMSO) was added to above 3 ml of nucleus suspension, final concentration was 250 μg/ml. The mixture was spread on a 100 mm plastic petri dish on ice and stirred for 30 min while exposed to a 365 nm UV light bulb at a distance of 2 cm. Then, nuclear suspensions were treated with proteinase K (20mg/ml) in the presence of SDS (0.5%), and DNA was isolated by phenol/chloroform (1:1) extraction and ethanol precipitation (3 Volume). The deproteinized sample was suspended in 9 ml of 10 mM TE buffer (Tris-Hcl, 1 mM EDTA PH7.5) and cleaved with Rsal (1250 units) and HinF1 (1250 units) in a buffer of 10 mM Tris ,10 mM $MgCl_2$, 30 mM NaCl , 1 mM DTT, and 100 μg/ml of bovine serum albumin for 12 hr at 37 °C. During the final hour, RNase (Pharmacia) was added to 20 μg/ml. The sample was then extracted one time with phenol : chloroform: isoamylalcohol = 50:49:1, precipitated with ethanol, and suspended in 3 ml of TE. The sample was purified by a 2.5 x 20 cm Bio-gel P-2 column and eluted by water at a flow rate of 0.2 ml/min. Fractions of 0.6 ml were taken and OD_{260} of each determined. First peak (fraction 5) are identified to contain nearly all of the telomere species by ^{32}P-labeled probes $(TTAGGG)_4$ and fluorescent labeled probes EB method [13].

2.5 AFM observation

A drop of the solution (5μl), was distributed on freshly cleaved mica compressed nitrogen gas was applied to the sample in such a way that the liquid spread out on the mica surface, and immediately dried under an infra-red lamp prior to the AFM observation [14]. Tapping mode images were obtained on a nanoscope III with a multimedia AFM using nanoprobe silicon tips. Microscopic analysis was carried out by using a commercial atomic force microscope (Digital instruments. Santa Barbara, CA). All AFM images were obtained in air at room temperature by using contact mode with a spring contact of 0.12nN nm^{-1}. The cantilever was 200 μM in length. The lateral dimensions of biological structures in AFM are overestimated because of the finite dimensions of the tops.

2.6 SNOM observation

The preparation of SNOM samples was carried out according to the method of Hausmann et al., [15], Spread samples were fixed 10 min in 3.7% acid-free formaldehyde, 0.1 M sucrose, pH 9.2, followed by air-drying. The preparations were extracted for 30 min with 0.2% Triton-X-1000/PBS, washed with PBS, incubated for 10 min in PBTG (PBS, 0.2% BSA, 0.1% Tween 20, 0.1% Gelatine, followed by the incubation at 4 ºC overnight with an antibody solution containing mouse anti-human TRF2 or p53 antibodies [16,17]. And then incubated again for 30 min at 37 ºC with mouse anti-ribbit

IgG, TRITC and the reaction was stopped by washing off the substrate.. Before SNOM, specimens were dehydrated through an ethanol series (70, 85, 95% for 5 min each) and air-dried.

The detection of fluorescent labelled samples were carried out with an advanced commercial SNOM (Aurora, ThermoMicroscopes, USA). A He-Ne laser beam with a wavelength of 543 nm was coupled into the SNOM probe via a glass fibre. The topographic scan was controlled by measuring the interaction of the lateral shear-force oscillation of the SNOM tip. The instrument was controlled by the NanoScope IIIa (Digital Instruments Vecco GmbH) controller. All near-field images were recorded and visualised in three dimensional topographic false colour plots using the NanoScope IIIa software (version 4.42r1) running under Windows on a PC.

3. RESULTS

3.1 Direct visualization of the loop structures in cells

The high resolution and cross sectioning measurement function of AFM make it convenient for us to observe telomere morphology and further to analysis its fine structure in cells. Fig 1 showed that there exactly exists a looped with a tail structures (Fig.1-A, B) and a well-known linear bead structure. (Fig.1-A, C) in the lysine-rich histone-depleted chromatin extract.

We have measured the contour lengths of the looped structures, the average length of the looped was 1 .1 $\pm$ 0.3 μM (n = 40) , which is longer than what (2.5 ± 0.5 kb, about 0.85 μM length) have been measured for the protein-deleted samples with UV-psoralen treatment [11]. Possible reason is that DNA was condensed after UV-psoralen treatment. The mean height of the loop-tail junction was 4.1 $\pm$ 1.5 nm (n = 40), suggesting a complex structure of DNA and proteins.

