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Intrinsic hTRF1 fluorescence quenching reveals details of telomere DNA binding activity: Impact of DNA length, structure and position of telomeric repeats

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Abstract

The myb-DNA binding domain is characterized by a 3- α helical bundle and three repeats of this domain drive sequence specific DNA-binding of the c-myb transcription factor. Human TRF1 contains a single myb-related domain and as a homodimer, enables the sequence specific binding of telomeric DNA. In this report we provide a kinetic assessment of hTRF1 DNA binding activity. Using intrinsic fluorescence quenching we present evidence that hTRF1 binds to both telomeric and non-telomeric DNA with kinetic discrimination to allow stable binding to telomeric tracts of DNA. The position of telomere repeats does not impact binding though the number of repeats and structure does impact binding. Kinetic analysis of DNA-dependent intrinsic tryptophan fluorescence quenching of hTRF1 revealed a two step binding process that is impacted by telomere repeat length, position, and structure. These data are consistent with existing structural and equilibrium binding data for hTRF1 recognition and binding of telomere DNA.

Keywords

c-myb; TRF-1; pre-steady state; kinetics; DNA binding; fluorescence quenching

INTRODUCTION

Human telomeric binding factor 1 (hTRF1) was first identified and characterized by the de Lange laboratory [1] and was shown to influence telomeric length in a series of overexpression studies of wild type hTRF1 and a dominant negative mutant [2]. In vitro, hTRF1 binds to and bends telomeric DNA to an angle of approximately 120°, which is thought to be important for telomeric maintenance [3]. Considering the higher ordered T-loop structure of mammalian telomeres, DNA bending is clearly a feature which could promote these structures [4]. Binding of hTRF1 to telomeric DNA is not cooperative, yet binding is length dependent, with longer telomeric tracts increasing hTRF1 binding as determined in vitro using electrophoretic mobility

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shift assays (EMSA) [5]. This evidence supports the hypothesis that the shortening of telomeres results in less hTRF1 binding. Since the original identification of hTRF1, additional telomere binding proteins have been identified including hTRF2, Pot1 (protection of telomeres 1) and Rap1 (Repressor/Activator Protein 1) in what has been termed the shelterin complex [6–9]. The complexity of DNA binding events is made even greater as a number of DNA repair proteins also can be localized to telomeres including Ku, the DNA-PKcs (DNA dependant protein kinase) catalytic subunit, ATM (ataxia telangiectasia mutated) and the MRN (MRE11, RAD50, and NBS1) complex [10–12]. The exact mechanisms by which hTRF1 and hTRF2 regulate telomere length are unknown. In vitro analysis has suggested a role in inhibiting DNA synthesis following telomerase catalyzed extension [13;14]. More recently, hTRF1 and hTRF2 have been shown to stall replication fork progression at telomeric repeats [15] suggesting the possibility of conserved mechanism of action of telomere repeat binding factors.

TRF1 forms homodimers and contains a c-terminal Myb-like three helix DNA binding domain [16]. This dimerization is essential for DNA binding as each monomer entails only part of the Myb-like domain. Studies have demonstrated that hTRF1 has high specificity for the mammalian telomeric repeat sequences [17–19]. Both solution and crystal structural data reveal amino acids within two separate alpha helices contact the six base TTAGGG sequence [16;20;21]. Though hTRF1 and hTRF2 share similarities in DNA binding domains, they do not heterodimerize because the N-terminus required for dimerization in hTRF1 is acidic and the N-terminus of hTRF2 is basic [22]. This supports the observation that these proteins have non-overlapping, distinct functions at telomeres which has been attributed to their differential ability to interact with other proteins [23;24]. The stability of hTRF1 has also been investigated and the prolyl-isomerase Pin1 has been demonstrated to impact stability in what has recently been shown to be a ubiquitin dependent process [25;26].

