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Biochemical and Biophysical Research Communications 328 (2005) 49–56
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DNA structure-dependent recruitment of telomeric proteins to single-stranded/double-stranded DNA junctions

Giscard H. Yanez, Sheik J. Khan, Alexandra M. Locovei, Ilene M. Pedroso, Terace M. Fletcher*

Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, FL, USA

Received 18 December 2004
Available online 5 January 2005

Abstract

Telomeres protect chromosome ends by assembling unique protein–DNA complexes. TRF2 is a telomere binding protein that is involved in protecting the G-strand overhang, a 3′, guanine-rich, overhang at the telomere terminus. TRF2 may protect the G-strand overhang by recognizing some organizational aspect of the telomeric single-stranded/double-stranded (ss/ds) DNA junction. This work demonstrates that TRF2, purified or in crude extracts, recognizes telomeric ss/ds DNA junctions containing wild type telomeric sequence in the ds region and a G-strand overhang with at least one telomeric repeat. Telomeric complexes containing TRF2 and pot1 assemble less efficiently when the G-strand overhang is in the form of an intramolecular G-quadruplex. However, recruitment of the DNA repair proteins, WRN, Mre11, and Ku86, is not inhibited by a G-quadruplex. This suggests that an intramolecular G-quadruplex has the potential to disrupt certain telomeric assemblies, but efficient recruitment of appropriate DNA repair proteins provides the means to overcome this obstacle.

Keywords: TRF2; Pot1; WRN; Telomere; G-quadruplex; G-tetraplex; G-quartet; DNA structure

Telomeres protect chromosome ends by interacting with telomere binding proteins to form higher-order structures that distinguish them from double-stranded DNA breaks. In addition to the binding of specialized proteins, telomere higher-order structure is most likely influenced by the unique properties of its DNA. Telomeres consist of short repeated sequences with one strand being guanine-rich (the G-strand) and the other cytosine-rich (the C-strand). In addition to double-stranded DNA, vertebrate telomere termini contain a long stretch of single-stranded DNA referred to as the G-strand overhang. The G-strand overhang is a 3′ overhang that extends 100–200 bases beyond the C-strand in human cells [1]. Evidence is gathering which suggests that the G-strand overhang is one of the crucial determinants of telomere structure and function [2].

Telomere protection depends on the presence of TRF2, an essential protein that interacts directly with double-stranded telomeric DNA [3]. Telomere disruption can occur through the loss of TRF2 function [4], is independent of telomere length, and is associated with G-strand overhang reduction [4]. TRF2 association with the G-strand overhang may involve modulation of telomeric high-order structure. For example, telomeric DNA can be remodeled by TRF2 in vitro into a looped structure referred to as the t-loop [5]. The t-loop has also been detected in telomeres isolated from many cell types and species [6,7]. Evidence of single-stranded character in the loop junction inspired a model in which the G-strand overhang could be protected by invading into the double-stranded region of the t-loop forming a displacement- or d-loop [5]. This structure may affect

* Corresponding author. Fax: +1 305 243 3955.
E-mail address: tfletcher@med.miami.edu (T.M. Fletcher).

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doi:10.1016/j.bbrc.2004.12.134

the interactions of another important telomere binding protein, pot1, which can bind to single-stranded telomeric DNA through oligonucleotide/oligosaccharide binding-folds (OB-fold) [8].

Another proposed structure associated with the G-strand overhang is a G-quadruplex [9]. The G-quadruplex involves Hoogsteen-type base pairing between guanines to form the G-tetrad [10]. These structures prefer to form with physiologically relevant cations such as K^+ and Na^+. Both intermolecular (two- and four-stranded) and intramolecular [10] G-quadruplex formation has been observed. Intramolecular structures, in particular, provide either opportunities or pose challenges for the cell since they are highly stable and can form spontaneously in a variety of DNA concentrations. Considering this, it is reasonable to believe that these structures affect telomere processes involving the long G-strand overhang.

