Telomere Repeat Binding Factor 2 Interacts with Base Excision Repair Proteins and Stimulates DNA Synthesis by DNA Polymerase β

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Abstract

The ends of linear chromosomes are capped and protected by protein-DNA complexes termed telomeres. Consequences of telomere dysfunction include genomic instability that can contribute to neoplastic transformation and progression. Telomere binding proteins interact with numerous proteins involved in DNA repair, underscoring the importance of regulating DNA repair pathways at telomeres. Telomeric DNA is particularly susceptible to oxidative damage, and such damage is repaired primarily via the base excision repair (BER) pathway. Using a screen for potential interactions between telomere repeat binding factor 2 (TRF2) and proteins involved in BER of oxidized bases in vitro, we found that TRF2 physically bound DNA polymerase β (Pol β) and flap endonuclease 1 (FEN-1). The interactions with endogenous proteins in human cell extracts were confirmed by coimmunoprecipitation experiments. The primary binding sites for both Pol β and FEN-1 mapped to the TRF2 NH2-terminal and COOH-terminal domains. We further tested the ability of TRF2 to modulate BER protein partners individually on a variety of substrates in vitro. TRF2 stimulated Pol β primer extension DNA synthesis on telomeric and nontelomeric primer/template substrates, resulting in up to a 73% increase in the proportion of longer products. TRF2 also stimulated Pol β strand displacement DNA synthesis in reconstituted BER reactions and increased the percent of long-patch BER intermediates on both telomeric and nontelomeric substrates. Potential roles of TRF2 in cooperation with BER proteins for DNA repair pathways at telomeres, as well as other genomic regions, are discussed. (Cancer Res 2006; 66(1): 113-24)

Introduction

Telomeres are protein-DNA complexes that protect the ends of linear chromosomes from inappropriate chromosome fusions by DNA repair pathways. Consequences of telomere dysfunction include chromosome end fusions and genomic instability, which promote the emergence of malignant phenotypes and cancer development (reviewed in ref. 1). Telomere dysfunction may also induce cellular senescence or apoptosis (2, 3). The DNA component of human telomeres consists of 5 to 15 kb of TTAGGG tandem repeats and a 3′ single-strand G-rich tail that serves as a substrate for telomerase. This tail can loop back and invade the duplex telomeric DNA, forming a large telomere loop (t-loop) that is stabilized by a displacement loop (D-loop) structure (4). Maintenance of the precise telomeric sequence is critical for proper telomere function (5) and is required for association of telomeric proteins. Telomere repeat binding factors 1 and 2 (TRF1 and TRF2) bind duplex TTAGGG repeats (2, 6) and protection of telomeres 1 (POT1) binds single-stranded TTAGGG repeats (7, 8). These proteins regulate telomere length and access of the 3′ telomeric tail (reviewed in ref. 9). Loss of TRF2 at the telomeres induces loss of the telomeric tail, telomere end fusions, and an ataxia telangiectasia mutated (ATM)- and p53-mediated DNA damage response which triggers apoptosis or senescence (2). Telomeric proteins also recruit other critical proteins to the telomeres.

Increasing evidence supports the importance of regulating DNA repair at telomeres. Numerous reports indicate a strong relationship between telomeric proteins and proteins involved in cellular responses to, and repair of, DNA damage. Several proteins that act in DNA double-strand break repair associate with the telomeres, and defects in these proteins lead to telomere dysfunction, genomic instability, and cancer predisposition (reviewed in refs. 10, 11). TRF2 interacts directly with the MRE11 complex (12), ATM (13, 14), Ku (15), Werner syndrome (WRN; 16–18), and Bloom syndrome (BLM; 19, 20) proteins and modulates many of their activities. In addition, TRF2 regulates and suppresses double-strand break repair at the telomeres; i.e. nonhomologous end joining (21) and deletions of telomeric t-loops by homologous recombination (22). TRF2 was recently reported to localize to sites of double-strand breaks outside the telomeres (13), raising the possibility that TRF2 may function in regulating repair in nontelomeric DNA.