It is well established that hTRF1 and hTRF2 impart their biologic effect via binding telomere DNA. Previous biochemical analysis of DNA binding activity has largely relied on steady state measurements. More detailed analysis of the minimal c-myb DNA binding domain has been performed and yielded insight into the kinetics and thermodynamics of the interaction [27–29]. These types of analyses have not been extended to the full length c-myb proteins or other full length c-myb domain containing proteins. To ascertain how the kinetics of the c-myb DNA binding domain of hTRF1 imparts its specificity kinetically to achieve telomere-specific-binding, we undertook the analysis of purified full length hTRF1 and assessed its interactions with a series of synthetic DNA substrates.

Methods and Materials

Cloning and virus production

The hTRF1 cDNA was amplified from the Human Full Length cDNA kit (Panomics, Redwood City, CA) using the hTRF1 sense primer (5'ACGGCTAGCATCGAGCCATTTAAC 3') and hTRF1 antisense primer (5' GATCTGCAGAGCTTTTACAAACAC 3'). The 1.6 kbp PCR product was cloned into the pCR®-Blunt II-TOPO® vector (Invitrogen, Carlsbad, CA) and subsequently cloned into the pRSET B vector (Invitrogen, Carlsbad, CA) to add an N-terminal poly-histidine tag. The [His]₆-hTRF1 construct was then cloned into the pBacPAK 8 transfer vector for the creation of baculovirus. Recombinant baculovirus was prepared and plaque purified virus obtained and amplified.

Protein Expression and Purification

[His]₆-hTRF1 was produced by infecting a 100 ml culture of SF9 cells with recombinant baculovirus at an MOI of 10. The infection was allowed to proceed for 48 hours, at which time the cells were collected by sedimentation at 1000 × g. Cells were washed with cold 1X PBS

three times and spun down at $1000 \times g$ after each wash. The cell pellet was weighed and resuspended in 10 volumes of buffer R (50 mM NaPi, pH 7.4, 1M KCl, 0.2% Triton X-100, 7 mM β -ME) supplemented with 5 mM imidazole. The cells were disrupted by dounce homogenization using pestle A, and sonication. Nucleic acids were precipitated with PEI (0.1%) and the extract was stirred for 10 minutes at 4°C. The nucleic acids were sedimented at $11,000 \times g$ for 30 minutes at 4°C. The supernatant containing [His]₆-hTRF1 was then fractionated on a Ni-NTA matrix (Qiagen, Valencia, CA) that was pre-equilibrated in buffer R supplemented with 5 mM imidazole. The crude extract was added to the Ni-NTA matrix and allowed to rotate at 4°C for 30 minutes. The Ni-NTA and extract slurry was then poured into a column and the flow-through collected separately as the column was washed with 10 column volumes of buffer R + 5 mM imidazole. The bound protein was eluted using buffer R + 350 mM imidazole. Fractions containing [His]₆-hTRF1 were identified via Coomassie Blue stained SDS gels and pooled and diluted in buffer R without KCl to reduce the salt concentration to below 100 mM. The diluted protein was then loaded onto a 1 ml heparin-Sepharose column equilibrated in 100 mM KCl buffer. The protein was then eluted off of the column using a linear gradient from 0.1 M to 1 M KCl. Fractions were analyzed for the presence of [His]₆-hTRF1 and were dialyzed against buffer contained 20 mM HEPES pH 7.5, 1 mM BME, 500 mM KCl, and 20% glycerol). Fractions were stored at -80°C until use.

Fluorescence spectroscopy

[His]₆-hTRF1 fluorescence was measured using a Cary Eclipse Fluorescence spectrophotometer (Varian, Palo Alto, CA) in 400 μ l reaction volumes in the presence of 1X binding buffer (10 mM Tris-HCl pH 7.5, 5 mM MgCl₂, and 7.5 mM DTT). Fluorescence emission scans and excitation scans were performed on hTRF1 to determine the optimum emission and excitation wavelengths, 277 and 335 nm respectively at a slit width of 10 nm. Kinetic fluorescence quenching studies were performed with 500 nM DNA and initiated by the addition of 100 nM protein. Fluorescence was monitored at 0.2 second interval for 15 minutes. Individual experiments were repeated at least 3-times and the quenching data was then averaged and plotted using the SigmaPlot software (Systat, Point Richmond, CA). Control reactions without DNA were also performed to account for photo-bleaching. The reduction in fluorescence due to bleaching observed in the controls was used to correct the data sets for reactions in the presence of DNA. Data was normalized to the maximum fluorescence signal for each DNA and fit to either a single exponential decay (Equation 1) or a double exponential decay (Equation 2) using SigmaPlot software (Jandel Scientific).