Evidence for a function for G-quadruplexes in various processes is mounting. Recently, electron microscopy and probing with a G-quadruplex specific nuclease reveals that G-quadruplexes can stabilize looped structures of telomeric DNA in *Escherichia coli* [11]. However, G-quadruplex formation appears to be refractory to telomerase [12,13] and DNA pol δ [14] activity in vitro, suggesting a role in negatively regulating telomere length. This is supported by the finding that antibodies raised against a specific hypotrichous ciliate, intramolecular G-quadruplex structure, targeted the abundance of chromosome ends in the macronucleus [15] but were conspicuously absent from the replication band. A cellular mechanism may be required for removing or preventing the occurrence of these structures during replication. The RecQ helicases, associated with the replication machinery, preferentially unwind G-quadruplexes [16] and their activity in vitro can be stimulated by interactions with TRF2 [17]. In addition, a putative helicase, Rtel, has recently been shown to be required for telomere length maintenance and genomic stability in *Mus spretis* ES cells [18]. Its homology to DOG-1 raises the possibility that the role of Rtel at telomeres is to resolve G-quadruplex structures since deletion of DOG-1 in *Caenorhabditis elegans* results in deletions of large stretches of G-rich DNA [19].

The requirement for a G-strand overhang in telomere maintenance and structure combined with the presence of double-stranded and single-stranded DNA binding proteins makes the telomeric ss/ds DNA junction a crucial region for telomere function. Although structures such as d-loops and G-quadruplexes have been proposed to exist in this region, no direct studies have addressed the structural requirements for formation of telomeric complexes at ss/ds DNA junctions. This is particularly important when considering the participation of this region in structures such as the t-loop. In addition, a drug design approach to telomerase inhibition has been to target and stabilize telomeric G-quadruplexes [9,20,21].

To address how telomeric complexes assemble at structurally diverse telomeric ss/ds DNA junctions, we have set up an in vitro system for the side-by-side determination of DNA structure and protein recruitment to defined DNA substrates. Our results show that telomeric ss/ds DNA junctions are capable of forming intramolecular G-quadruplexes and that these structures inhibit the assembly of TRF2- and pot1-containing telomeric complexes.

Materials and methods

Oligonucleotide sequence. The DNA sequences are as follows: T2T0 is 5′-CCCTAACCCTAACGTCTCAGCGTCG[Biotin-TEG]CATCG TCTCATGCGTTAGGGTTAGGG-3′ with complementary 5′-CCCT AACCCTAACGCATGAGACGATGCGACGCTGAGACGTTAGG GTTAGGG-3′. T2T2–T2T4 substrates have 2–4 additional 5′-TTAG GG-3′ repeats on the 3′ end. N2N4 is 5′-CGAGATCGGTTGCGTCT CAGCGTCG[Biotin-TEG]CATCGTCTCATGCGCTACAGCACAG ATTCACAATTAAGCTCTGCCATCAG-3′ with complementary 5′-TCTGTGCTGTAGCGCATGAGACGATGACGCGCTGAGACGC ATCCGATCTCGTGGAGCAGTAGTCTGTAGAGTGCG-3′. Hairpin T2T0 is 5′-CCCTAACCCTAACGCATGAGACGATG-[Biotin-TEG]-CATCGTCTCATGCGTTAGGGTTAGGG-3′. Hairpin T2T2–T2T4 substrates have 2–4 additional 5′-TTAGGG-3′ repeats on the 3′ end. Hairpin N2N4 is 5′-TCTGTGCTGTAGCGCATGAGACGATG [Biotin-TEG]-CATCGTCTCATGCGCTACAGCACAGATTCACA ATTAAGCTCTGCCATCAG-3′.

Electrophoretic mobility shift assay. For each substrate DNA, equimolar amounts of complementary strands of non-biotinylated oligonucleotides were annealed at a final concentration of 1 μM, in 5 mM $MgCl_2$ and 40 mM Tris–HCl (pH 8), 1 mM EDTA (TE). The annealing mixtures were heated to 95 °C for 5 min and then cooled slowly until they reached room temperature, followed by placing on ice for 15 min. Annealed oligonucleotides (1.5 pmol) were incubated in TE with 100 mM KCl, NaCl or LiCl (10 μl final volume) for 30 min at RT. Bromophenol blue/xylene cyanol loading dye was added and samples were subjected to 15% (29:1) native polyacrylamide gel electrophoresis in 1× TBE buffer and ±100 mM indicated salts at 5 V/cm until the samples ran ~15 cm into the gel (16–24 h). Gels were pre-run for 30 min before loading. The DNA was visualized by staining with SYBR Gold (Molecular Probes).

Dimethyl sulfate protection assay. Annealed oligonucleotides (1 μM) were 5′ ^{32}P end labeled and incubated in TE ±100 mM KCl for 30 min at RT. The mixture was reacted with dimethyl sulfate (final concentration 1%) for 15 min at RT followed by ethanol precipitation. Each sample was resuspended in 10% piperidine and heated to 95 °C for 15 min, followed by addition of formamide loading dye and electrophoresis in a 15% sequencing gel.

