The human telomeric protein hTRF1 induces telomere-specific nucleosome mobility

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ABSTRACT

Human telomeres consist of thousands of base pairs of double-stranded TTAGGG repeats, organized by histone proteins into tightly spaced nucleosomes. The double-stranded telomeric repeats are also specifically bound by the telomeric proteins hTRF1 and hTRF2, which are essential for telomere length maintenance and for chromosome protection. An unresolved question is what role nucleosomes play in telomere structure and dynamics and how they interact and/or compete with hTRF proteins. Here we show that hTRF1 specifically induces mobility of telomeric nucleosomes. Moreover, Atomic Force Microscopy (AFM) imaging shows that hTRF1 induces compaction of telomeric DNA only in the presence of a nucleosome, suggesting that this compaction occurs through hTRF1–nucleosome interactions. Our findings reveal an unknown property of hTRF1 that has implications for understanding telomere structure and dynamics.

INTRODUCTION

Linear eukaryotic chromosomes end in specialized nucleoprotein complexes named telomeres (1,2). In humans, telomeres consist of 10–15 kb of double-stranded TTAGGG repeats, and 100–200 nt 3′ protrusions of the G-rich strand. The establishment of a capping structure at telomeres protects chromosomes from degradation and recombination, however the structure of telomeres remains elusive. The proteins hTRF1 and hTRF2 specifically bind human duplex telomeric DNA as homodimers (3–5), whereas hPOT1 recognizes the 3′ single-stranded overhangs (6). These three proteins, together with TIN2, hRAP1 and TPP1, form the complex named shelterin (7,8). It has been proposed that the shelterin complex stabilizes a lariat structure named t-loop (9), presumably deriving from invasion of the upstream telomeric double-stranded DNA by the single-stranded G-overhang (10). Telomeric DNA in higher eukaryotes is organized into a chromatin structure characterized by regularly and unusually closely spaced nucleosomes (11–13), separated by short linkers that in humans are only ~10 bp long (11,12). Electron microscopy (14) and micrococcal nuclease (MNase) footprinting experiments (15) indicate that nucleosomes are also present in the terminal t-loop.

Due to the complex functions they perform, telomeres are likely to interconvert between different and still poorly defined conformations. Whereas the functions associated to telomeric proteins have been widely studied, the role played by nucleosomes in telomere structure and function is almost completely unexplored (16). Several in vitro studies have shown that nucleosomes assembled on telomeric DNA have particular features. As a consequence of the 6 bp sequence repeat of human telomeric DNA that is out of phase with the helical periodicity of DNA (13,17,18), telomeric nucleosomes are the least stable nucleosomes so far studied and occupy multiple isoenergetic positions without rotational phase (13,17,19,20). In our view, it seems very likely that the competition between histones and TRF proteins for telomeric DNA binding is involved in regulating telomere dynamics.

Recently, we showed that hTRF1 forms specific and stable ternary complexes with nucleosomes containing human telomeric repeats (21). hTRF1 binding causes alterations in the nucleosome structure, without dissociation of histone subunits (21). Similarly, hTRF2 interacts with telomeric chromatin (22), albeit with a lower affinity for nucleosomal binding sites than hTRF1 (A.G., M.S., S.C., unpublished results). Photobleaching experiments have shown that nucleosome dissociation is very slow (23), compared with that of H1 and several other
binding proteins, including TRF1 and TRF2 (24,25). Biochemical probing experiments have shown that nucleosomes are dynamic complexes in equilibrium between wrapped and partially unwrapped states (26), and capable of spontaneous short-range movements along DNA (27,28). Significantly, as a consequence of the peculiar features of telomeric DNA sequences, telomeric nucleosomes are intrinsically more mobile than bulk nucleosomes (29). An attractive hypothesis arising from these observations is that hTRF1 binding may result in nucleosome repositioning rather than in nucleosome disruption or loss.

Here, we show that binding of hTRF1 causes the sliding of the telomeric nucleosome. Furthermore, TRF1 seems to interact with the mobilized nucleosome inducing the DNA to fold back. Our results reveal an unknown sequence-specific remodeling activity of hTRF1 that allows binding in the context of chromatin and provides an insight into the dynamic structure of telomeres.