A 'beads-on-a-string' pattern fored by nucleosomal cores and linker DNA is a characteristic feature of lysine-rich histone-depleted chromatin. This topical appearance of nucleosomal chains was descried previously by electron microscopy [18]. The nucleosomes have an average diameter of ≈34 $\pm$ 5 nm (n = 40) and a mean height of 4.1 $\pm$ 1.1 nm (n = 40). Nucleosomes in native chromatin from *Tetrahymopna* therrnophila is 31.2 ± 4.9 nm [19]; micleosomes in native chicken chromatin, 30 $\pm$ 3 nm, nucleosome cores assembled in vitro into chromatin fibres, ≈30-40 nm, with the smallest cores measuring 27 nm.

On the other hand, no any looped structures and a well-known linear bead structure was observed in the purified sample of removed protein (Fig.1-D). However, in sample of removed protein from the psoralen photo-cross cells, the loops were still observed duo to psoralen cross-linking T residues of opposite DNA strands randomly (Fig.3). It is suggests that telomeric protein is necessary for the formation of the loops in cells.

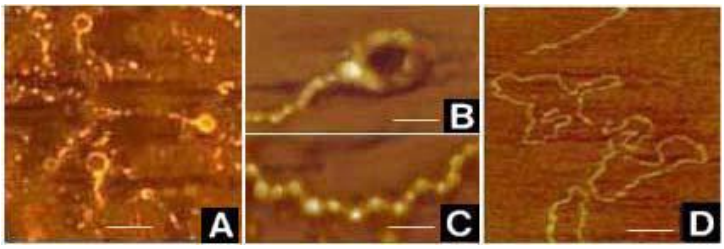

Fig.1, A, the looped structures exited in crude cell extract. Scale bars: 1.0 μm.B, a typical t-loop structure of telomeric DNA. Scale bars: 200 nm. C, a typical linear bead structure.. Scale bars: 200 nm. D, the loops disappear in the purified sample without protein. Scale bars: 1.0 μm.

3.2 Binding sites of proteins for looped structures in cells

In order to understand the binding proteins, TRF2 has been determined in the nanometer resolution regime by SNOM. SNOM imaging also revealed a typical loop structure at the chromosome end that contained TRF2 protein (Fig. 2 A,C). The fluoresecent labeled TRF2 protein was presented in the t-loop junction. The specialized telomere-binding protein, TRF2 protein, binds at the junction of the loop (Fig. 2 B), which may play this role in stabilizing or allowing formation of the loop. The exists of p53 at the junction of the loop were also observed (Fig. 2 D), which is similar to the results obtained by EM in vitro [11].

These results provide direct evidence for the presence of p53 at TRF2-bound t-loop junctions. The location of p53 binding within the junction, either to the displaced strand or the Holliday junction-like structure have observed most of p53 appeared in the tera-stranded junctions [20]. Our previous work evidenced the circular portion of loops was double-strand structure, the triplex or tetraplex structure for the loop-tail junction [6].

It suggests that the proteins are capable of binding to the junction simultaneously. SNOM images also demonstrate that the looped molecules must also contain TRF2 [23], p53 has a high affinity for the t-loop junction. All t-loop junctions that are labeled with fluorescence therefore, are indicated to contain both p53 and TRF2.

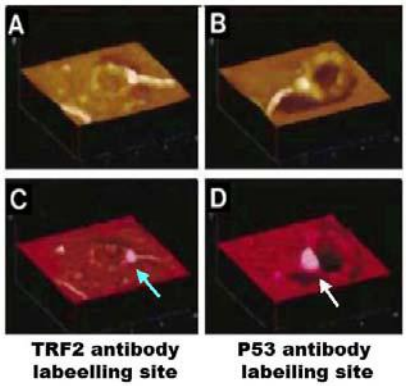

Fig.2, SNOM Images of telomeres associated with TRF2 or p53 proteim. A and B, the topographic images reveal a t-loop structure. C and D, C, TRF2 antibody labelling sites, as regions of high intensity of transmitted light at 543 nm (blue arrow); D, p53 antibody labelling sites(white arrow).Image area 4.0 X 4.0 μm.

3.3 Visualization of telomeric DNA loops isolated

Fig 3 is a typical AFM images of telomeric DNA isolated from the psoralen photo-cross cells. AFM showed that the average value of loop circle portion height is about 0.426 $\pm$ 0.172 nm. This value could illustrate that the loop circle portion is duplex DNA. The average height of the tail-loop junction area is about 0.851 $\pm$ 0.293 nm (Fig.3 A). Due to the height of triplex is two times that of duplex, so we conclude that the structure of junction area is triplex [21,22]. The height of some tail-loop junction is higher than above average height, it is 1.286 $\pm$ 0.051 nm, suggesting tetra-stranded structure may exist the tail-loop junction models of triplex or two kinds of tetra-stranded were proposed (Fig.3 B,C and D), Generally speak, the height of DNA duplex is about 0.36~0.76 nm in AFM. In this work, the average height of duplex DNA is 0.426 nm. The maximal is 0.768 nm, the minimal is 0.235 nm. There will appear different result of measure under the different condition of measure, such as tip, humidity of environment and so on. The difference of the

height of duplex loop may be the different humidity of air. So we should compare the height of different DNA loop in the same images to eliminate these influence.