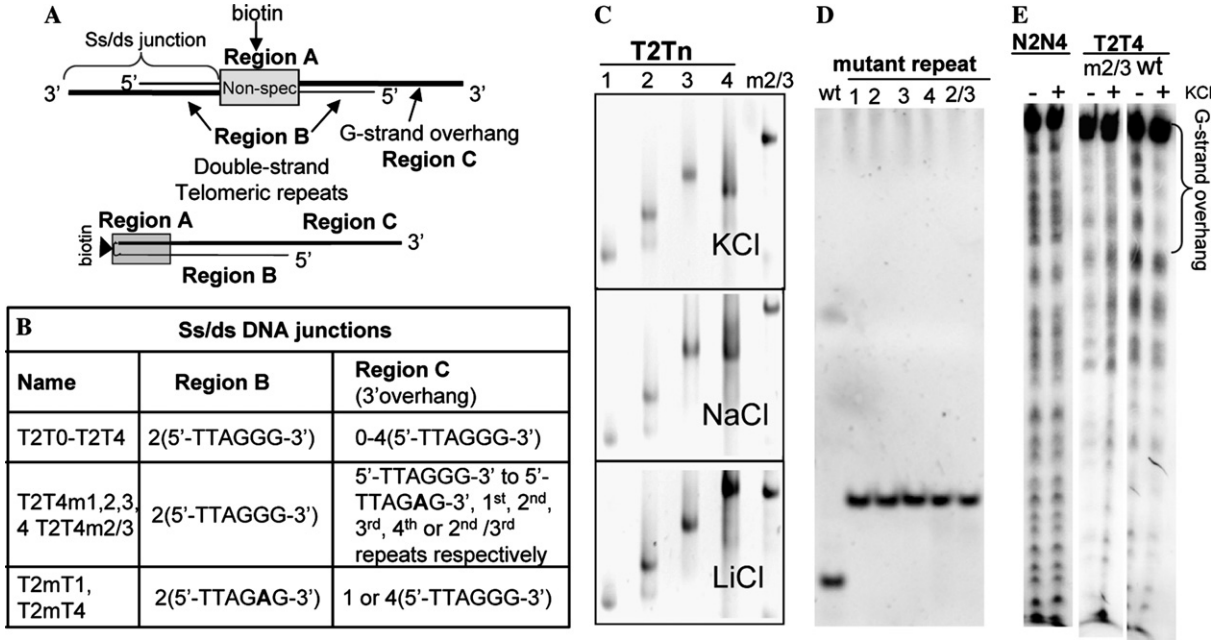
Telomere end recruitment assay. Annealed, biotinylated DNA (90 pmol) was incubated with 2 mg Dynal M280 streptavidin-coated paramagnetic beads in binding and washing (B&W) buffer, containing TE, 5 mM $MgCl_2$, and 0.1% Triton X-100 (final volume 350 μl) for 3–16 h at RT. The beads were washed twice with 350 μl of B&W buffer then stored in 350 μl B&W buffer with 0.1 mg/ml BSA and 0.1% thymersol. To insure that 100% of substrate DNA was immobilized, an aliquot of the immobilized DNA was eluted from the beads with formamide loading dye, run on a sequencing gel, and the SYBR Gold-stained band intensities of each target DNA were compared to DNA standards. Once the immobilized DNA was quantitated, 3 pmol was pre-incubated for 30 min in 20 μl telomere end recruitment assay

(TERA) buffer (20 mM Hepes, pH 7.3, 100 mM KCl, 10% glycerol, 0.5 mM EDTA, 5 mM $MgCl_2$, 0.1% Triton X-100, 1 mM DTT, and 1 mM AEBSF, 1 μg/ml of each of aprotinin, pepstatin, and leupeptin, with 2 mg/ml BSA) to allow G-quadruplex formation. The recruitment reaction was initiated by adding 32 μg HeLa-S3 whole cell [22] extract or indicated amounts of purified TRF2, 2 μg calf thymus DNA, and TERA buffer to a final volume of 100 μl. Reactions were incubated at 37 °C for 15–30 min. Protein recruitment levels were similar between 7.5 and 30 min but were reduced at 60 min. Immobilized DNA–protein complexes were isolated using a Dynal magnet, and washed once with TERA buffer lacking BSA. Proteins were eluted by heating to 95 °C in SDS loading dye, run on a 7.5% protein gel, and detected by Western blot analysis. The DNA was also released by heating in the SDS buffer allowing for loading precision to be checked by removing the bottom of the protein gel and staining with SYBR Gold. HeLa nuclear [23] extracts were also used in the TERA but the whole cell extracts provided stronger signals. Antibodies used for detection were as follows: TRF2 (mouse monoclonal, Upstate), human Pot1 (#978 kindly supplied by Dr. T. de Lange, Rockefeller), WRN (rabbit polyclonal, Novus Biologicals), Mre11 (rabbit polyclonal, Calbiochem), and Ku86 (rabbit polyclonal, Santa Cruz Biotechnology). Digital images of the Western blots were quantitated using the AlphaImage2000 program to obtain background-corrected integrated intensities of each band. The intensities for each DNA substrate on a particular gel were normalized to T2T4 to allow for comparison between experiments.