$$y=ae^{-bx} \quad \text{Equation 1}$$

$$y=ae^{-bx}+ce^{-dx} \quad \text{Equation 2}$$

Stopped flow kinetic analysis was performed essentially as we have previously described [30–32]. Briefly, DNA and protein were diluted in the identical buffer at 2X concentration in equal volumes and loaded into the injections syringes on an SX18MV stopped-flow reaction analyzer (Applied Photophysics, Leatherhead UK). Data was collected following excitation at 280 nm, and fluorescence monitored using a 350-nm-long pass cut on filter (LG-350, Corion, Franklin, MA). Data was collected at 25°C and Traces represent an average of at least 5 individual shots.

DNA substrates

The DNA substrates used for the kinetic studies of [His]₆-hTRF1 are presented in each figure. Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA), and were gel-purified via preparative denaturing polyacrylamide gel electrophoresis. Duplex DNA substrates were generated by mixing equal concentrations of complementary strands with annealing buffer (50 mM Tris-HCl pH 7.5, 10 mM magnesium acetate, and 5 mM DTT), heating to 95°C for 5 minutes, and cooling slowly to room temperature overnight.

RESULTS and DISCUSSION

Purification of hTRF1 displays intrinsic fluorescence that is quenched upon DNA binding

The hTRF1 gene was cloned from a human cDNA library using PCR. An N-terminal [His]₆ was added by cloning in frame into the pRSet B vector. The recombinant gene was then cloned into the pBacPak8 transfer vector for the creation of a recombinant baculovirus. Plaque purified virus was amplified and used to infect SF9 insect cells for protein production. A two column purification procedure was employed as described in materials and methods which resulted in highly purified [His]₆-hTRF1 as assessed by SDS-PAGE analysis [14] (Figure 1A). The lower molecular weight contaminants represent less than 5% of the total protein and do not react with an anti-TRF1 or anti-express antibodies suggesting that they are not degradation products of the full length hTRF1 protein (data not shown). The purified protein binds DNA in EMSA assays, which demonstrates that it is indeed a dimer (data not shown).

While significant information regarding Myb binding domains has been obtained from structural and equilibrium binding studies, very little is known about the kinetics of these interactions. To study the kinetics of DNA binding in a solution based assay we investigated the potential that intrinsic tryptophan fluorescence could be used as a measure of DNA binding activity. The structural analysis of the hTRF1 DNA binding domain revealed two tryptophan residues positioned close to the sites of DNA interaction, yielding the possibility that their fluorescence may be quenched upon DNA binding (Figure 1B). To determine if intrinsic protein fluorescence could be used, excitation and emission spectra were taken over the ranges expected for tryptophan fluorescence. The peak excitation wavelength of hTRF1 was found to be 277 nm and peak emission determined to be 335 nm, typical of tryptophan excitation and emission we have observed in DNA binding protein [30; 31] (Figure 1C). To determine if quenching of this intrinsic fluorescence occurred upon DNA binding, we performed equilibrium binding experiments with a telomeric DNA containing three 5'-TTAGGG-3' repeats with a 10-bp non-telomeric extension at each termini. We used this internal repeat design with unique termini to ensure proper alignment of the repeats as well as to minimize any end-effects that may influence binding. The results presented in Figure 2 demonstrate that in reactions containing 100 nM [His]₆-hTRF1 and 500 nM telomeric DNA, significant quenching is observed. Surprisingly a small but reproducible degree of quenching was also observed upon incubation with a DNA substrate where the three telomeric repeats were replaced with non-telomeric sequence. As reactions were performed in fairly low ionic strength, we performed a salt titration to determine the stability of the interactions. The quenching results with both DNA substrates were very consistent with minimal effect of ionic strength on the observed quenching up to 500 mM NaCl (data not shown). These solution-based equilibrium binding analyses confirm TRF-1 telomere DNA binding activity and extend previous results which have employed EMSA analysis and suggest that there is an interaction, albeit weak, between TRF-1 and non-telomeric DNA.

