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DNA structure-dependent recruitment of telomeric proteins to single-stranded/double-stranded DNA junctions

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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

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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