MATERIALS AND METHODS

DNAs and proteins

The Biotin-TG580 DNA fragment was prepared by PCR with the primers 5'-Biotin-TGA ACC ATC ACC CTA ATC AAG-3' and 5'-GTT GTG TGG AAT TGT GAG CG-3', using as a template the plasmid pBIKSK-TG580Ade (29). The PCR product was then digested with Adel and gel-purified. To obtain the DNA fragments hTel27 and TAND1, the plasmids pUC-hTEL27Ade and pUC-TAND1Ade (29) were cut with NheI. The linearized plasmids were digested with AdeI and gel-purified. To obtain the DNA fragments hTel27 and TAND1, the plasmids pUC-hTEL27Ade and pUC-TAND1Ade (29) were cut with NheI. The linearized plasmids were dephosphorylated with calf intestinal phosphatase and the 5' protruding ends were filled in with Klenow enzyme in the presence of 100 μM each of dATP, dCTP, dTTP and ddGTP. For the restriction enzyme (RE) assay, filling in of 5' protruding ends was performed in the presence of 50 μCi of [α-32P]dCTP. Finally the plasmids were cut with AdeI and gel-purified.

Nucleosome and purified histone octamer were prepared from chicken erythrocytes as previously described (19). Recombinant, His6-tagged full-length hTRF1 was baculovirus expressed in Sf9 cells and purified as previously described (30).

Nucleosome reconstitution

For the RE assay, nucleosomes were assembled onto radiolabeled hTel27 and TAND1 DNA fragments by the octamer exchange method as described (20). For AFM imaging nucleosome were assembled by the salt dialysis technique (29,31). Briefly, 2 μg of hTel27 or TAND1 were mixed with 1.8 μg of purified histone octamer from chicken erythrocytes in 50 μl of 10 mM Tris–HCl (pH 7.6), 2.0 M NaCl, 0.5 mM EDTA, 0.5 mM PMSF, 1 mM benzamidine. Samples were stepwise dialyzed against buffers containing decreasing NaCl concentrations (1.2, 1.0, 0.8, 0.6, 0.2 M NaCl), and finally dialyzed overnight against 5 mM Tris–HCl (pH 7.6), 0.5 mM EDTA.

RE assay of nucleosome mobility

Radiolabeled nucleosomes (1 μg) were ligated to equimolar amounts of Biotin-TG580 by using 5 U of T4 DNA ligase at 4°C overnight. To prevent self-ligation, the DNA fragments have asymmetric protruding ends derived by cutting with AdeI. Moreover, the opposite ends have been dephosphorylated and filled in with a terminal deoxyxynucleotide (see the section DNAs and proteins). Samples were then incubated for 5 h at 4°C on a rotating wheel with streptavidin-coated paramagnetic beads (M280 streptavidin-dynabeads), in order to remove unligated nucleosomes. After attachment, beads were washed three times with 100 μl WBN (20 mM Tris–HCl, pH 7.6, 1 mM EDTA, 0.15 M NaCl, 0.1% Nonidet-P40, 1 mM MgCl2, 1 mM DTT, 0.1 mg/ml BSA, 40 μg/ml oligonucleosomes). Bound DNAs and nucleoparticles (concentration 25–50 nM) were then resuspended in WBN and incubated with hTRF1 (final concentration 1 μM) for 1 h at 25°C on a rotating wheel. Finally, samples were washed three times with RE buffer, digested for 20 min with SmaI or BseGI, and run on a 4.5% native polyacrylamide gel. The percentage of mobilized nucleosome (% Mobil. Nuc.) has been calculated using the equation

\[
\text{% Mobil. Nuc.} = 1 - \left( \frac{\% \text{Nuc. Sample}}{\% \text{Nuc. Ref.}} \right)
\]

where \% Nuc. Ref. is the fraction of nucleosomal band in the reconstituted nucleosome or in the ligated complex before incubation at 25°C. As shown in Supplementary Figure S1, after ligation of the nucleosome assembled on hTel27 to Biotin-TG580 and before incubation at 25°C, the fraction of nucleosome released by SmaI (Supplementary Figure S1, lane 4) is equivalent to that present in the reconstituted sample (Supplementary Figure S1, lane 1).

