

An Intramolecular G-Quadruplex Structure Is Required for Binding of Telomeric Repeat-Containing RNA to the Telomeric Protein TRF2

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S Supporting Information

ABSTRACT: Telomeric repeat-containing RNA (TERRA) is important for telomere regulation, but the structural basis for how TERRA localizes to chromosome ends is unknown. Here we report on studies exploring whether the TERRA G-quadruplex structure is critical for binding to telomeres. We demonstrate that the telomeric protein TRF2 binds TERRA via interactions that necessitate the formation of a G-quadruplex structure rather than the TERRA sequence per se. We also show that TRF2 simultaneously binds TERRA and telomeric duplex or G-quadruplex DNA. These observations suggest that the TERRA G-quadruplex is a key feature of telomere organization.

Telomeres are essential for maintaining genome stability in eukaryotes. Human telomeres comprise tandem TTAGGG repeats¹ and a protective protein complex² that includes the telomeric repeat binding factors TRF1 and TRF2.³ Telomeres are transcribed into telomeric repeat-containing RNA (TERRA)^{4,5} consisting of tandem UUAGGG repeats with variable subtelomeric sequences.^{4–6} TERRA is localized to telomeres and is considered to be important for the maintenance of telomere structure, heterochromatinization, and replication.^{4,5,7,8} While synthetic TERRA oligonucleotides fold into a G-quadruplex structure,^{9–11} it is not known whether endogenous TERRA adopts such a structure in cells, and if so, whether this is functionally important. Herein we report that TRF2 binds to TERRA through an interaction that is dependent on the G-quadruplex structure. We also show that TRF2 mediates the simultaneous binding of the TERRA intramolecular G-quadruplex and telomeric DNA, suggesting that the G-quadruplex structure is a key element for TERRA association to the TRF2–telomere complex.

While it has been reported that TERRA binds to TRF2,⁷ the structural basis for this interaction is unknown. We studied whether TRF2 recruits TERRA to telomeres through a G-quadruplex interaction. To explore this possibility, we used circular dichroism spectroscopy (CD) and UV melting experiments to validate that a TERRA oligonucleotide, TERRA-wt [sequence (UUAGGG)₄], folds into a G-quadruplex structure. The CD spectrum of TERRA-wt (Figure 1) displays a positive peak around 260 nm and a negative peak near 240 nm that are K⁺ dependent [Figure S1 in the Supporting Information (SI)] and consistent with a parallel G-quadruplex.⁹ We employed TERRA-mut, with two mutated Gs

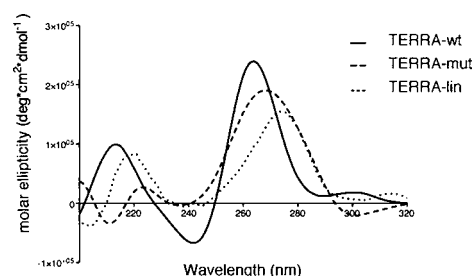


Figure 1. Biophysical analysis of TERRA oligonucleotides. Shown are CD spectra for TERRA-wt, TERRA-mut, and TERRA-lin oligonucleotides in the presence of 100 mM KCl. The TERRA-wt spectrum is characteristic of a parallel G-quadruplex.

in each repeat [sequence (UUACCG)₄], and TERRA-lin, which lacks the three Gs within the fourth repeat [sequence (UUAGGG)₃UUA], as negative controls. For both controls, the CD spectra did not show peaks characteristic of G-quadruplex structure formation, with a positive peak shifted away from 260 nm and the absence of a 240 nm negative peak (Figure 1).

Thermal difference spectral analysis confirmed that TERRA-wt folds into a G-quadruplex, as the spectrum shows a characteristic negative peak at 295 nm¹² that is absent in the controls TERRA-mut and TERRA-lin (Figure 2A). Additionally, UV melting experiments demonstrated that the TERRA-wt G-quadruplex structure is stable at 10 mM potassium, with a melting temperature (T_m) of 64.4 °C (Figure 2B). No melting transition was observed for TERRA-mut or TERRA-lin. Our results corroborate that TERRA forms a stable parallel G-quadruplex structure under near physiological conditions.