Kinetics of hTRF1 binding internal telomere tracts of DNA

Having established that hTRF1 fluorescence can be quenched in the presence of DNA we examined the pre-steady state interactions of hTRF1 with DNA. Substrates containing 3–4

telomeric repeats were selected for analysis based on equilibrium DNA binding studies which showed that three repeats are the minimum required to detect binding in an EMSA assay [2]. The first attempts at measuring the pre-steady state binding of hTRF1 to telomeric DNA was performed on a stopped flow instrument, which allows for measurement of intrinsic fluorescence quenching in the millisecond time scale. Quenching of hTRF1 fluorescence could not be detected over a 500 millisecond time range (data not shown). However, upon increasing the measurement time to 10 seconds, the first fluorescence quenching of hTRF1 due to binding of DNA could be measured (Figure 2B). Interestingly the fluorescence continued to decrease over the entire time frame and suggested a continued slow binding process which necessitates a longer reaction time. Quenching was also observed with non-telomeric DNA over this short time frame which indicated the need for longer reaction times (data not shown).

To enable analysis to be carried out over multi-minute time frames we modified the cuvette-based equilibrium methodology to allow kinetic measurements. The data presented in Figure 3 shows the analysis of the non-telomeric 38-bp substrate and the DNA containing the three internal telomeric repeats. The non-telomeric DNA exhibits a quenching for the first two minutes (Figure 3A), but then there is no further fluorescence quenching. This data was fit to a single exponential decay curve indicating a single binding event. Results obtained with the substrate containing three internal telomeric repeats exhibited a continued decrease in fluorescence after this first two minute window (Figure 3A). The quenching data for the internal three telomeric repeat DNA did not fit to a single exponential equation but was very well described by a double exponential decay curve, suggesting that a minimum of two events are being observed. The amplitude of the decay describing the interaction with the non-telomeric substrate was less than that observed for the telomeric DNA interaction while the rates of decay were significantly greater for the interaction with the telomeric DNA (Table 1). In a two step binding reaction observed for the interaction with the DNA containing three telomeric repeats, one of these events obviously is the binding of the DNA molecule to TRF1 as also was observed with non-telomeric DNA. The second event may very likely be a conformational change that allows hTRF1 to more fully engage telomeric DNA or represent a translocation to a second telomeric repeat. There is also the possibility that the second subunit of the TRF-1 dimer exhibits different binding kinetics such that the first monomer-DNA interaction is relative slow followed by a fast second interaction. Interestingly, analysis of the c-Myb binding domain alone has revealed a two step binding process to the consensus binding motif for the transcription factor [33].

Effect of telomere tract length on binding kinetics

Based on high resolution structural analysis of the hTRF1 myb domain, two repeats should be sufficient for binding a single hTRF1 homodimer. To investigate the effect of repeat length on binding we prepared a substrate with four internal TTAGGG repeats and analyzed the kinetics of binding via fluorescence quenching. The results presented in Figure 3B demonstrate that upon hTRF1 binding to the DNA containing four internal repeats, fluorescence quenching was observed and the data fit a single exponential decay. The single amplitude of the decay observed for the four repeat containing DNA is nearly identical to the combined amplitude of quenching for both steps observed in the interaction with the three-repeat containing substrate (Table 1). Interestingly, the overall rate of decay is less for the four repeat containing DNA compared to the non-telomeric DNA and the three-repeat containing DNA. This data suggests that one possibility for the two step process observed in binding DNA with three telomeric repeats may be a “non-productive” interaction with either non-telomeric DNA or binding of hTRF1 out of register with the repeat tract, an event not observed in substrates with an even number of telomeric repeats.