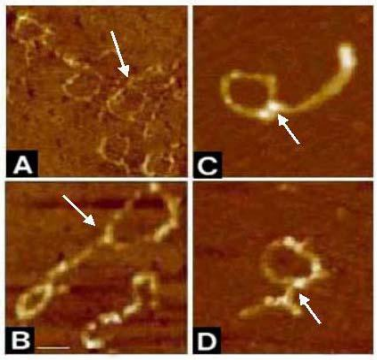

Fig.3. the typical t-loop structure of telomeric DNA and its corresponding height. A. the average value of loop circle portion height and the tail-loop junction area (white arrow) is about 0.426 ± 0.172 nm and 0.851 ± 0.293 nm (n = 41), respectively. B and C the tail-loop junction area (white arrow) is about 1.288 ± 0.051 nm (n = 14). D. the tail-loop junction area (white arrow) is about 1.295 ± 0.062 nm (n = 5).

Fig. 4 shows that SNOM imaging of a isolated telomeric DNA with EB label, which revealed a typical DNA t-loop.

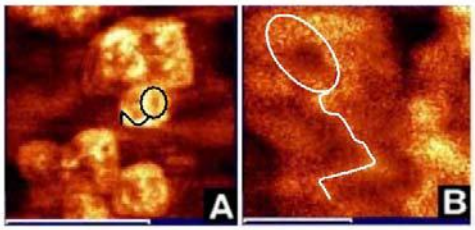

Fig.4, SNOM fluorescence images of telomere DNA looped structure with ethidium Bromide. A. the fluoresence images of DNA looped structure, Scale bars:5.0 μm. B. a typical t-loop structure of telomeric DNA, Scale bars: 1.0 μm..

4. DISCUSSION

Telomeres are essential nucleoprotein structure at the ends of all eukaryotic chromosomes. Studies on interaction of telomeres and proteins have received particular attention for its possible significance to aging and cancer [1]. In this work, the telomere DNA and its complex of human TRF2 and p53 has been determined in the nanometer resolution regime by atomic force microscopy (AFM) and Scanning near-field optical microscopy (SNOM).We have observed the telomere looped DNA (it is unstable) and its complex of human TRF2 and p53 in chromosome extract without any treatment, such as deprotein, enzyme digest and purification.. AFM and SNOM images showed that t-Loops were generated by interaction of the telomere DNA with TRF2. TRF2 proteins bound specially at the t-loop junction through intramolecular interactions.

During the extraction of telomere, due to the possible isolation of protein and telomere DNA could result in the unstable structure of telomere, such observed DNA structure might be formed during preparation. To overcome this difficult, we

used chromosome containing proteins and used a photosensitive probe called psoralen, which could intercalate into a duplex or triplex DNA and upon UVA irradiation preferentially cross-link T residues of opposite strands [11]. Maybe the position of psoralen works is the same as TRF2 [5,11]. So psoralen cross-linking of the DNA strands is predicted to preserve the t-loops after removal of protein. According to above same method, no any loop with a tail was observed in the samples without treatment of psoralen-UVA (see Fig.1-D). Psoralen cross-linking could stabilize t-loops that might exist in vivo before removing proteins from the DNA (see Fig.1-d).

Above results showed that both proteins are present in a complex at the t-loop Junction in cells. These results suggest a more specific role for p53 in t-loop formation/stabilization [6]. There are important biological significant roles for p53 localization to the t-loop junction. It reported that induction of apoptosis by telomere 3' overhang-specific DNA [24] and that the tumor suppressor protein p53 is involved in the maintenance of telomeric tract length in mice [20]. Normally, p53 translocates from the cytoplasm to the nucleus at the G1/S transition and is shuttled back to cytoplasm shortly thereafter. The presence of p53 at the t-loop junction just prior to DNA replication may promote resolution of the junction to facilitate telomere replication. It is also possible that p53 is sequestered at the t-loop junction to allow immediate recognition of any loss of end protection by p53. Cell cycle arrest or apoptosis then can be triggered. Additionally, other protein factors may be recruited to the chromosome end through interactions with other protein such as p53. These factors may be essential for telomere structure and function. It demonstrated that by senescence in primary cells is induced by altered telomere state, not telomere loss [25] or decreasing telomerase activity [26]The changes of the telomere structure state (cap or uncap) may signals p53 directly through association with the t-loop junction, then then to trigger apoptosis through the p53/ATM-dependent DNA damage checkpoint pathway [27].

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