To determine whether TRF2 can associate with a TERRA G-quadruplex, we compared the binding of glutathione S-transferase (GST)-tagged full-length TRF2 (Figure S3) to TERRA-wt, TERRA-mut, and TERRA-lin. We employed an enzyme-linked immunosorbent assay (ELISA) in which incubation of the immobilized target oligonucleotide with a range of GST–TRF2 protein concentrations (0–200 nM) was followed by quantification using an anti-GST antibody (see Methods in the SI). Using this system, we demonstrated that TRF2 binds to TERRA-wt with low-nanomolar affinity (dissociation constant $K_d = 2.5 \pm 0.8$ nM) and shows no detectable binding to TERRA-mut and TERRA-lin controls

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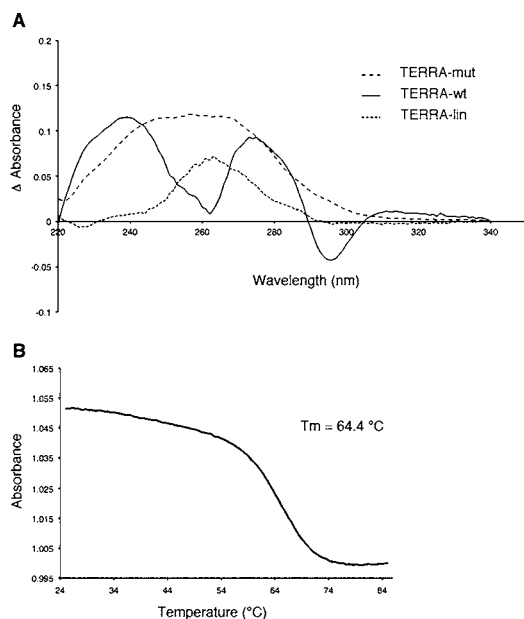


Figure 2. UV spectroscopy for TERRA oligonucleotide characterization. (A) Thermal difference spectra of TERRA-wt, TERRA-mut, and TERRA-lin oligonucleotides in the presence of 10 mM KCl. The TERRA-wt spectrum shows a negative peak at 295 nm typical of G-quadruplex structures. (B) UV melting curve of TERRA-wt in the presence of 10 mM KCl. The calculated melting temperature (T_m) is indicated.

(Figure 3). This demonstrates that TRF2 binds to TERRA via a G-quadruplex-dependent interaction. To consolidate this, we also demonstrated that TRF2 binds tightly to two other previously documented RNA G-quadruplex structures from the NRAS and BCL2 5' untranslated regions^{13,14} (Figure S4). Importantly, these findings highlight that it is the G-quadruplex structure, rather than the sequence per se, that is key for high-affinity TRF2 binding.

TRF2 binds to telomeric duplex DNA³ and to telomeric G-quadruplex DNA.¹⁵ We therefore considered that if the TERRA G-quadruplex is a bona fide target for TRF2, then it would be expected to exhibit comparable binding affinities. We characterized the affinity of TRF2 for TERRA-wt, the telomeric DNA G-quadruplex hTELO [sequence GG-(TTAGGG)₄TTAG; Figure S5], and also the telomeric duplex DNA hTELO-dup [5'-GG(TTAGGG)₄TTAG-3'/3'-C-(AATCCC)₄AAT-5'; Figure S6]. The data in Figure 4 show that TRF2 binds to hTELO with an affinity ($K_d = 1.6 \pm 0.3$

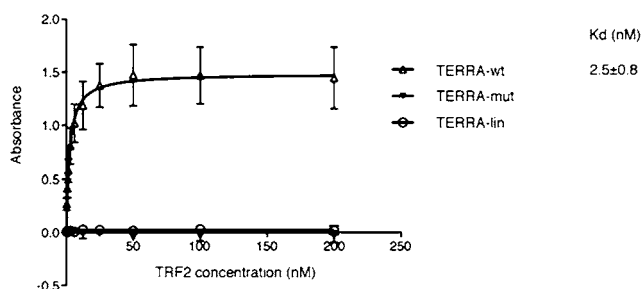


Figure 3. TRF2 binds to TERRA via a G-quadruplex-dependent interaction. TRF2 binding curves for the TERRA-wt, TERRA-mut, and TERRA-lin oligonucleotides are shown, and the dissociation constant (K_d) for TERRA-wt is indicated.