TRF2 expression and purification. The baculovirus containing the construct for his-tagged TRF2 [3] was obtained from Dr. Titia de Lange (Rockefeller University) for expression in Sf9 cells. Generally, cells were lysed 48 h after infection (M.O.I. of 5) with twice cell pellet volume of extraction buffer (20 mM Tris–HCl, 500 mM NaCl, 10% glycerol, 1% Triton X-100, 2.5 mM imidazole (pH 8.0) 1 mM AEBSF, and 1 μg/ml of each of aprotinin, pepstatin, and leupeptin). TRF2 was purified using TALON resin (BD Biosciences Clontech) and the manufacturer's batch purification protocol. Briefly, 250 μl of resin pre-equilibrated in wash buffer (50 mM sodium phosphate, 300 mM NaCl) was used to purify TRF2 from 1 ml extract. After washing thrice with wash buffer, TRF2 was eluted with 500 μl elution buffer (50 mM sodium phosphate, 300 mM NaCl, and 150 mM imidazole).

Results

The goal of this work was to identify components of multi-protein assemblies on defined ss/ds DNA junctions. The ss/ds junction is a key structural motif not only at telomere ends but also at the replication fork where considerable amounts of G-strand are in a single-stranded form during lagging strand synthesis. Mini-telomeric DNA substrates were tested for their ability to form non-Watson–Crick secondary structures and to recruit specific DNA binding proteins using a TERA. Each target DNA (Fig. 1) contained a region (Region A) of double-stranded DNA of non-specific sequence (Non-spec.). For the TERA purposes, this region of the DNA contained a centrally located, biotin-label for immobilization to streptavidin beads. Immobilization through the Region A portion of each DNA substrate reduces the chances of bead-induced steric hindrance of protein binding in the telomeric region. Region A DNA was flanked on each side by



B Ss/ds DNA junctions

Name	Region B	Region C (3'overhang)
T2T0-T2T4	2(5'-TTAGGG-3')	0-4(5'-TTAGGG-3')
T2T4m1,2,3, 4 T2T4m2/3	2(5'-TTAGGG-3')	5'-TTAGGG-3' to 5'-TTAGAG-3', 1st, 2nd, 3rd, 4th or 2nd /3rd repeats respectively
T2mT1, T2mT4	2(5'-TTAGAG-3')	1 or 4(5'-TTAGGG-3')

Fig. 1. The structure of telomeric ss/ds DNA junctions. (A) Telomeric ss/ds DNA junctions composed of either two strands (top) or one hairpin strand (bottom). The basic DNA sequences are in Materials and methods and variations in (B). Non-spec. refers to the central region containing DNA of non-specific sequence. (B) Sequence of telomeric ss/ds DNA junctions. (C) Native polyacrylamide gels (15%) in the indicated salts (100 mM) of the two stranded DNA (A,B) where *n* refers to number of repeats in the G-strand overhang and m2/3 refers T2T4m2/3. (D) Native polyacrylamide gel (15%) in 100 mM KCl buffer containing $(5'\text{-TTAGGG-}3')_4$ DNA with wild type telomeric sequence (wt) or 5′-TTAGGG-3′ replaced with 5′-TTAGAG-3′ in telomeric repeats 1, 2, 3, 4 or 2/3 as indicated. (E) DMS protection assay of T2T4 wild type (wt) or mutated T2T4m2/3 (m2/3), and non-specific DNA sequence (N2N4) as described in Materials and methods. The (−) and (+) refer to the absence or presence of 100 mM KCl.

combinations of double-stranded 5′-TTAGGG-3′ or 5′-TTAGAG-3′ DNA (Figs. 1A and B) and 3′, G-strand overhangs with either 5′-TTAGGG-3′ or 5′-TTAGAG-3′ sequence (Region C). G-strand overhangs with 4 telomeric repeats have the potential to form an intramolecular G-quadruplex [13]. Negative controls consisted of replacing telomere DNA with non-specific sequence DNA (N) of the same length. Each target DNA was composed of either two annealed strands or one strand that can form a hairpin (Fig. 1A).