In addition to double-strand break repair, accumulating evidence indicates that base excision repair (BER) is also important at the telomeres. BER is the primary pathway for repairing oxidized, alkylated, deaminated, and hydrolyzed bases (23) induced by both endogenous and exogenous sources including chemotherapeutic agents. BER plays a pivotal role in maintaining genomic integrity and BER enzyme deficiencies are associated with cancer (reviewed in ref. 24). Telomeres are particularly susceptible to oxidative and alkylating DNA damage (reviewed in ref. 25). We recently reported that 8-oxo-guanine disrupts binding by TRF1 and TRF2 to telomeric DNA (26), indicating a necessity for repairing DNA lesions in the telomeres to maintain the optimal association of telomeric proteins. In general BER, specific DNA glycosylases
to oxidative damage (29, 30), we screened for TRF2 interactions with BER proteins are not understood. Telomere metabolism. The functional importance of, and roles for, TRF2 and Pol β were investigated by co-immunoprecipitation and identified a novel interaction with FEN-1. We further tested the ability of TRF2 to modulate BER protein partners individually on various substrates and in reconstituted BER reactions in vitro. TRF2 stimulated Pol β nucleotide incorporation on primer/template and BER substrates. The degree of Pol β stimulation by TRF2 was similar on telomeric and nontelomeric substrates.

Materials and Methods

Recombinant proteins. Recombinant histidine-tagged human WRN, TRF2, and TRF1 proteins were purified as previously described (17). Purification of recombinant human proteins DNA Pol β (31), FEN-1 (32), APE1 (33), and OGG1 (34) was as previously reported. Purified human recombinant PARP1 and p53 proteins were generously provided by Dr. Gilbert de Murcia (Université Louis Pasteur, Strasbourg, France) and Dr. Kent Soe (Institute of Molecular Biotechnology, Jena, Germany), respectively.

Screen for protein interactions. The DiscoverLight Protein Array Kit (Pierce, Rockford, IL) was used to screen for interactions between purified TRF2 and select BER proteins according to the protocol of the manufacturer. Briefly, various protein concentrations (as indicated in Fig. 1A) were spotted in replicate on the membranes, which were then dried and blocked in PBS-T (1X PBS, 0.1% Tween 20) containing 5% bovine serum albumin (BSA) for 1 hour at room temperature. Next, the membranes were incubated with TRF2 (10 ng/mL) for 1 hour at room temperature. A duplicate membrane was incubated with blocking buffer alone as a control for possible cross-reactivity with the TRF2 antibody. After washing with PBS-T, the membranes were probed with monoclonal mouse anti-TRF2 antibody (1:500, Imgenex, Sorrento Valley, CA) overnight. After washing, the membranes were incubated with secondary antibody [1:10,000, antimouse immunoglobulin G (IgG)-horseradish peroxidase; Vector, Burlingame, CA] for 1 hour at room temperature, washed again, and then developed with the SuperSignal West Pico chemiluminescent kit (Pierce).

Table 1. Oligonucleotides used for substrate preparations

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>TFLP</td>
<td>(T)12CAAAATCATCCTGACCTCGAGACC</td>
</tr>
<tr>
<td>TUS</td>
<td>CTATCTCGAGCTAGTTGAGGT</td>
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<td>TTP</td>
<td>CTAACACATGCTGCGTC GCCAGGGATTAGGGTTAGG</td>
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<tr>
<td>TelH0</td>
<td>TGACCCGGGATCTCGGAGGC</td>
</tr>
<tr>
<td>TelO8</td>
<td>GTGAGTCTGAGTCTGAG</td>
</tr>
<tr>
<td>TelC</td>
<td>TCGAGTCTGAG</td>
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NOTE: *X, 8-oxo-guanine.

Figure 1. Physical interactions between TRF2 and BER proteins. A, increasing concentrations of various proteins were spotted in duplicate (0.6, 0.8, 0.9, and 1 ng) on a grid of the Discover-Light Protein Array membrane and then hybridized with TRF2 protein (10 ng/mL). After washing, bound TRF2 protein was detected by Western blotting with an anti-TRF2 antibody. B, communoprecipitation of TRF2 and Pol β. HeLa whole-cell extracts (500 ng) were immunoprecipitated with either rabbit anti-TRF2 (lane 3) or control IgG (lane 4) antibodies. The communoprecipitates were analyzed by SDS-PAGE and Western blot analysis with anti–Pol β or anti-TRF2 antibodies as indicated. TRF2 (lane 1) and Pol β (lane 6) were loaded as markers and positive controls. Input, 10% loaded (lane 2). C, coimmunoprecipitation of TRF2 and FEN-1. HeLa whole-cell extracts (500 ng) were immunoprecipitated with either rabbit anti-TRF2 (lane 3) or control IgG antibodies (lane 1). The immunoprecipitates were probed with anti-FEN-1 or anti-TRF2 antibodies as indicated. Input, 10% loaded (lane 2).