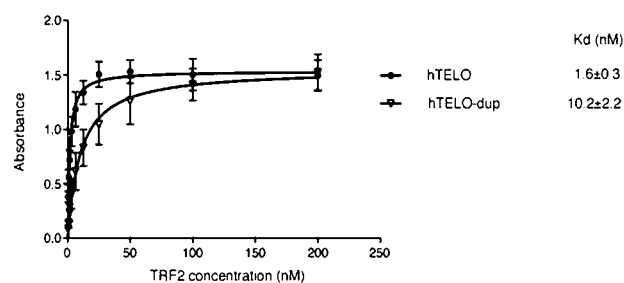


Figure 4. TRF2 binds more tightly to a telomeric DNA G-quadruplex structure than to a duplex, as shown by TRF2 binding curves for hTELO and hTELO-dup oligonucleotides. The corresponding K_d values are indicated.

nM) comparable to that for TERRA-wt ($K_d = 2.5 \pm 0.8$ nM; Figure 3) and has approximately 6-fold lower affinity for hTELO-dup ($K_d = 10.2 \pm 2.2$ nM). As TRF2 binds to telomeric duplex DNA in cells,³ these results suggest that TRF2 also binds to endogenous TERRA G-quadruplexes.

We considered that in addition to forming intramolecular G-quadruplex structures, endogenous TERRA could directly interact with telomeric DNA to form hybrid intermolecular G-quadruplexes. Indeed, the formation of a telomeric DNA–RNA G-quadruplex has been demonstrated *in vitro*,¹⁶ and therefore, we evaluated whether TRF2 could also bind to this hybrid structure. A hybrid TERRA–hTELO G-quadruplex (hTELO-hyb) was generated as described in Methods. The results in Figure 5 show that TRF2 binds to hTELO-hyb with an affinity ($K_d = 14.5 \pm 3.3$ nM) lower than that for TERRA-wt and hTELO but similar to that for hTELO-dup (Figures 3 and 4).

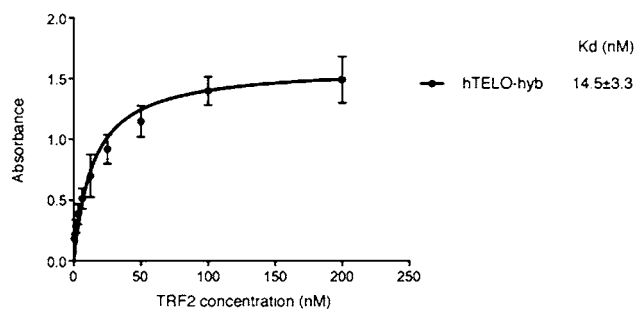


Figure 5. TRF2 binds to a hybrid telomeric DNA–TERRA G-quadruplex structure, as shown by the TRF2 binding curve for hTELO-hyb oligonucleotide. The K_d value is indicated.

It has been proposed that TRF2 mediates the association of TERRA and telomeric DNA.⁷ To address whether the TERRA intramolecular G-quadruplex plays a role in this interaction, we sought direct evidence of complex formation between the TERRA G-quadruplex, TRF2, and telomeric duplex DNA by exploring whether TRF2 could bind simultaneously to TERRA-wt and hTELO-dup. TRF2 protein (0–200 nM) was first allowed to bind to immobilized hTELO-dup. Next, unbound TRF2 was washed away, and digoxigenin-labeled TERRA-wt (200 nM) was added. This was followed by a final detection step with addition of an antidigoxigenin antibody (see Methods). The results showed that TRF2 binds simultaneously to both hTELO-dup and TERRA-wt (Figure 6). No signal was detected in the ELISA when TRF2 was absent, confirming that the TERRA-wt does not interact directly with hTELO-dup and