The G-strand overhang at telomeric ss/ds DNA junctions forms secondary structures

Although G-quadruplex structures of various smaller sized, single-stranded oligonucleotides have been extensively studied, ss/ds DNA junctions of the size shown in Fig. 1 have not been analyzed. We tested for the presence of G-quadruplexes in our ss/ds DNA junctions using native polyacrylamide gel electrophoresis (PAGE) and a dimethyl sulfate (DMS) protection assay.

Mini-telomeres (Fig. 1A) containing two repeats of telomeric DNA in Region B (T2Tn) with G-strand overhangs (*n*) ranging from 1 to 3, telomeric repeats (T2T1–T2T3), had progressively slower mobilities in native polyacrylamide gels (Fig. 1C) as the size of the G-strand overhang increased. However, DNA with 4 telomeric repeats in the G-strand overhang (T2T4) resulted in a faster moving, compact structure in 100 mM KCl and NaCl but not LiCl gels. This cation preference is consistent with G-quadruplex formation [24]. Alteration of 5′-TTAGGG-3′ to 5′-TTAGAG-3′ in the 2nd and 3rd TTAGGG repeats (T2T4m2/3) abrogated the compact structure. In addition, replacing the central G with A in any one of the four repeats (Fig. 1D, T2T4m1, 2, 3, or 4) prevented formation of the compact structure. This is a novel finding and suggests that a central guanine is essential in all 4 telomeric repeats for stabilization of an intramolecular G-quadruplex under these experimental conditions.

To further validate that the compact structure is a G-quadruplex, dimethyl sulfate protection (DMS) assays were performed. N7 is protected from DMS when it participates in hydrogen bonding in the G-tetrad [25]. The guanines in the G-strand overhang of T2T4 were protected from methylation by DMS only in the presence of K^+ (Fig. 1E), strongly suggesting that the compact structure detected in the electrophoretic mobility assay represents a G-quadruplex. Again, replacing the T2T4 sequence with T2T4m2/3 destabilized the G-quadruplex allowing for methylation of the guanines by DMS.

The G-strand overhang directs DNA–protein complex assembly at ss/ds DNA junctions

Proteins from HeLa cell crude extracts recruited to telomeric ss/ds DNA junctions in a 100 mM KCl-containing buffer were identified utilizing the TERA. This assay involved immobilization of biotinylated, telomeric substrates on streptavidin beads. The immobilized DNA substrates were first incubated for 30 min at room temperature in TERA buffer containing 100 mM KCl to insure G-quadruplex structure formation followed by addition of HeLa extracts. DNA–protein complexes were isolated and proteins were detected by Western blot analysis. Representative Western blots for TRF2 are shown in Fig. 2A demonstrating TRF2 binding to telomeric ss/ds DNA junctions with variations in the G-strand overhang length or sequence. Western blots were quantitated and the integrated intensities, normalized to T2T4, are shown in Fig. 2B. TRF2 appeared to have an approximately 3-fold recruitment preference for DNA with G-strand overhangs of 1, 2 or 3 telomeric repeats (T2T1, T2T2, and T2T3) relative to DNA with 4 telomeric repeats in the G-strand overhang (T2T4). In addition, the lack of TRF2 binding to a blunt-ended substrate (T2T0) strongly suggests that optimal assembly of TRF2-containing complexes requires a G-strand overhang with a minimum of 1,5′-TTAGGG-3′ repeat. Telomeric complexes containing TRF2 were incapable of assembling onto ss/ds DNA junctions lacking telomeric DNA (N). Since only T2T4 is capable of folding into an intramolecular G-quadruplex, the data imply that G-quadruplex formation destabilizes TRF2-containing telomeric complexes. If TRF2 complex formation on ss/ds DNA junctions was inhibited by the presence of a G-quadruplex, replacing 5′-TTAGGG-3′ with 5′-TTAGAG-3′ to destabilize the G-quadruplex in Fig. 1 should remove this inhibitory effect. However, alterations in the G-strand overhang sequence resulted in a further reduction in TRF2-containing complex assembly (Figs. 2A and B, T2T4m2/3, T2T4m1). As expected, altering the sequence in the double-stranded telomeric region (T2mT4 and T2mT1) completely abrogated TRF2 recruitment.

