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Biochemical and Biophysical Research Communications 363 (2007) 44–50
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# Interactions of TRF2 with model telomeric ends

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Received 7 August 2007
Available online 30 August 2007

**Abstract**

Telomeres are DNA–protein complexes at the ends of eukaryotic chromosomes, the integrity of which is essential for chromosome stability. An important telomere binding protein, TTAGGG repeat factor 2 (TRF2), is thought to protect telomere ends by remodeling them into T-loops. We show that TRF2 specifically interacts with telomeric ss/ds DNA junctions and binding is sensitive to the sequence of the 3′, guanine-strand (G-strand) overhang and double-stranded DNA sequence at the junction. Association of TRF2 with DNA junctions hinders cleavage by exonuclease T. TRF2 interactions with the G-strand overhang do not involve the TRF2 DNA binding domain or the linker region. However, mobility shifts and atomic force microscopy show that the previously uncharacterized linker region is involved in DNA-specific, TRF2 oligomerization. We suggest that T-loop formation at telomere ends involves TRF2 binding to the G-strand overhang and oligomerization through both the known TRFH domain and the linker region.


*Keywords:* TRF2; Telomere; DNA structure; G-quadruplex

Telomeres are unique DNA–protein complexes at the ends of eukaryotic chromosomes that preserve genomic integrity and stability. They protect the ends from degradation and fusions and they also allow the complete replication of chromosomes, through the concerted action of telomerase, a telomere-specific DNA polymerase [1]. Telomeres contain regions of non-coding double-stranded DNA with multiple repeats of the sequence TTAGGG that end in a 3′ single-stranded overhang referred to as the ''G-strand overhang'' [2]. The single strand overhang of telomeres can reach several hundred bases, and is likely formed and maintained by both nuclease degradation of the C-strand and DNA polymerization [3]. The G-strand overhang may also contribute to telomere higher-order structures that play an important role in protecting chromosomal ends [4].

The telomere maintenance, nucleoprotein complex(es) includes TRF1 (TTAGGG repeat binding factor-1) [5] and TRF2 (TTAGGG repeat binding factor-2) [6] which bind to double-stranded telomeric DNA through a myb-homeodomain or 'telo-box' motif. TRF2 plays an important protective role at the telomere. Cells expressing a dominant negative version of this protein have reduced G-strand overhangs, exhibit end-to-end chromosome fusions [7] and experience ATM- and p53-mediated apoptosis [8]. Overexpression of TRF2 in primary cells causes telomere shortening without accelerating senescence [9]. Interestingly, overexpression of TRF2 in telomerase deficient mice accelerates epithelial carcinogenesis along with increased chromosomal instability and DNA damage [10].

Electron microscopy has revealed that TRF2 possesses the ability to remodel linear telomeric DNA into large duplex T-loop structures *in vitro* [11]. T-loops were also observed in psoralen-crosslinked telomeres isolated from cells [11]. The G-strand overhang has been shown to be

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doi:10.1016/j.bbrc.2007.08.122

essential for T-loop formation involving the end of telomeric DNA constructs [12]. In addition, TRF2 stimulates the invasion of single-stranded DNA into plasmids containing telomeric DNA and alters DNA topology [13]. This raises important questions concerning the structure of the T-loop and the role of TRF2 in its formation. How does TRF2 recognize a telomere end to form a T-loop? The crystal structure of the DNA binding domain of TRF2 shows the ability to bind to duplex DNA [14]. TRF2, however, contains other domains (Fig. 3A) such as a homodimerization domain (TRFH), a basic N-terminal domain, and an uncharacterized linker region. These regions may be involved in the ability of TRF2 to form T-loops. TRF2 has recently been shown to recognize 4-way junctions and replication fork intermediates through the N-terminal domain [15]. Other recent literature suggests that TRF2 induces strand invasion via its TRFH homo-dimerization domain and either N- or C-terminal DNA binding domains [13].