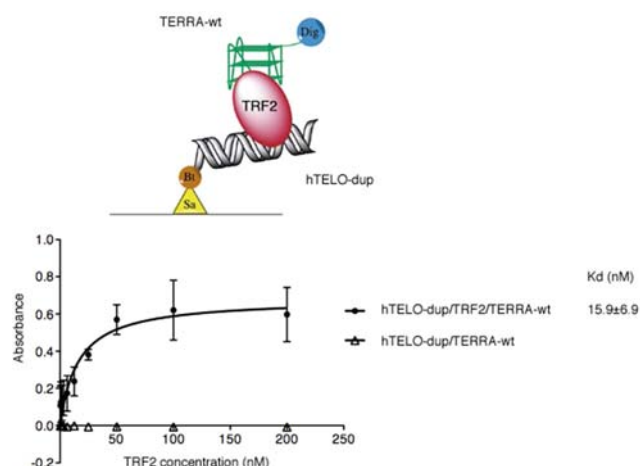


Figure 6. The TERRA RNA G-quadruplex and telomeric duplex DNA bind simultaneously to TRF2. A binding curve showing that TERRA-wt forms a complex with TRF2 bound to hTELO-dup as assessed by an ELISA assay is presented. Controls without added TRF2 are also shown. The diagram depicts the different components used.

that the measured binding is dependent on the TRF2 concentration. Figure 6 shows that the TERRA-wt binding affinity for the preformed TRF2–hTELO-dup complex (apparent $K_d = 15.9 \pm 6.9$ nM) is comparable to that of TRF2 for hTELO-dup (Figure 4).

A complementary experiment was also carried out to test the possible simultaneous binding of TERRA-wt and hTELO to TRF2 protein. Figure 7 shows that DNA and RNA G-quadruplex structures are also able to bind to TRF2 simultaneously.

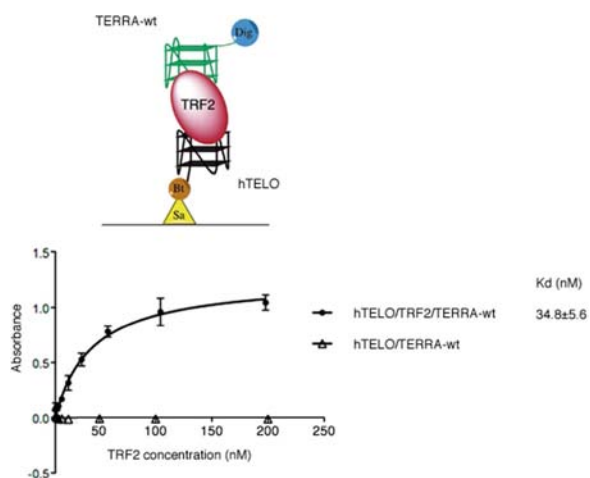


Figure 7. The TERRA and telomeric DNA G-quadruplexes bind simultaneously to TRF2. A binding curve showing that TERRA-wt forms a complex with TRF2 bound to hTELO as assessed by an ELISA assay is presented. Controls without added TRF2 are also shown. The diagram depicts the different components used.

In conclusion, we have shown that the formation of a RNA G-quadruplex structure is essential for the interaction of TERRA with TRF2. Since TRF2 binds to TERRA in cells, our data suggest a mechanism of in vivo association of these two components wherein the RNA G-quadruplex structure plays a central role. Long TERRA molecules have been proposed to fold simultaneously into several G-quadruplexes,¹⁷

and therefore, on the basis of our data, one TERRA molecule may well interact with many TRF2 proteins at telomeres. Our study has also shown that TRF2 is a key player in mediating TERRA association, via a G-quadruplex structure, to telomeric DNA, providing new insights into the molecular interactions between nucleic acids and proteins at chromosome ends.

■ ASSOCIATED CONTENT

📄 Supporting Information

Experimental procedures and additional figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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