Is the DNA recruitment preference of TRF2 in extracts a DNA binding property of TRF2? To address this, his-tagged TRF2 was baculovirus-expressed and purified. Purified TRF2 also had a preference for interacting with certain ss/ds DNA junctions (Figs. 2C and D). Not surprisingly, TRF2 did not interact with DNA lacking telomeric sequence (N). As observed with HeLa extract, purified TRF2 was recruited less efficiently to telomeric DNA lacking a G-strand overhang (Figs. 2C, T2Tn = 0 and D, T2T0). However, unlike that observed with HeLa extracts, TRF2 was recruited as efficiently to T2T4 as to T2T1, T2T2, and T2T3 (Figs. 2C and D). This demonstrates that TRF2 recognizes telomeric ss/ds DNA junctions, even if the G-strand overhang forms an intramolecular G-quadruplex.

Taken together, these results demonstrate that TRF2 interacts directly with telomeric ss/ds DNA junctions that have at least one, single-stranded, 5′-TTAGGG-3′

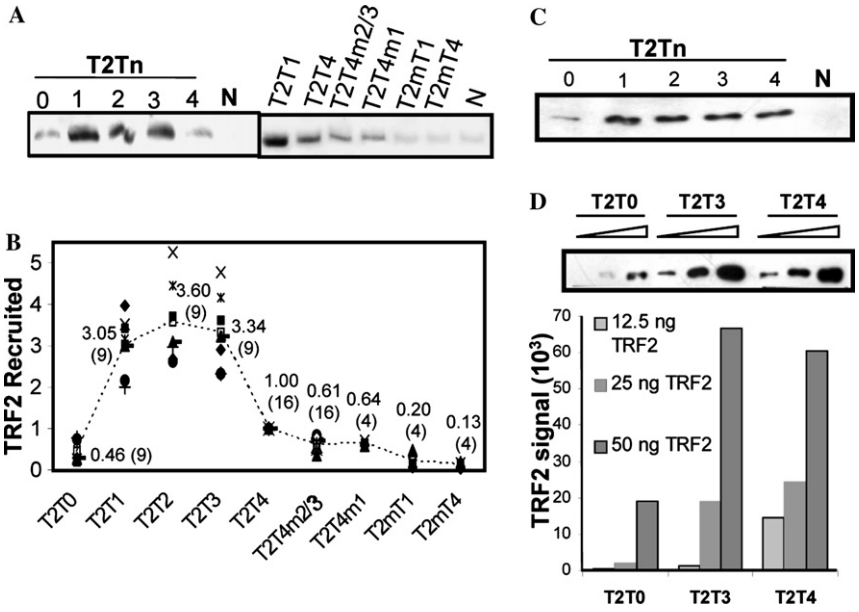


Fig. 2. Recruitment of TRF2 to telomeric ss/ds DNA junctions. (A) A Western blot from a TERA detecting TRF2 recruitment from 32 μg HeLa whole cell extract to 3 pmol of bead-immobilized DNA with variable sized G-strand overhangs ($n = 0$–4) and altered sequence. This particular blot represents a TERA with the hairpin DNA (Figs. 1A and B) of indicated composition. N refers to DNA with non-specific sequence. (B) Relative TRF2 recruitment to telomeric DNA. Western blots were quantitated as described in Materials and methods, and the background-corrected, integrated intensities were normalized to T2T4 DNA. Data were obtained from both two-stranded and hairpin DNA. The dotted line runs through the average relative binding for each DNA substrate. The numbers beside the line for each DNA substrate indicate the average relative binding with the number of determinations in parentheses. (C) Interactions of purified TRF2 with telomeric ss/ds DNA junctions. Western blot detecting purified TRF2 (50 ng) binding to hairpin telomeric DNA (3 pmol) with variable G-strand overhang lengths ($n = 0$–4) in the TERA. N refers to the DNA of non-specific sequence. (D) Western blot detecting purified TRF2 (12.5, 25, and 50 ng) binding to indicated DNA (3 pmol) in the TERA. The bar graph represents the quantitation as described in Materials and methods. The TRF2 signal refers to the background-corrected intensities of the bands.