A first step in elucidating T-loop formation is to understand how TRF2 recognizes its substrates. In this study, we examine TRF2 binding interactions with sequence-specific DNA substrates that mimic the very ends of telomeres. Because telomere sequences can spontaneously form non-Watson Crick structures, such as the G-quadruplex, our studies also include DNA substrates which have the ability to form G-quadruplexes. We show that TRF2 interacts specifically with single-stranded/double-stranded telomeric DNA junctions. The data from this work is the first to suggest that TRF2 can oligomerize upon DNA binding, to some extent, independently of its homodimerization domain, TRFH. This may be functionally useful in forming oligomers along the telomere. In addition, TRF2 can recruit another telomere binding protein, Rap1, to the model telomere end but this complex competes with Ku complex.

## Materials and methods

*DNA substrates*. Oligonucleotide substrates both 5′ end biotinylated and unbiotinylated were synthesized and purchased from Sigma. Oligonucleotides were annealed according to Fig. 1A in 1× TE (Tris–EDTA, pH 8) buffer with 1 mM $MgCl_2$, by heating for 5 min at 95 °C and allowing to cool down to room temperature. Annealed double-stranded oligonucleotide substrates were 5′ end-labeled with T4 PNK and [$\gamma$-$^{32}$P]ATP and verified on a 15% native PAGE gel. Biotinlyated substrates were immobilized on streptavidin beads according to the method of Yanez et al. [16] for use in the mini-telomere recruitment assay. The pRST5 plasmid [12] with large telomere tracts, (approximately 96 TTAGGG repeats) was obtained from Dr. Jack Griffith and linearized with restriction enzyme BamHI.

*Protein expression*. His-tagged TRF2 was expressed in *Sf9* cells using a baculovirus construct donated from Dr. T. de Lange (Rockefeller University) and affinity purified with $Co^{2+}$ beads (Talon) as described [16]. Constructs for the truncation mutants of TRF2, amino acids 301–500 (DBD + linker) and amino acids 440–500 (DBD) were generated from GenScript and cloned into pET-15b expression vectors which carry an N-terminal His Tag (Novagen). Both proteins were expressed in *Escherichia coli* Rosetta 2 (DE3) cells (Novagen), followed by $Ni^{2+}$ affinity purification (Qiagen). All proteins used were verified on protein gels with Coomassie Blue or silver staining (Bio-Rad) and western blotting with a TRF2 antibody raised against full length protein (Upstate) or directed against the C-terminus (US Biological #T2400-12) as appropriate.

*Electrophoretic mobility shift assay (EMSA)*. TRF2 binding reactions (10 μl) with 10 nM labeled oligonucleotides were performed with 1 μg sheared calf thymus DNA in EMSA buffer (20 mM Hepes pH 7.8, 150 mM KCl, 1 mM $MgCl_2$, 20% glycerol) for 30 min at 20 °C. Protein–DNA complexes were resolved on an 8% native polyacrylamide gel and analyzed with a phosphoimager (Molecular Dynamics). The concentration of TRF2 required to shift 50% ($C_{50}$) of the oligonucleotide was determined by fitting the isotherm % oligonucleotide bound *vs.* [TRF2] to the Hill equation. Competition studies were performed with the same reaction conditions (360 nM TRF2) using labeled T5T1 oligonucleotide and titrating unlabeled competitor oligonucleotide.

*Exonuclease T digestion*. TRF2 binding reactions (5 nM oligonucleotide, $^{32}$P end-labeled on the G-strand) were performed in the same manner as above for 15 min in EMSA buffer with or without TRF2 (360 nM). Exonuclease T (ExoT) (NEB) was added (2 U/μl final concentration) and the digestion was stopped at different time points (0, 2.5, 5, 7.5 min) by addition of EDTA and sodium acetate (final concentrations 50 and 300 mM, respectively). Digested products were phenol chloroform extracted, ethanol precipitated, separated on a 7%, 7 M urea sequencing gel and visualized by phosphoimaging (Molecular Dynamics).