Briefly, various protein concentrations (as indicated in Fig. 1A) were spotted in replicate on the membranes, which were then dried and blocked in PBS-T (1X PBS, 0.1% Tween 20) containing 5% bovine serum albumin (BSA) for 1 hour at room temperature. Next, the membranes were incubated with TRF2 (10 ng/mL) for 1 hour at room temperature. A duplicate membrane was incubated with blocking buffer alone as a control for possible cross-reactivity with the TRF2 antibody. After washing with PBS-T, the membranes were probed with monoclonal mouse anti-TRF2 antibody (1:500, Imgenex, Sorrento Valley, CA) overnight. After washing, the membranes were incubated with secondary antibody [1:10,000, antimouse immunoglobulin G (IgG)-horseradish peroxidase; Vector, Burlingame, CA] for 1 hour at room temperature, washed again, and then developed with the SuperSignal West Pico chemiluminescent kit (Pierce).

1 D. Wilson and J. Fan, unpublished results.
control, overnight at 4°C. Samples were then incubated with protein A-sepharose beads (40 µl) at 4°C for 1 hour, followed by multiple washes. Bound proteins were eluted by boiling in sample buffer and were analyzed by SDS-PAGE and Western blotting using mouse anti-TRF2 (1:1,500; Imgenex), mouse anti–Pol β (1:1,000; Trevigen, Gaithersburg, MD), or rabbit anti-FEN-1 (1:1,000; Bethyl Laboratories, Montgomery, TX) antibodies, followed by chemiluminescent analysis (Pierce).

**Glutathione S-transferase-TRF2 fragments binding assay.** Recombinant human TRF2 protein fragments were generated by PCR cloning using the human TRF2 cDNA from the baculovirus DNA construct, kindly provided by Dr. Titia de Lange (Rockefeller University, New York, NY), as a template. The PCR products were subcloned into the BamHI and EcoRI sites of the p-GEX-6P-2 (Amersham Pharmacia) expression vector. The glutathione S-transferase (GST)-TRF2 fragments were expressed in _E. coli_ as previously described (26). The purity of single-step purified fragments was determined by SDS-PAGE and Coomassie staining. The HeLa nuclear extract preparation and pull-down assays with the GST-tagged TRF2 fragments were essentially as previously described (16). Briefly, the GST-TRF2 fragments were bound to glutathione beads and then incubated with HeLa nuclear extracts (400 µl). After washing, total protein was eluted in sample buffer by boiling and was analyzed by SDS-PAGE and Western blotting with mouse monoclonal anti–Pol β (1:1,000 dilution; Trevigen) or rabbit polyclonal anti-FEN-1 (1:1,000 dilution; Trevigen) antibodies. Equal loading of GST protein and the various GST-TRF2 fragments was verified by amido black staining of the precipitated proteins on the membrane.

**ELISA.** ELISA was as previously described (16) with some modifications. The blocking and binding steps were done in PBS containing 3% BSA and 0.1% Tween 20. Wells were coated with 35 mmol/L Pol β or FEN-1 diluted in carbonate buffer (50 µl) or with BSA as a background control by incubating for 2 hours at 37°C. After blocking, various TRF2 concentrations were added (50 µl; see figure legends) and incubated for 2 hours at 37°C. Following washes, primary antibody [1:1,000 rabbit anti-TRF2 (H-300); Santa Cruz Biotechnology] was added and incubated for 1 hour at 37°C. Wells were washed and secondary antibody (1:10,000, antirabbit IgG-horseradish peroxidase; Vector) was added for 1 hour at 37°C. After washes, bound TRF2 was detected with o-phenylenediamine dihydrochloride following termination with 3 mol/L H2SO4. Absorbance was read at 490 nm. Values for wells coated with Pol β or FEN-1 were corrected for the background signals obtained with anti-TRF2 antibody alone. To ensure that the interaction was not mediated by DNA, control reactions included either 5 µg/ml DNase I (Calbiochem, San Diego, CA) or 10 µg/ml ethidium bromide during the binding step. To determine the dissociation constant (K_d) for the TRF2-FEN-1 and TRF2-Pol β complexes, the fraction of immobilized FEN-1 or Pol β bound by TRF2 was calculated and the data were analyzed by Hill plot and Scatchard binding theory as previously described (36).