AFM imaging

After ligation, samples were incubated with hTRF1 (final concentration 1 μM) at 25°C for 1 h, and crosslinked by dialysis against 0.1% glutaraldehyde, 10 mM Tris–HCl (pH 7.6), 1 mM EDTA for 3 h at 4°C. After fixation, samples were diluted ~4-fold and immediately applied on spermidine (1 mM)-treated mica surface (SPD-mica). Imaging was performed using a Nanoscope IIIa equipped with E-scanner (Digital Instruments Inc., Santa Barbara, CA), operating in air under Tapping Mode™ at room temperature using canonical sharp silicon tips. Images have been recorded with a scanning rate of 3–4 Hz. Raw SFM images have been processed only for background removal (flattening). Volumes have been derived by area and height of the molecules, measured by using the microscope manufacturer’s image processing software. To evaluate contour lengths, AFM images were converted from Nanoscope format into TIF files and processed using SigmaScan Pro software (SPSS Inc., Chicago, IL). To measure the \( L_1 \) parameter and the apparent contour length, we used the read-through
method, assuming that DNA passes through the center of the protein (32). The histograms were fitted to Gaussian or multi-Gaussian functions by using the open source QtiPlot software.

RESULTS
hTRF1 binding induces the sliding of telomeric nucleosomes in a sequence-specific way

To investigate whether human TRF1 induces nucleosome mobilization, we used an experimental system that we previously designed to study nucleosome thermal repositioning [Figure 1A, ref. (29)]. The principle of this system is that a nucleosome reconstituted on a DNA fragment containing telomeric repeats is ligated to a naked DNA fragment and then incubated with the telomeric DNA binding factor hTRF1. The position of the nucleosome before and after hTRF1 binding is then investigated through restriction site mapping.

An *in vitro* reconstituted nucleosome on a terminally labeled DNA sequence (hTel27 or TAND1) was ligated to a 580-bp-long DNA sequence (Biotin-TG580) containing the TG-pentamer nucleosome positioning sequence (33) close to the ligation site. This DNA fragment binds the histone octamer with higher affinity than hTel27, a 207 bp DNA fragment containing 27 tandem TTAGGG repeats (29), and than TAND1, a 182-bp-long DNA fragment which we have used as a standard of average sequence DNA (17,34). The free energy difference of nucleosome formation of TG-pentamer is of $-2.20 \text{kcal/mol}$ of nucleosome with respect to TAND1 and of $-3.27 \text{kcal/mol}$ of nucleosome with respect to hTel27 [see ref. (17) and Supplementary Figure S2]. Therefore, the TG-pentamer should act as a nucleosome trap, preventing histone octamers from sliding back towards the original terminal position.