*Mini-telomere recruitment assay*. The recruitment assay was performed as described [16]. Briefly, 5 pmol of immobilized biotinylated telomere DNA was incubated with 32 μg of HeLa whole cell extract, 2 μg calf thymus DNA, indicated amounts of recombinant TRF2 (rTRF2) in recruitment 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, 1 mM AEBSF, 1 μg/ml of each of aprotinin, pepstatin, leupeptin, with 2 mg/ml of BSA) at a final volume of 100 μl, for 30 min at 37 °C. DNA–protein complexes were isolated using a Dynal magnet, and proteins of interest were detected by western blot with specific antibodies; Ku86 (Santa Cruz), hRap1 (Imgenex), and TRF2 (Upstate).

*AFM sample preparation and imaging*. DBD + linker (6.4 nM) and pRST5 DNA (1.68 nM DNA) were incubated in 10 μl binding buffer (20 mM Hepes, 150 mM KCl, 1 mM $MgCl_2$, pH 7.8) for 30 min. The samples were then diluted to 100 μl with pure water and deposited onto 2 μM glutaraldehyde-treated AP-mica followed by incubation for 30 min as described [17]. For the PRST5 DNA sample alone, 50 μl (16.8 fmol/μl DNA) in 10 mM NaCl, 5 mM phosphate, was deposited onto 2 μM glutaraldehyde-treated AP-mica for 30 min [17]. For the DBD + linker sample alone, 9.6 nM DBD + linker in 10 mM NaCl, 5 mM phosphate was deposited onto 2 μM glutaraldehyde-treated AP-mica for 30 min [17]. In situ imaging was carried out with a Macmode PicoSPM (Agilent Technologies) equipped with triangular $Si_3N_4$ Cantilevers (Molecular Imaging) with a spring constant of 0.1 N/m. Measurements were performed at ~8 kHz driving frequency. The scanning rate was 1.78 Hz.

## Results

### *Interactions of TRF2 with telomeric DNA are stimulated by a G-strand overhang*

A higher affinity for telomeric ends would facilitate the TRF2 protective function by sequestering the G-strand overhang within the t-loop structure. To directly test the affinity of TRF2 for telomere ends, several telomeric constructs were designed with a focus on telomeric ss/ds DNA junctions (Fig. 1A). The length or composition of double-stranded 5′-TTAGGG-3′ repeats (Tx) and the G-strand overhang (Ty) were varied (Fig. 1A). In addition, one construct had 7 base pairs of non-telomeric DNA (N) inserted between the double-stranded 5′-TTAGGG-3′ repeats and the G-strand overhang (T5NT1). Another construct had a double-stranded, 5′-TTAG-3′ insertion proximal to the overhang (T5T1.5).

TRF2-DNA complexes were detected by electrophoretic mobility shift assays (EMSA) in the presence of excess calf thymus DNA to reduce observable TRF2 interactions with non-telomeric DNA. Since TRF2 is known to oligomerize [18], we verified the EMSA data with a pulldown assay [19] (Supplementary Fig. S1).

As expected, the apparent affinity of TRF2 was greater for substrates with 5 compared to 3 double-stranded 5′-TTAGGG repeats-3′ (Fig. 1B and C). However, substrates containing a 3′ overhang of 4, 5′-TTAGGG-3′ repeats (T5T4 and T3T4) were preferred when compared to the absence of an overhang (T5T0 and T3T0) with $C_{50}$'s of 51 and 213 nM for T5T4 and T5T0, respectively (Fig. 1F). Reducing the overhang to 1,5′-TTAGGG-3′ repeat only slightly reduced the binding affinity. This was further verified using competition assays with unlabeled DNA (Fig. 1G).