**FEN-1 incision.** The 10-nt 5′ flap substrate was prepared as previously described (32). The 15-nt 5′ flap substrate was constructed by annealing oligonucleotides TF1P, TUS, and TTPL (Table 1) in a 1:1:2:2 molar ratio, respectively. The flap was 5′ end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. The reactions (10 µl) were done in 50 mmol/L HEPES (pH 7.5), 50 mmol/L KCl, 5% glycerol, 10 mmol/L MgCl2, 0.05% Triton X-100, and 100 µg/ml BSA at 37°C for 10 minutes and stopped with stop dye (32). DNA substrate and protein concentrations were as indicated in the figure legends. The samples were heated at 95°C and loaded on an 18% denaturing polyacrylamide gel. The reactions were visualized by Phosphorimager analysis and quantitated with ImageQuant software (Amersham Pharmacia). Incision activity was calculated as (amount of product) / (total amount of radioactivity in each lane). Relative incision activity is based on a percentage comparison between FEN-1 alone (normalized to 100%) and the incision reactions with TRF2.

**DNA polymerase primer extension.** Reactions were done as previously described (37) with some modifications. Substrates were prepared by annealing a radiolabeled primer strand to a complementary template strand as described (37). Briefly, reactions (10 µl) were done in BER buffer [20 mmol/L Tris-HCl (pH 7.4), 4 mmol/L MgCl2, 1 mmol/L DTT, 40 µg/ml

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**Figure 2.** Mapping the sites of Pol β and FEN-1 interactions with TRF2. A, schematic of the known domains and structural motifs of TRF2 and the borders of the various GST-tagged fragments. B, basic NH2 terminus; TRFH, dimerization domain; Myb, Myb-like telomere DNA binding domain. Numbers indicate the amino acid sequence. C, Coomassie staining of the recombinant GST-tagged TRF2 fragments (2 µg each) used in the binding assay after single-step purification and SDS-PAGE. C, TRF2 domains that interacted with Pol β and FEN-1. HeLa nuclear extracts (400 µl) were incubated with either GST alone (lanes 2 and 9) or GST-tagged TRF2 fragments (lanes 3-6 and 8) that were prebound to glutathione beads. Eluted proteins were separated by SDS-PAGE, transferred to a membrane, and stained with amido black to ensure equal loading of the various TRF2 fragments. The membrane was probed with mouse anti–Pol β or rabbit anti-FEN-1 antibodies.

**Communnoprecipitation assay.** HeLa whole-cell extracts were prepared as previously described (35) and were precleared with protein A-sepharose beads (Amersham Pharmacia). Extracts (500 µg each) were incubated with either 5 µg of rabbit anti-TRF2 (H-300; Santa Cruz Biotechnology, Santa Cruz, CA) or 5 µg of rabbit IgG (Santa Cruz Biotechnology) as a negative control, overnight at 4°C. Samples were then incubated with protein A-sepharose beads (40 µl) at 4°C for 1 hour, followed by multiple washes. Bound proteins were eluted by boiling in sample buffer and were analyzed by SDS-PAGE and Western blotting using mouse anti-TRF2 (1:1,500; Imgenex), mouse anti–Pol β (1:1,000; Trevigen, Gaithersburg, MD), or rabbit anti-FEN-1 (1:1,000; Bethyl Laboratories, Montgomery, TX) antibodies, followed by chemiluminescent analysis (Pierce).
BSA] and contained 1 mmol/L ATP, 40 μmol/L each of dATP, dGTP, dTTP, and dCTP, and either Pol β or Klenow polymerase, together with TRF2 as indicated in the figure legends. Reactions were initiated by adding DNA substrate (25 nmol/L; Table 1) and were incubated at 37°C for 15 minutes, followed by termination with equal volume stop dye (37). Samples were heated at 90°C for 5 minutes and then run on 20% denaturing polyacrylamide gels. The reactions were visualized using a Phosphorimager and quantitated with ImageQuant (Molecular Dynamics). The percent of each product with a given number of nucleotides incorporated was calculated as follows: % of total = (amount of radioactivity associated with each dNMP addition / total radioactivity) × 100.