repeat. However, TRF2 recruitment from HeLa extracts has additional recruitment specificities suggesting that optimal TRF2 recruitment may require the presence of other telomeric proteins. This concept was addressed by Western blot detection of various proteins known to be associated with telomeres in the TERA (Fig. 3). As expected, the single-stranded, telomeric DNA binding protein, pot1 (Figs. 3A and B), was not detected on either non-specific DNA (N) or telomeric DNA lacking a G-strand overhang (T2T0). Interestingly, like TRF2, optimal pot1 recruitment was reduced when the G-strand overhang was capable of forming an intramolecular G-quadruplex (T2T4). Another interesting finding is that pot1 was recruited efficiently to T2T1, even though optimal binding of purified pot1 requires a minimum of 1.5 to 2, single-stranded, telomeric repeats [26,27]. This, in addition to the significant reduction in pot1 binding to DNA with alterations in the double-stranded telomeric region (Fig. 3, T2mT1 and T2mT4), suggests that pot1 recruitment to these ss/ds DNA junctions is directed by more than pot1-DNA interactions. In fact, the binding preferences of pot1 strongly mimic that of TRF2, implicating a structural preference for the formation of telomeric assemblies at ss/ds telomeric DNA junctions.

To further elucidate the nature of telomeric assemblies, DNA repair proteins shown to be associated with TRF2 [28–30] were detected with the TERA (Fig. 3C). In general, recruitment of DNA repair proteins appeared to be less affected by manipulation of the target DNA composition compared to that of TRF2 and pot1. For example, unlike TRF2 and pot1, the DNA repair proteins, WRN, Mre11, and Ku86, were efficiently recruited to the ss/ds junction of non-specific sequence (N). In addition, the presence of a G-quadruplex (T2T4) or the absence of a G-strand overhang (T2T0) did not impair recruitment of any of the DNA repair proteins. Interestingly, altering the sequence of either the telomeric double-stranded region or the G-strand overhang resulted in a slight reduction of WRN, Mre11, and Ku86 recruitment. Although DNA repair protein recruitment was not greatly affected by target DNA composition, protein signals were not due to non-specific interactions of DNA repair proteins with streptavidin beads since mock TERAs with beads lacking DNA showed no protein recruitment (Fig. 3C, beads alone).

Discussion

Through a combination of structural analysis and a telomeric end recruitment assay with defined substrate DNA composition, we have determined a set of structural requirements for assembly of telomeric

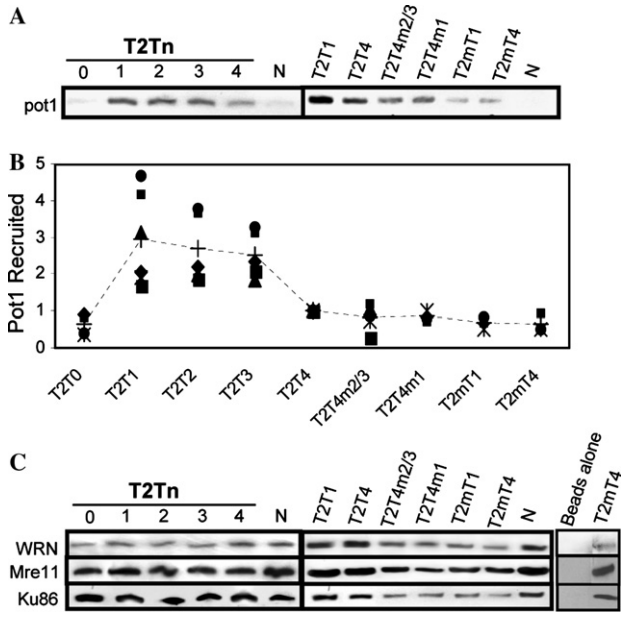


Fig. 3. Interactions of pot1 and DNA repair proteins with telomeric ss/ds DNA junctions. (A) A Western blot detecting human pot1 recruitment from HeLa extract to DNA in the TERA as described in Fig. 2. (B) Relative pot1 recruitment to telomeric DNA. Western blots were quantitated as described in Materials and methods and Fig. 2B and intensities normalized to T2T4. Data were obtained from hairpin DNA. The dotted line runs through the average relative binding for each DNA substrate. (C) Western blots detecting WRN, Mre11, and Ku86 recruitment to the indicated telomeric and non-specific (N) DNA in the TERA. Mock TERA was performed with beads lacking DNA (beads alone).

protein–DNA complexes at this functionally important region of the telomere. Importantly, we have shown that telomeric complexes assemble less efficiently on ss/ds telomeric DNA junctions with the G-strand overhang in the form of an intramolecular G-quadruplex. In addition, we have shown that purified TRF2 has the ability to recognize specific telomeric ss/ds DNA junctions. The data on purified TRF2 are consistent with previously published findings. Hints of TRF2 recognition of telomeric ss/ds junctions can be found in the detection of TRF2, through electron microscopy, at the end of a large stretch of telomeric DNA [5]. DNase I footprinting of oligonucleotides containing 4, 5′-TTAGGG-3′ repeats and a short G-strand overhang demonstrates a slightly stronger footprint more proximal to the G-strand overhang [31]. Moreover, t-loop formation involving the very end of the telomere requires a G-strand overhang with a minimum of one 5′-TTAGGG-3′ repeat [5]. Our data show that TRF2 binding to ss/ds telomeric DNA junctions requires a G-strand overhang with one, telomeric repeat proximal to the ds region but can also interact with target DNA containing a G-quadruplex revealing a new binding characteristic of this protein.