To determine the requirements for the higher binding affinity, several single-strand/double-strand constructs were designed and binding was determined. As expected, altering all 5′-TTAGGG-3′ repeats in the double-stranded region to 5′-TTAGAG-3′ (T5MT1) substantially reduced

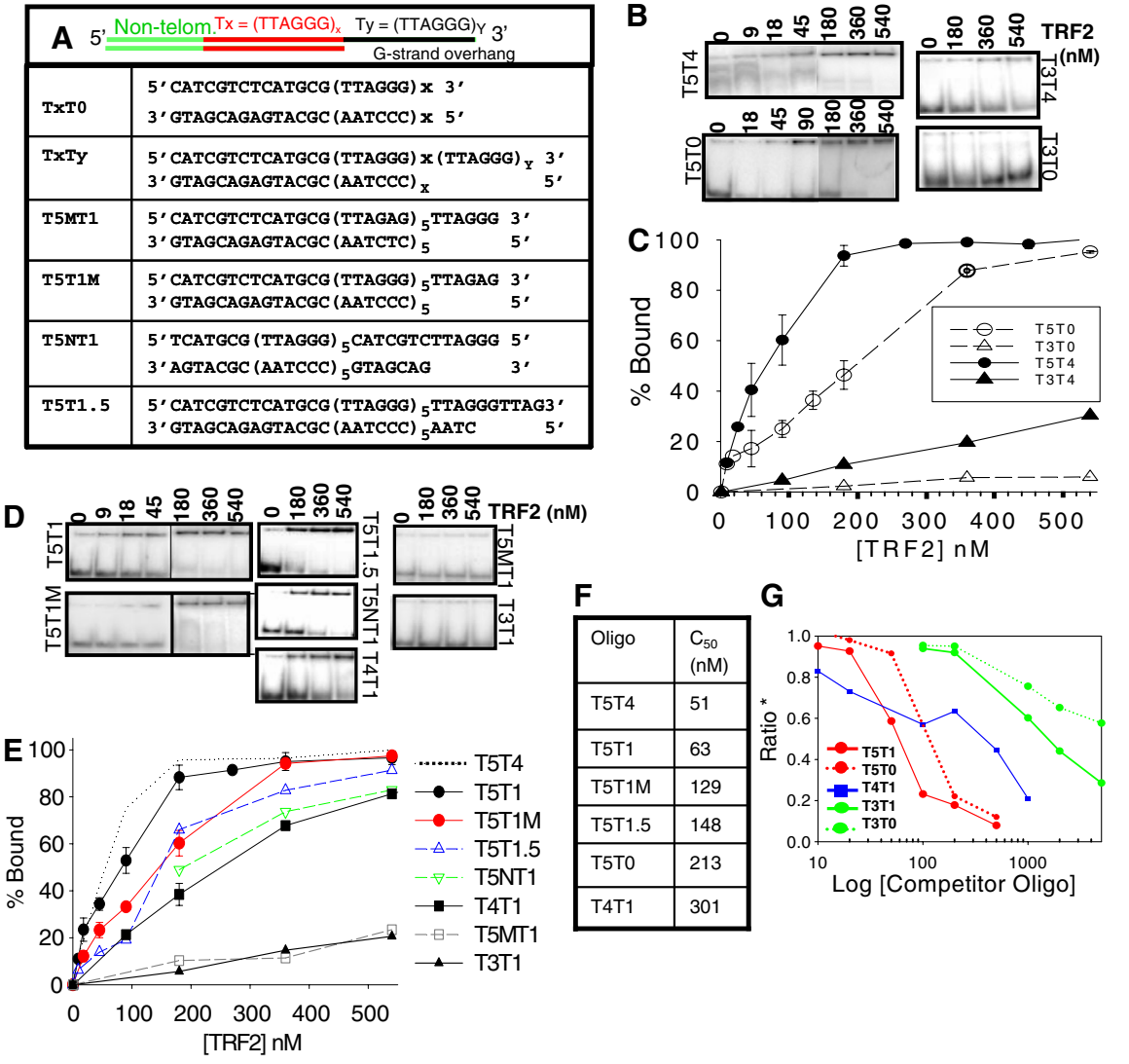


Fig. 1. TRF2 binds to telomeric ss/ds DNA junctions. (A) Schematic representation and sequence of DNA constructs used in this study. Tx, number of duplex TTAGGG repeats (red). Ty, number of TTAGGG repeats in the G-strand overhang (black). All constructs contain non-telomere DNA sequence (green). (B) Electrophoretic Mobility Shift Assay (EMSA) for T5T4, T5T0, T3T4, T3T0 performed with 10 nM labeled DNA substrates as indicated and indicated concentrations of recombinant TRF2 (nM). Protein: DNA complexes were resolved on 8% native PAGE. (C) Binding isotherms for T5T4, T5T0, T3T4, T3T0. Error bars represent 1 SD for 3–4 determinations. (D) Electrophoretic Mobility Shift Assay (EMSA) for additional oligonucleotide constructs, as indicated, performed under the same conditions as (B). (E) Binding isotherms for EMSA experiments represented in (D). (F) Amount of TRF2 required shift 50% of the telomeric substrates ($C_{50}$). (G) Binding isotherms for the competition assay performed as described in Materials and methods where TRF2 (360 nM) bound to labeled T5T1 was competed with unlabeled competitor as represented in the graph. Ratio * represents the ratio of : % shift with competitor divided by the % shift without competitor. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