The ligation products (BioTG-Tel/Nuc and BioTG-TAND/Nuc) were then purified from unligated nucleosomes by binding to streptavidin-coated paramagnetic beads and then incubated with hTRF1. The nucleosome position before and after hTRF1 binding was determined by separate digestions with the REs SmaI and BseGI (RE digestion assay, detailed in Figure 1A). Figure 2A shows a RE digestion assay of the complex BioTG-Tel/Nuc after 1h incubation at 25°C in presence and absence of hTRF1. Digestion with SmaI, which cuts immediately downstream of the TG-pentamer sequence, generates two bands corresponding respectively to a naked 233-bp-long DNA fragment and to the nucleosomal complex. Estimations of the relative fraction of the nucleosomal band before and after incubation at 25°C or with hTRF1 (see Figure 2 and Supplementary Figure S1) allows the percentage of nucleosome sliding to be calculated (29,35). After incubation at 25°C, the fraction of naked DNA released by SmaI increases with respect to that present in the reconstituted sample (Figure 2A, lanes 3 and 2), indicating that part of the nucleosome population shifted inward, leaving ~70% of the nucleosomes still in the starting position. When hTRF1 was added to the reaction, the nucleosomal band almost completely disappears (Figure 2A, lane 5), suggesting that nucleosomes are repositioned by hTRF1 binding. It can be seen that after incubation with hTRF1, a fraction of the sample that remains in the well increases (see Figure 2A, lane 5 compared with lane 3), indicating that part of the nucleosome population slides onto the SmaI restriction site, thus inhibiting digestion. The invariance of the relative percentage of the 469 bp naked DNA band when the complex is cut with the enzyme BseGI, whose recognition site is located upstream of the TG-pentamer sequence, rules out the possibility that hTRF1 causes nucleosome dissociation (Figure 2A, lanes 4 and 6). The induction of nucleosome sliding by hTRF1 is further manifested by the increase of the low-mobility nucleosomal band that corresponds to a nucleosome centered on the fragment released by BseGI (Figure 2A, lanes 4 and 6). Moreover, the fraction of undigested complex that remains in the well is higher when the sample is incubated with hTRF1 (see Figure 2A, lanes 4 and 6); as for the SmaI digests, this indicates that digestion is inhibited by the sliding of the nucleosome onto the restriction site. To test that the hTRF1 remodeling activity is specific to telomeric nucleosomes, the RE assay was performed on the BioTG–TAND/Nuc
complex (Figure 2B). In this case, incubation with hTRF1 has no effect on nucleosome position, since the nucleosome remains on the terminal TAND1 DNA (Figure 2B, lanes 3 and 5).

Results for at least three experiments for each construct are summarized in Figure 2C, which reports the percentages of mobilized nucleosomes calculated by measuring the relative intensities of the nucleosomal and the naked DNA bands after SmaI digestion. The results show that at 25°C, ~25% of telomeric nucleosomes slide inwards (Figure 2C). Significantly, nucleosome sliding in the presence of hTRF1 increases to ~95%. It is also very likely that this value is slightly underestimated, since in calculating the percentage of mobilized nucleosomes we do not take into account the increase of material in the well of the gel, which is likely to derive from mobilized nucleosomes that hinder RE digestion. Instead, nucleosomes assembled on the TAND1 DNA remain mostly in the starting position, independently from hTRF1 presence. In conclusion, hTRF1 binding induces nucleosome repositioning away from the telomeric DNA repeats.
AFM imaging shows that hTRF1-induced nucleosome mobilization is coupled to DNA compaction

As a complementary method to study nucleosome mobility, we used the single-molecule imaging technique AFM (36). By measuring a series of parameters—contour length, volume, distance from the DNA ends—AFM allows the estimation of nucleosome positions relative to DNA (29) and to obtain information on shape and position of the ternary complexes (37). The experimental strategy is shown in Figure 1B. After incubation with hTRF1, the complexes were fixed by glutaraldehyde crosslinking and samples deposited on spermidine-treated mica and imaged by AFM (see Figure 3 for representative AFM fields).