Kinetics of TRF1 end binding activity

The distribution of telomere binding proteins along a telomere is largely unknown. To determine if TRF1 displays similar kinetics upon terminal repeat binding we constructed a substrate with three terminal duplex TTAGGG repeats and assessed binding via fluorescence quenching (Figure 4A). Interestingly the kinetics of this interaction was similar to the DNA substrate containing three internal repeats, though the rates of each decay was reduced compared to the internal repeat substrate suggesting that there is an “end effect” on binding of TRF-1 to telomeric repeats. Surprisingly when the end repeat length was increased to four, (Figure 4B) the kinetics of the interaction was different compared to the three repeats with a very fast decay with a small amplitude being observed followed by a slow decay over the majority of the quenching time course (Table 1). The kinetic trace observed with this substrate also revealed an exacerbated fluorescence deflection in the 6–9 minute time period (Figure 4B). This anomaly was also observed in other traces though these were not as prominent. Fitting the data to the identical equation over a shorter time frame, up to 6 minutes, did not appreciably alter the rate constants, and simply omitting the 6–9 minute data, again did not change the rate and amplitude values significantly, though an increase in the adjusted r-squared value for the fit was increased, as expected (data not shown). Thus the rate constants obtained accurately reflect the interaction of hTRF1 with these DNA substrates. The differential kinetics observed as a function of repeat length at the ends of DNA is similar to that observed for the internal repeat analysis presented in Figure 4. Interestingly, the kinetic data for the four terminal repeats did not fit a single exponential decay as was observed with the four internal repeats. Instead a double exponential fit was obtained with dramatic differences between the two calculated exponents.

In vivo telomeres contain 3'-TTAGGG extensions that can range up to a few hundred bases in length [34]. As TRF1 is a duplex DNA binding protein, we reasoned that only the first few single strand repeats would be accessible to hTRF1 bound to the terminal duplex repeat. Therefore we constructed a DNA containing a single-strand double strand junction to assess the effect of the single strand overhang of hTRF1 binding. This DNA contained two duplex repeats followed by two single strand repeats. Kinetic analyses again revealed a double exponential decay (Figure 4C). Compared to the results obtained with the substrate with four duplex repeats which displayed a single exponential decay, the four repeats of which only two are duplex show binding consistent with the two-step process observed for the three-repeat DNA containing substrates. However, the differential between the components of the fit, rate and amplitude, were more similar to the three-repeat end binding data than four repeat terminus (Table 1).

These results demonstrate that the kinetics of TRF1 binding to telomeric DNA is impacted not only by repeat length, but by position of the repeats as well as the extent of duplex structure. These observations reveal that the relationship between hTRF1 and DNA is much more complex than previously thought. One cannot simply state that hTRF1 only binds TTAGGG repeats, as we have shown that hTRF1 does interact with non-telomeric DNA. However, through the complex kinetics of binding and a potential conformational change, hTRF1 binds telomeric DNA more favorably than non-telomeric DNA. This potentially can explain why, in equilibrium assays such as EMSA's, the binding to non-telomeric DNA is not observed. In this manuscript, we have provided the first kinetic evidence suggestive of a conformational change due to binding of telomeric DNA, which was previously predicted by structural studies (reference). However, further studies are needed to demonstrate a true correlation between the parameters we observe and a conformational change. Furthermore, the positioning of the telomeric tract in the larger DNA context also affects TRF1's interaction with it, further complicating the binding process. Further studies are needed to determine whether hTRF1 is also sensitive to DNA architecture, as well as having sequence specificity.