TRF2 binding, demonstrating that binding to the double-stranded region was sequence specific. However, altering the 3′ overhang from 5′-TTAGGG-3′ to 5′-TTAGAG-3′ (T5T1M) reduced binding but not to the levels of T5T0 suggesting that there is a slight preference for an overhang with telomeric sequence. This reduction of binding was also observed on smaller ss/ds DNA junctions such as T3T1M (data not shown). Interestingly, TRF2 binding was significantly reduced when the G-strand overhang was separated from the double-stranded telomeric DNA by insertion of 7 bases of non-telomeric DNA (T5NT1). Binding was also reduced when the double-stranded region contained an additional 5′-TTAG-3′ and the overhang was comprised of 5′-GGTTAG-3′ instead of the 5′-TTAGGG-3′ (T5T1.5).

To further characterize TRF2 interactions with the telomeric ss/ds DNA junction, the G-strand overhang of TRF2/DNA complexes was subjected to cleavage by Exonuclease T (Fig. 2A), a 3′–5′ single-stranded DNA exonuclease that can efficiently cleave short 3′ overhangs. In the absence of TRF2, a time course of Exo T cleavage revealed digestion of the T5T1, 3′ overhang with slight pauses at the second T and the first G of the 5′-TTAGGG-3′ overhang (Fig. 2A, top panel). However, TRF2 inhibited efficient cleavage of T5T1 by Exo T resulting in a significant pause or block at the last G of 5′-TTAGGG-3′. In addition, the pauses at the second T and first G seen in the absence of TRF2 were accentuated. Altering the sequence of the 3′ overhang to 5′-TTAGAG-3′ (T5T1M, Fig. 2A, middle panel) removed the Exo T block at the last G. However, TRF2 binding to T5T1M still caused an exaggerated pause at the second T and first G similar to that seen when TRF2 bound to T5T1. The TRF2-dependent changes in Exo T cleavage were not present on a DNA substrate that binds TRF2 very poorly (T5MT1, Fig. 2A, bottom panel).