**In vitro BER.** The telomeric (Table 1) and nontelomeric (38) BER substrates were prepared as previously described and the 8-oxo-guanine-containing strand was 5’ end labeled. Substrates were pretreated with 128 nmol/L human OGG1 in 40 mmol/L HEPES (pH 7.6), 75 mmol/L KCl, 1 mmol/L DTT, 0.1 μg/μL BSA, and 5% glycerol for 20 minutes at 37°C. Repair reactions (10 μL) contained 100 nmol/L pretreated substrate in 40 mmol/L HEPES (pH 7.6), 75 mmol/L KCl, 1 mmol/L DTT, 5% glycerol, 10 mmol/L MgCl2, 4 mmol/L ATP, 20 μmol/L each of dATP, dGTP, dTTP, and dCTP, and 3.4 ng/μL APE1. Reactions were initiated by adding Pol β and TRF2; amounts were as indicated in the figure legends. The reactions were incubated for 25 minutes at 37°C and terminated by adding stop dye. After heating at 95°C, the reactions were run on 20% denaturing polyacrylamide gels and visualized using a Phosphorimager and quantitated with ImageQuant (Molecular Dynamics). Under these reaction conditions, the incised abasic site produced by the sequential actions of OGG1 and APE1 was ~90% for both substrates (data not shown). Percent of each product with a given number of nucleotides incorporated was calculated as follows: % of total = (amount of radioactivity associated with each dNMP addition / total radioactivity) × 100. Values were corrected for background in the no enzyme control.

**Results**

**TRF2 interacts with proteins that function in BER.** Individual members of the network of proteins that act in BER interact both physically and functionally with other members (reviewed in ref. 24). To screen for interactions between telomeric protein TRF2 and select key factors in BER of oxidative DNA damage, a nitrocellulose membrane was spotted with various concentrations (in replicate) of purified recombinant human proteins: p53, PARP1, WRN, FEN-1, Pol β, and hOGG1 (Fig. 1A). Recombinant human TRF1 was blotted as a negative control because TRF2 does not interact with TRF1 and recombinant human TRF2 served as a positive control (6). The spotted membrane was blocked with BSA.
and then incubated with TRF2 in binding buffer (see Materials and Methods). Western blot detection of TRF2 indicated that WRN and Pol β bound to TRF2 and revealed FEN-1 as a novel TRF2 interacting protein (Fig. 1A). No interaction with TRF1 was detected as expected. Increasing protein (up to 50 ng) did not yield any additional binding partners in this assay (data not shown). However, because this assay screened select proteins, the possibility that TRF2 interacts with other BER proteins remains open.

Next, we determined whether TRF2 interacts with Pol β and FEN-1 in human cells. The incubation of HeLa whole-cell extracts with an antibody against TRF2 specifically immunoprecipitated endogenous TRF2 as expected (Fig. 1B and C, lane 3, bottom) but also coprecipitated endogenous Pol β and FEN-1 (Fig. 1B and C, respectively, lane 3, top). In contrast, IgG alone did not precipitate any significant amounts of TRF2, Pol β, or FEN-1 [Fig. 1B (lane 4) and C (lane 1)].

To map the site(s) of protein interaction between TRF2 and either Pol β or FEN-1, a battery of recombinant TRF2 protein fragments (Fig. 2A and B) was used in binding assays. The fragments were expressed as GST fusion proteins, bound to glutathione beads, and then mixed with HeLa nuclear extracts in the presence of DNase I to prevent DNA-mediated interactions. Precipitates were analyzed by SDS-PAGE and Western blot. Neither Pol β nor FEN-1 was precipitated by GST protein alone (Fig. 2C, lane 2). However, both proteins precipitated with the TRF2 fragment lacking the NH2-terminal domain (ΔB; lane 3) but not with the fragment lacking both the NH2 terminus and the COOH-terminal domain that binds telomeric DNA (ΔBΔM; lane 4). The TRF2 NH2-terminal fragment (amino acids 1-45) precipitated both proteins (lane 5). These results suggest that Pol β and FEN-1 bind to the TRF2 NH2-terminal and COOH-terminal domains. Consistent with this, the TRF2 COOH-terminal fragment (amino acids 437-501) was sufficient to precipitate both Pol β and FEN-1 (lane 8). In addition, Pol β bound weakly to the fragment containing only the TRF2 dimerization domain (lane 6). This fragment may reveal an interaction site that is masked in the TRF2 ΔBΔM fragment. In summary, Pol β and FEN-1 bind primarily to the NH2-terminal and COOH-terminal domains of TRF2.