Since hTRF1 is a regulator of telomeric length, these data provides initial kinetic analysis of how TRF-1 interacts with telomeric and non-telomeric DNA. The first and most dramatic conclusion is that the formation of a stable complex is extremely slow taking place over a period of minutes as opposed to other DNA binding proteins with rates of association on the order of tenths of seconds. The two step binding reactions are consistent with two individual trp-DNA quenching interactions which are dictated by the number and position of the telomeric repeats. A second mechanism involves one direct DNA-dependent quenching interaction with the second decay being attributed to a protein conformational change. Alternatively, two separate DNA-dependent conformational changes in the protein could result in quenching, each influenced again by the telomeric repeat length and position. The sequence independent quenching observed in the time frame of seconds most likely represents diffusion limited events the majority of which are non-productive and do not lead to stable binding. The data obtained is also consistent with an initial interaction followed by a two-dimensional sliding model to locate sequence specific sites as the position of the telomeric repeats were found to influence the rates of both trp- quenching kinetics observed over the 15 minute time frame. A more detailed study to distinguish between these possibilities is warranted and to determine how TRF1 interactions with DNA impacts telomere maintenance and function.

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Figure 1. hTRF1 purification and intrinsic fluorescence

A, Coomassie stained SDS polyacrylamide gel showing the final purified hTRF1 protein. The hTRF1 is running with the 66 kDa molecular weight marker as expected. B, NMR structure of the myb domain of hTRF1 interacting with a telomeric DNA duplex [19]. The helix-loop-helix of the myb domain (yellow) is interacting with the major groove of the DNA. The two tryptophan residues potentially responsible for intrinsic fluorescence inside the myb domain of hTRF1 are highlighted (magenta). C, Excitation and emission spectra of hTRF1. The excitation spectrum (red trace) peaks at 277 nm, and the corresponding emission spectrum (black trace) peaks at 335 nm.

Figure 2. Steady state and pre-steady state fluorescence quenching of hTRF1

A, DNA-dependent quenching of TRF1 intrinsic fluorescence. Baseline fluorescence in the absence of DNA (black bar) was measured in binding reactions containing 100 nM hTRF1. Upon the addition of DNA to a final concentration of 500 nM, telomeric (grey bar) and non-telomeric (white bar) DNA intrinsic tryptophan fluorescence was measured. Statistical significance was measured via a paired t-test and p values presented for each comparison. B, Pre-steady state binding of hTRF-1 to DNA. The interaction between DNA (100 nM) and hTRF1 (100 nM) were analyzed via stopped-flow analysis. Fluorescence was measured over a 10 second time frame mixing either a buffer control (black trace) or with 100 nM DNA (gray trace). The data were collected and represent an average of at least five separate shots as we have previously described (31). The data were normalized to the maximum fluorescence intensity for each individual DNA substrate.

Figure 3. Time and DNA-Dependent fluorescence quenching of hTRF1

Buffer and DNA were added to a cuvette and the reaction was initiated by the addition of hTRF1. The intrinsic fluorescence of hTRF1 was measured over a 15- minute time period. A) Binding to non-telomeric DNA (light trace) was measured and the data was fit to a single exponential decay depicted as the black line. Binding to the telomeric DNA with three internal TTAGGG repeats (dark grey trace) and data fit to a double-exponential decay. B) Binding to a DNA containing 4 internal TTAGGG repeats was also measured and the data fit to a single exponential decay. The individual traces were all normalized to the maximum fluorescence value obtained with each individual DNA substrate. The sequence and structure of the DNA substrates are depicted above the graph.

Figure 4. Influence of position and structure on TRF1 binding kinetics

A, DNA-dependent quenching of hTRF1 intrinsic fluorescence was measured using a blunt ended substrates containing A) three terminal telomeric repeats; B) a substrate with a 3' telomeric overhang; C) a DNA with four terminal repeat. The data were normalized to the maximum fluorescence value for each individual DNA substrate. Each data series was fit to double exponential decay and the sequence and structure of the DNA substrates are depicted above the graph.

Table 1

Rate constants for DNA-dependent hTRF-1 quenching.

DNA	Fit	1st decay		2nd decay	
		Rate	Amplitude	Rate	Amplitude
non tel	Single	0.5457	0.0263		
3-tel internal	double	3.6266	0.0391	0.1313	0.0355
4-tel internal	single	0.2843	0.0699		
3-tel end	double	1.3820	0.0324	0.0871	0.0742
4-tel end	double	7.4340	0.0178	0.0014	0.972
2+2 end overhang	double	0.5115	0.0491	0.0098	0.3655