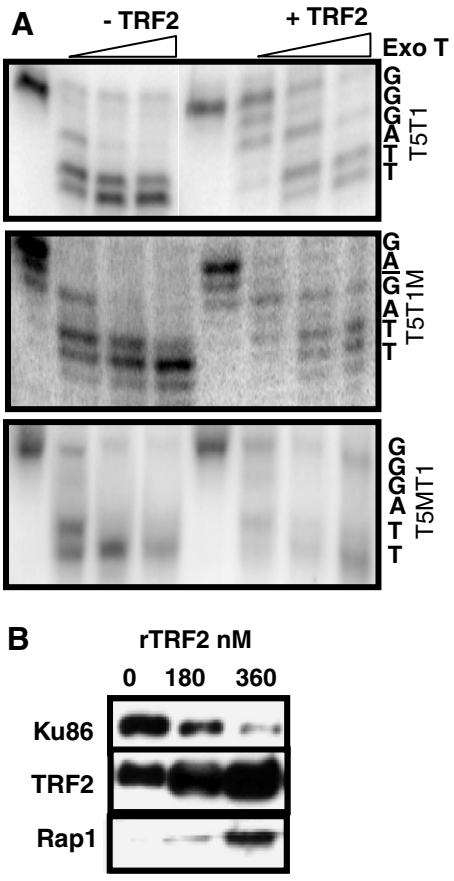


Fig. 2. TRF2 interacts with the G-strand overhang, blocks nuclease digestion and affects protein recruitment. (A) The effect of TRF2 (360 nM) on the time course (2.5, 5, and 7.5 min) for Exonuclease T (2 U/μl) digestion of T5T1 (top panel), T5T1M (overhang altered to TTAGAG, middle panel), and T5MT1 (double-stranded DNA altered to TTAGAG, bottom panel). (B) Relative recruitment of Rapl and Ku86 from HeLa cells with increasing recombinant TRF2 (rTRF2) as indicated. Mini-telomere recruitment assay with 50 nM magnetic bead-immobilized T3T1 and 32 g of HeLa whole cell extract performed as described in Materials and methods. Proteins were detected by western blot with indicated antibodies.

### *TRF2 alters the recruitment of proteins to telomeric ss/ds DNA junctions*

In addition to blocking exonuclease cleavage, TRF2 recognition of telomeric ss/ds DNA junctions affects multi-protein complexes that are recruited to these model substrates such as shelterin [20,21]. Telomeric protein–DNA complexes containing TRF2 and pot1 have previously been isolated from HeLa cell extracts and recruited to structurally characterized model mini-telomeric ends using a biotinylated, mini-telomere recruitment assay [16]. Here we show that adding purified recombinant TRF2 increases the recruitment of another shelterin protein, rap1, from extracts to the telomeric ss/ds DNA junction, T3T1 (Fig. 2B). These results are consistent with findings that TRF2 and rap1 are in the same complex within the telosome [22].

Human Ku70/86, involved in non-homologous end joining, has a slight preference for interacting with telomeric ends relative to non-telomeric DNA *in vitro* [23]. In Fig. 2B, we show that the amount of Ku86 from extracts recruited to the substrates was reduced with increasing recombinant TRF2 suggesting that TRF2-dependent complexes compete with Ku complex for certain ss/ds DNA junctions.

### *The DNA binding domain and linker region of TRF2 are not involved in interacting with the G-strand overhang but the linker region is involved in oligomerization*

To determine the TRF2 domain requirements for telomeric ss/ds DNA junction recognition, the binding of truncated forms of TRF2 were observed through mobility shift analysis. Unlike full-length TRF2, incremental mobility shifts were observed as the concentration of the DNA binding domain (Fig. 3A, DBD, amino acids 440–500) was

increased (Fig. 3B). In addition, no apparent differences in affinity of the DBD for T5T1 and T5T0 were observed. The DBD + linker (Fig. 3A, amino acids 301–500) version of TRF2 appeared to form larger complexes with both T5T1 and T5T0 (Fig. 3C). At low concentrations, a small shift was observed but increasing the DBD + linker concentration resulted in the formation of large complexes that barely enter 8% polyacrylamide gels. No intermediate complexes were observed even at more narrow DBD + linker concentration ranges (data not shown). These results suggest that the linker region participates in cooperative multimerization of TRF2 on telomeric DNA in addition to the already characterized TRFH domain [24]. However, the affinity of the DBD + linker region for T5T1 did not appear to be greater than T5T0 suggesting that interactions with the G-strand overhang did not involve the DBD or the linker region of TRF2.