ELISA was done to compare the affinities of TRF2 for Pol β and FEN-1. Wells of microtiter dishes were coated with BSA (control), Pol β, or FEN-1, followed by addition of full-length TRF2 protein. Bound TRF2 was detected using a specific antibody and colorimetric analysis (see Materials and Methods). TRF2 addition to wells coated with BSA yielded an A490 nm of 0.040 ± 0.003, similar to BSA alone (0.039 ± 0.002), indicating no significant TRF2 binding to BSA as seen previously (16). The colorimetric signal increased as a function of increasing TRF2 concentrations for both FEN-1 (Fig. 3A) and Pol β (Fig. 3C) and began to plateau at ~5 nmol/L TRF2. The calculated apparent dissociation constants (Kd) for TRF2-FEN-1 and TRF2-Pol β binding were 1.4 and 1.7 nmol/L, respectively, indicating similar and relatively strong binding affinities as measured by ELISA. For comparison, the apparent Kd for the TRF2-WRN interaction measured by ELISA was 2.3 nmol/L (16). To ensure that the interactions were not mediated by DNA, we tested a point in the linear portion of the curve (1.6 nmol/L TRF2) for binding in the presence of DNase I or ethidium bromide. Neither reagent significantly altered the signal obtained for TRF2 binding to Pol β or FEN-1 (Fig. 3B and D).

FEN-1 incision of 5’ flaps in the presence of TRF2. Next, we determined whether TRF2 influences FEN-1 endonuclease activity. FEN-1 incubation with a 10-nt 5’ flap nontelomeric substrate generated the expected incised 10-nt product (Fig. 4A, lane 2). The addition of up to a 7.5-fold molar excess of TRF2 did not significantly alter the yield of FEN-1 incised product (lanes 3-7). Similar results were obtained with a 1-nt flap structure (data not shown). To determine whether TRF2 binding to the substrate would influence FEN-1 activity, a 15-nt 5’ flap substrate with (TTAGGG)4 sequence in the duplex region 5’ to the flap (10 nmol/L) FEN-1 was incubated alone (lane 2) or together with increasing TRF2 concentrations (9, 18, 90, 180, and 900 pmol/L; lanes 3-7, respectively) at 37°C for 10 minutes. The relative percent incision activity was calculated as described in Materials and Methods and normalized to the FEN-1 alone control (lane 2). Values represent the average and SD of at least three independent experiments. B, FEN-1 incision of a telomeric flap substrate. Reactions contained 10 pmol/L FEN-1 incubated with a 15-nt flap substrate harboring telomeric sequence 5’ to the flap (10 nmol/L). FEN-1 was incubated alone (lane 2) or together with increasing TRF2 concentrations (100, 300, and 1,000 pmol/L; lanes 3-5) at 37°C for 10 minutes. The relative percent incision activity was calculated as in (A).

**Figure 4.** FEN-1 incision activity in the presence of TRF2. A, FEN-1 incision of a 10-nt flap substrate. Reactions contained 120 pmol/L FEN-1 incubated with a 10-nt flap substrate (100 nmol/L) either alone (lane 2) or together with increasing TRF2 concentrations (9, 18, 90, 180, or 900 pmol/L; lanes 3-7, respectively) at 37°C for 10 minutes. The relative percent incision activity was calculated as described in Materials and Methods and normalized to the FEN-1 alone control (lane 2). Values represent the average and SD of at least three independent experiments. B, FEN-1 incision of a telomeric flap substrate. Reactions contained 10 pmol/L FEN-1 incubated with a 15-nt flap substrate harboring telomeric sequence 5’ to the flap (10 nmol/L). FEN-1 was incubated alone (lane 2) or together with increasing TRF2 concentrations (100, 300, and 1,000 pmol/L; lanes 3-5) at 37°C for 10 minutes. The relative percent incision activity was calculated as in (A).