Although we have previously successfully studied thermal repositioning by AFM imaging (29), studying hTRF1-dependent nucleosome mobilization by AFM raises additional difficulties because of the presence of at least three nucleoprotein complexes: the nucleosomal TG580/hTel27 construct (TG-Tel/Nuc), hTRF1 bound to the naked TG580/hTel27 DNA (TG-Tel/TRF1) and the ternary complex with hTRF1 bound to the nucleosomal construct (TG-Tel/Nuc/TRF1). Therefore, to characterize the different complexes, populations of TG-Tel (naked TG580/hTel27 DNA), TG-Tel/TRF1, TG-Tel/Nuc, were analyzed separately as well as the mixed population obtained by incubating the nucleosomal construct with hTRF1 to form the ternary complex TG-Tel/Nuc/TRF1. We measured several parameters for every molecule: the contour length, C; the distances between the ends of the construct and one border of the nucleoprotein complex, L1 and L2, and the volume of the nucleoprotein complex, V (Figure 4A). In order to estimate a reliable value for DNA lengths, we fitted all the obtained distributions with either Gaussian or multi-Gaussian functions (32). In this way, it is possible to make a comparison in terms of mean values of distributions, corresponding to the most probable value of contour length. Figure 4 reports the values from the measurements of the contour length of the protein-free construct TG-Tel, of the complex TG-Tel/TRF1 and of the complex TG-Tel/Nuc; in Figure 4C are also reported the L1 and L2 values of the TG-Tel/TRF1 complex. Data are presented as interpolations of the frequency histograms and Gaussian fittings. The corresponding frequency histograms are shown in Supplementary Figure S3. The measured mean value for the contour length of the protein-free construct TG-Tel is 250 ± 12 nm (Figure 4B), corresponding to a DNA helical rise of 0.32 nm/bp (38). The mean value of the contour length of the complex TG-Tel/TRF1 is 246 ± 23 nm (Figure 4C), a value only slightly smaller than that of the naked DNA, indicating that the binding of hTRF1 does not significantly shorten the construct, in agreement with previous observations (39). Furthermore, L1 and L2 lengths confirm the specific binding of hTRF1 to TTAGGG repeats. The L1 mean value of TG-Tel/TRF1 is 48 ± 17 nm, very close to the theoretical value of 52 nm corresponding to the length of the 27 telomeric repeats. Consistent with these results is the obtained mean value of the L2 parameter, 193 ± 17 nm, in good agreement with the theoretical length of the TG580 sequence (Figure 4C).

Differently from hTRF1 binding to telomeric DNA, the formation of a nucleosome reduces the contour length of the DNA molecule, consistent with the wrapping of 147 bp of DNA around the histone octamer. In the case of the
TG-Tel/Nuc complex, the mean value of the contour length is 203 ± 25 nm (Figure 4D), 47 nm shorter than that of the naked DNA.

We then measured the area and the height of the ellipsoid representing the nucleoprotein complexes, and calculated the volumes. The mean value of nucleosome volumes corresponding to TG-Tel/Nuc samples is of 579 ± 164 nm³. This finding is in good agreement with the x-ray crystallography volume of nucleosome (532 nm³) (40) and with previous AFM studies on mononucleosome (41). In the case of the TG-Tel/TRF1 complex, the presence of 27 telomeric repetitions gives rise to an extremely broad distribution of volumes deriving from multiple hTRF1 binding. For this reason the mean value corresponding to this distribution is statistically irrelevant. Considering that the molecular weight of the proteins and their volume obtained by AFM are linearly correlated (42), we inferred the volume of hTRF1 homodimer bound to DNA (MW: 110 kDa) on the basis of AFM data for the nucleosome (MW: 200 kDa). Consequently, the estimated volume of hTRF1 homodimer is 318 nm³.

In order to analyze the mixed population of complexes obtained after incubation of hTRF1 with TG-Tel/Nuc, we only considered the molecules fulfilling the following parameters to be ternary complexes: (i) contour lengths <203 nm, indicating the presence of the nucleosome onto the construct, (ii) volumes of the nucleoprotein complex >897 nm³, indicating the presence of the nucleosome and of at least one hTRF1 homodimer.

The specific binding of hTRF1 to telomeric DNA at one end of the construct (Figure 4C), allows us to identify the nucleosome position by measuring the distance L₂ from the opposite DNA end to the border of the nucleoprotein complex (Figure 4A). Figure 5 shows the distributions of nucleosome positions after incubation at 25°C in presence and in absence of hTRF1) and at 47°C. Nucleosome positions are expressed as L₂ values and are reported as interpolated data, multi-Gaussian fitting and single-Gaussian fitting (see also Supplementary Figure S4 that reports L₂ values in histogram form, and Supplementary Figure S5 in which are summarized the frequencies of the various forms found in the AFM experiments). From the comparison of the L₂ distribution of
TG-Tel/Nuc/TRF1 (Figure 5A, green line and Figure 5B, right panel; $L_2$ most probable value: 153 ± 21 nm) and the $L_2$ distribution of TG-Tel/Nuc (Figure 5A, blue line and Figure 5B, left panel; $L_2$ most probable value: 186 ± 21 nm) it emerges that hTRF1 induces nucleosome repositioning onto adjacent sequences, in agreement with the data from the RE assay reported in Figure 2. Interestingly, nucleosome repositioning induced by hTRF1 binding at 25°C/C is higher than thermal repositioning obtained by incubating at 47°C/C (Figure 5A, red line and Figure 5B, central panel; $L_2$ most probable value: 172 ± 23 nm). From the distributions of nucleosome positions reported in Figure 5A, it emerges that ~98% of nucleosomes move inward as a consequence of hTRF1 binding, in very good agreement with the conclusions drawn from biochemical analysis.