TRF2 multimerization on telomeric DNA has already been visualized by both electron and atomic force microscopy [11,12,18]. However, the binding of the DBD + linker form of TRF2 to telomeric DNA has not been imaged by either of these methods. We found that the DBD + linker appeared as small spherical monomers in the absence of DNA (Fig. 3D, panel ii). However, it clearly oligomerized when bound to a linearized DNA plasmid that contained 550 bp of telomeric DNA (Fig. 3D, panel iii). In addition, DNA condensation and small loops appear in the images. The height and width of which was $0.49 \pm 0.10$ nm and $27.2 \pm 2.6$ nm, respectively (Fig. 3D, panel iii).

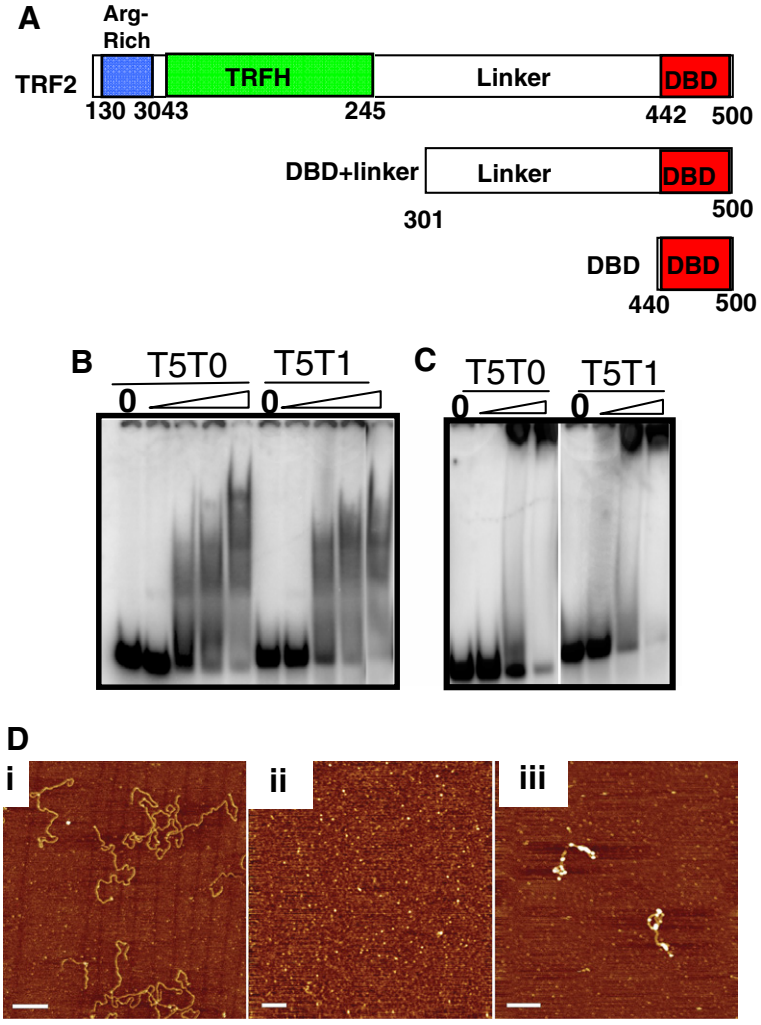


Fig. 3. TRF2 DNA binding domain and linker regions do not interact with the G-strand overhang. (A) Domain organization of TRF2 and truncation constructs. The top figure shows full length TRF2 amino acids 1–500 with three characterized domains; the arginine-rich, N-terminal domain (amino acids 13–30, blue), the homo-dimerization (TRFH) domain (amino acids 43–245, green), the DNA binding domain (DBD, amino acids 442–500, red) and an uncharacterized "linker region" (amino acids 245–442, white). The middle and bottom figures show the DBD + linker (amino acids 301–500) and DBD (amino acids 440–500) constructs. (B) EMSA of truncated TRF2 forms. 10 nM of labeled T5T0 and T5T1 DNA was used in all the assays. [DBD] concentrations are 1.4, 14, 140, and 1400 nM. (C) [DBD + linker] concentrations are 0, 4, 48, and 480 nM. (D) Oligomerization of DBD + linker on linear pRST5 DNA visualized by atomic force microscopy (i) DNA alone, (ii) DBD + linker alone, (iii) DBD + linker binding to DNA. The bar represents 200 nm. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

## Discussion

In order to protect the very end of the telomeres, TRF2 must somehow communicate with the ss/ds DNA junction. Our data show that TRF2 specifically recognizes telomeric ss/ds DNA junctions by interacting with the G-strand overhang. This interaction depends on both the sequence of the G-strand overhang and the sequence of the double-stranded DNA most proximal to the junction. The association of TRF2 with the G-strand overhang would facilitate T-loop formation involving this region of the telomere and would allow for the G-strand overhang to be sequestered into a protective structure.