TRF2 stimulates Pol β primer extension. Next, we determined whether TRF2 modulates Pol β activity by examining primer extension DNA synthesis. A nontelomeric substrate was constructed by annealing a 15-mer primer strand to a 34-mer template (mix15/mix34). Pol β (0.5 nmol/L) extended the majority of the substrates (69%) by 6 to 10 nt and extended a minor population (15%) further...
[up to 15 nt; Fig. 5A (lane 2) and B]. TRF2 addition (0.5-3 nmol/L) caused a shift in the product distribution to longer products (Fig. 5A, lanes 3-5). At the highest TRF2 concentration tested, the percent of substrates extended 6 to 10 nt was decreased to 30% whereas the population of longer products (>11 nt) was increased to 62%; 2.3-fold and 4-fold difference, respectively, compared with Pol β alone (Fig. 5B). In contrast, heat-inactivated TRF2 did not alter Pol β activity (Fig. 5A, lanes 6-7), indicating that the Pol β stimulatory factor was heat labile. Furthermore, TRF2 alone did not alter the substrate (Fig. 5A, lanes 8-9), confirming that TRF2 does not possess inherent primer extension activity nor does the TRF2 protein preparation contain contaminating DNA polymerization activity. These results indicate that TRF2 stimulates nucleotide incorporation by Pol β on a nontelomeric primer/template substrate.

To determine whether TRF2 stimulation of DNA synthesis was specific for Pol β, we tested the ability of human TRF2 to stimulate the bacterial E. coli Klenow DNA polymerase. First, we determined an appropriate Klenow concentration at which polymerase stimulation could be detected (Fig. 5C). Although Klenow is more processive than Pol β, an increase in the percent of primers extended, as well as the proportion of longer products, could be detected as a function of Klenow concentration. At 0.32 nmol/L Klenow polymerase, increasing TRF2 concentrations (0.32-3.8 nmol/L) had no detectable effect on Klenow primer extension (Fig. 5D, lanes 1-6). In contrast, reactions done in the same experiment showed that TRF2 stimulated Pol β primer extension (Fig. 5D, lanes 7-8). These results attest to the specificity of the TRF2 stimulation of DNA synthesis for Pol β.

TRF2 stimulation of Pol β on telomeric substrates. Because TRF2 binds duplex telomeric DNA, we asked if TRF2 influenced Pol β activity on a primer/template substrate containing telomeric DNA. For this, a 15-mer primer was annealed to the sequence (AATCCC)₃ in the single-strand region (mix15/tel34) so that Pol β DNA synthesis would generate the telomeric duplex binding sites for TRF2. We repeated Pol β primer extension of the mix15/mix34 substrate in a side-by-side reaction with the mix15/tel34 substrate (Fig. 6) using less Pol β (0.25 nmol/L) to enhance the ability to detect stimulation. Whereas mix15/mix34 was a better substrate for Pol β DNA synthesis compared with mix15/tel34 (85% versus 21% extended, as well as the proportion of longer products, could be detected as a function of Klenow concentration. At 0.32 nmol/L Klenow polymerase, increasing TRF2 concentrations (0.32-3.8 nmol/L) had no detectable effect on Klenow primer extension (Fig. 5D, lanes 1-6). In contrast, reactions done in the same experiment showed that TRF2 stimulated Pol β primer extension (Fig. 5D, lanes 7-8). These results attest to the specificity of the TRF2 stimulation of DNA synthesis for Pol β.

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TRF2 stimulates Pol β strand displacement DNA synthesis on BER substrates. Next, we asked whether TRF2 modulated Pol β activity in the context of BER. For this, we reconstituted a portion of the repair reaction using a 34-bp nontelomeric substrate containing an 8-oxo-guanine residue at position 17 (Fig. 8A). OGG1 removes the 8-oxo-guanine residue, leaving an apurinic/apyrimidinic site. The DNA strand is cleaved at the apurinic/apyrimidinic site either on the 3′ side by the OGG1 lyase activity or on the 5′ side by APE1 activity (24). APE1 was added to all the reactions either to remove the 3′ aldehyde produced by the OGG1 lyase activity or to increase the percent of 5′ incised substrates. The incubation of the BER substrate labeled at the 5′ end of the 8-oxo-guanine–containing strand with OGG1 and APE1 generated an incised substrate that migrated as a 16-mer fragment on a denaturing gel (Fig. 8B, lane 1). Up to 90% of the substrates were incised (data not shown). Next, we examined