Finally, a noteworthy result emerges from the analysis of the contour lengths of the complexes TG-Tel/Nuc/TRF1. hTRF1 binding to the nucleosomal complex causes a sharp decrease of the mean value of the contour length, that is 37 nm shorter in the case of the ternary complex ($171 \pm 26$ nm) than that of TG-Tel/Nuc ($203 \pm 25$ nm) (Figure 6 and Supplementary Figure S6). This effect is not an artifact due to the experimental methodology, since hTRF1 binding on naked DNA results in only a small decrease of the contour length (Figure 4C). We attribute these results to the formation of additional interactions of hTRF1 with the mobilized nucleosome that lead to DNA compaction.

**DISCUSSION**

Nucleosome mobility and remodeling are key events in the regulation of biological processes such as transcriptional activation and repression, replication, differentiation, cell cycle progression (43). Several regulative proteins are able to recognize their binding sites on the nucleosome (44,45), yet generally this event is not coupled to nucleosome
repositioning (46,47). Nucleosome mobility usually requires ATP-dependent chromatin remodeling complexes, which alter nucleosome structure and positioning and favor access to DNA of regulative proteins or enzymatic complexes. Despite the dynamic nature of telomeres, ATP-dependent remodeling has not been associated to telomere function in higher eukaryotes. Only recently have chromatin remodeling complexes been implicated in telomere positioning effects and in telomere elongation in budding and fission yeast (48,49), suggesting that ATP-dependent remodeling enzymes are involved in telomere dynamics, although their role in telomere function of higher eukaryotes is yet to be demonstrated. Nevertheless, differently from other chromosome regions, telomeric chromatin possesses inherent dynamical properties that can be ascribed to intrinsic features imparted by the peculiar sequence of telomeric DNA (20,29).

The data presented in Figures 2 and 5 demonstrate that hTRF1 binding induces sliding of telomeric nucleosomes. This ATP-independent chromatin remodeling is telomere-specific, since hTRF1 is unable to shift nucleosomes formed on a reference DNA sequence. Moreover, nucleosome mobilization by hTRF1 is much greater than thermal repositioning (Figure 5A). The most probable explanation for these results is that hTRF1 binding enhances the intrinsic mobility of telomeric nucleosomes. According to this finding, although the histone octamer could slide randomly along telomeric DNA in a both forward and backward direction, the binding of hTRF1 onto telomeric DNA prevents the histone octamer to slide backwards, establishing a directional nucleosomal movement towards internal sequences of the construct. It is also possible that hTRF1 plays an active role in telomeric chromatin remodeling. In addition to promoting nucleosome sliding, hTRF1 binding induces alterations of nucleosome structure (21) that could represent the first step in nucleosome remodeling and hence contribute to nucleosome sliding. Finally, the AFM imaging presented here shows that hTRF1 not only shifts telomeric nucleosomes, but also causes a marked decrease in the DNA contour length in the complexes. An explanation for the observed compaction is that hTRF1 binding induces telomeric DNA to fold back and interact with the mobilized nucleosome, maybe through non-specific interactions between the acidic N-terminal domain of hTRF1 and the basic residues of histone tails (Figure 5B).

What do these results suggest about the interplay between hTRF1 and nucleosomes in vivo? In vitro hTRF1 interacts with telomeric nucleosomes in a number of different ways: it can bind to nucleosomal binding sites, induce nucleosome sliding, and form a bridge between telomeric DNA and nucleosomes. These features suggest that hTRF1 has the ability to regulate telomeric chromatin structure, whilst preserving the packaging of telomeric DNA into nucleosomes.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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