The interactions with the G-strand overhang do not appear to involve the DNA binding domain or the linker region of TRF2. The N-terminal region of TRF2 is very arginine-rich and a peptide pertaining to this region has been shown to interact with 4-way junction DNA [15]. In our study, optimal interactions with the G-strand overhang require a stretch of 3 guanines which would provide more electronegativity and hydrogen bond acceptors for the arginine-rich N-terminus. Furthermore, TRF2 binds slightly better to ss/ds telomeric DNA junctions with a G-strand overhang of 4 telomeric repeats even though we have previously found that the G-strand overhang of 4 telomeric repeats in a ss/ds telomeric DNA junction can form a G-quadruplex [16]. Additional studies are required to determine how TRF2 interacts with G-quadruplex structures. In addition, previous studies from our laboratory demonstrate that recruitment of TRF2 and pot1 from HeLa cellular extracts to telomeric ss/ds DNA junctions is inhibited when the G-strand overhang is in the form of a G-quadruplex [16]. Since we have also detected Rap1 recruitment (Fig. 2B), we suggest that we have isolated some form of shelterin complex which requires more than just TRF2 binding for optimal assembly.

The interactions of TRF2 with unusual DNA structures are not the only interesting property of this protein. Unlike TRF1, TRF2 oligomerizes extensively in the presence of DNA [18]. Part of this oligomerization is due to the dimerization (TRFH) domain [24]. Our results show that the linker region is an additional region of the protein involved in oligomerization. Although TRF1 and TRF2 share some sequence and a large degree of structural homology in the TRFH domain, their linker regions are not homologous. This may explain how TRF2 can stabilize T-loops whereas TRF1 cannot. It was recently shown that strand invasion by TRF2 *in vitro* involves the TRFH domain [13] and suggests that the TRFH domain is involved in T-loop formation. It would be interesting to see if the linker region is also involved in strand invasion or if this activity resides exclusively in the TRFH domain.

## Acknowledgments

We thank Drs. Paul Boehmer and Amjad Farooq for their cell culture and protein purification facilities. We are also grateful to Dr. Jack Griffith for providing us the pRST5 plasmid, and Asmaa Baker for linearizing the plasmid. We thank Dr. Titia de Lange for supplying the TRF2-expressing baculovirus. S. Khan is a predoctoral fellow funded by AHA predoctoral award # 0515164B. This work was also supported by the James and Esther King, Florida Biomedical Research Program 04NIR-06.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.08.122.

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