The recruitment patterns of TRF2 from HeLa extracts most likely reflect the differences in efficiency in telomeric complex assembly on the various DNA structures. This suggests that optimal assembly of certain complexes may require protein–protein interactions in addition to DNA binding ability. The finding that pot1 recruitment has the same DNA preference pattern as TRF2 suggests that these proteins either assemble together in complexes on telomeric DNA or that TRF2 and pot1 exist in separate complexes that happen to have the same substrate specificities. TRF2, TRF1, and pot1 can be co-immunoprecipitated from extracts demonstrating that they exist together in complexes [32,33]. A bridging protein in TRF2, TRF1, and pot1 complexes appears to be TIN2 (TRF1-interacting protein 2), originally thought to be involved in negatively regulating telomere length but now appears to have a role in general telomeric complex assembly [32,34]. In addition, a recently discovered protein, PTOP (or PIP1), appears to directly recruit pot1 to telomeres by interacting with pot1 and TIN2 [33,35]. Protein–protein interactions must be a major form of recruitment of pot1 to telomeres since a mutant pot1 lacking the ability to bind DNA is capable of co-localizing with TRF1 to telomeres in cells [36]. Our study demonstrates that at least some of these aspects of telomeres can be recapitulated in vitro on model telomeric DNAs. For example, a reduction in binding of a single-stranded binding protein such as pot1 caused by alterations in the double-stranded telomeric DNA sequence (Fig. 3) must reflect a loss in recruitment through a double-stranded telomeric binding protein. In addition, the results in this study extend the concept that, like pot1, optimal TRF2 recruitment also requires other proteins since TRF2 recruitment is affected by the structure and composition of the G-strand overhang. At this point, we have not been able to detect TRF1 binding to any of the telomeric ss/ds DNA junctions. This could reflect a preference for recruitment of a particular TRF2/pot1 complex. Interestingly, size exclusion chromatography revealed complexes containing TIN2, TRF2, PTOP(-PIP1), and pot1 but not TRF1 [32,33]. TRF1 was found to be in a separate larger molecular weight complex along with tankyrase [32].

Although efficient telomeric complex assembly is inhibited by G-quadruplexes, recruitment of DNA repair proteins (WRN, Mre11, and Ku86) is not affected by this structure (Fig. 3C). These proteins have been shown to interact with TRF2, suggesting that they are recruited to telomeres through TRF2 [28–30]. WRN binding and helicase activity have been shown to preferentially occur on unusual DNA structures such as G-quadruplexes [16]. Since our results suggest that this structure inhibits optimal telomeric complex formation, perhaps one role of WRN, in addition to resolving stalled replication forks [37], is to assist in telomeric

complex assembly in this region by removing intramolecular G-quadruplexes. As long as intramolecular G-quadruplexes can be removed by certain DNA metabolic activities such as WRN, the telomere is stable. Otherwise, the presence of intramolecular G-quadruplexes at the telomere could represent a dysfunctional telomeric structure.

Promoting the dysfunctional telomeric state could be achieved through small molecules. If certain G-quadruplex structures influence telomere structure, telomerase inhibitors designed to interact with and stabilize G-quadruplexes [9,20,21] may affect both telomerase positive and negative cells [38,39]. Certain G-quadruplex stabilizing drugs also inhibit WRN activity creating an additional challenge in telomere length maintenance and stability [40]. These findings raise the possibility that telomere structures are dynamic and can be manipulated by small molecules.

Acknowledgments

HeLa cell pellets were obtained by the National Cell Culture Center (Minneapolis, Mn). We thank Dr. Titia de Lange for providing the TRF2-expressing baculovirus and the anti-human pot1 antibody. We are also grateful for Dr. Paul Boehmer and his laboratory's assistance in obtaining and growing Sf9 cells. This work was supported, in part, by the Florida Biomedical Research Program 04NIR-06 (Florida Department of Health) and the Stanley Glaser Research Foundation.

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