Figure 6. Comparison of TRF2 stimulation of Pol β on telomeric and nontelomeric template substrates. A, reactions contained Pol β (0.25 nmol/L) alone or together with increasing TRF2 concentrations (0.125, 0.25, 0.75, and 1.5 nmol/L). Reactions were initiated by adding 25 nmol/L substrate with either nontelomeric template sequence (mix15/mix34; lanes 1-6) or telomeric template sequence (mix15/tel34; lanes 7-12) and were incubated for 15 minutes at 37°C, followed by analysis on a 20% denaturing gel. Quantitation and calculation of primer extension products for the nontelomeric (B) or telomeric (C) template substrates was as described in Materials and Methods. Columns, mean from three independent experiments; bars, SD.
nucleotide incorporation with increasing Pol β concentrations (Fig. 8B, lanes 2-5). Without the addition of DNA ligase, a 17-mer product represents single nucleotide incorporation and short-patch BER. Longer products represent Pol β strand displacement DNA synthesis and long-patch BER intermediates. In agreement with previous results (37, 40), Pol β primarily added single nucleotide gap filling (Fig. 8D). However, limited strand displacement DNA synthesis was observed as a function of Pol β concentration (Fig. 8B, lanes 2-5). Adding a 6:1 molar ratio of TRF2/Pol β resulted in an increase in the proportion of longer products at each concentration tested (compare lanes 2 and 6, 3 and 7, 4 and 8, then 5 and 9). Furthermore, stimulation of strand displacement DNA synthesis was also observed at the lower molar ratios of 1:1 and 3:1 TRF2/Pol β (Fig. 8C). Quantitation of the reactions with 5 nmol/L Pol β and 30 nmol/L TRF2 (Fig. 8B and C) indicate that TRF2 addition decreased the percent of short-patch BER intermediates from 26% to 6% and increased the long-patch BER intermediates (2-6 nt added) from 56% to 85% (Fig. 8D). Analysis of the individual long-patch BER bands shows that TRF2 addition increased the percent of the longest products (extended 5 and 6 nt) by 7- and 6-fold, respectively, whereas the percent of substrates extended by only 2 nt decreased (Fig. 8E). These results indicate that TRF2 stimulates Pol β strand displacement DNA synthesis on nontelomeric BER substrates.

To examine whether TRF2 influences Pol β-mediated BER on a telomeric substrate which could be bound by TRF2, we reconstituted the BER reactions with a 39-bp substrate containing an 8-oxo-guanine residue at position 17 within two tandem telomeric repeats (Fig. 9A). Similar to the nontelomeric substrate, Pol β primarily added a single nucleotide, and limited strand displacement DNA synthesis was observed as a function of Pol β concentration (Fig. 9B, lanes 2-5). TRF2 addition resulted in the appearance of longer products at each concentration tested (compare lanes 2-5 and lanes 6-9, respectively). Up to 4 to 5 additional nucleotides were added in the presence of TRF2 compared with Pol β alone. At 1.2 nmol/L Pol β, the degree of stimulation achieved by TRF2 (Fig. 9C, lane 3) was comparable to that achieved with FEN-1 (lane 5), an enzyme previously reported to stimulate Pol β strand displacement DNA synthesis (41). Heat-inactivated TRF2 had no detectable effect on Pol β (Fig. 9C, lane 4). TRF2 increased Pol β long-patch BER intermediates (2-7 nt added) from 6% to 16% in the presence of TRF2 (2.7-fold increase; Fig. 9D). Analysis of the individual long-patch BER products indicates a 6.6-fold increase in the longest detectable product in the presence of TRF2; products extended 7 nt increased from 0.3% to 2% (Fig. 9E). Adding both TRF2 and FEN-1 did not result in a synergistic or additive level of stimulation, in contrast to previous reports with WRN and FEN-1 (37). In summary, TRF2 increased the proportion of Pol β generated long products (up to 7-fold) on both nontelomeric and telomeric BER substrates. TRF2 may particularly enhance the repair of damaged bases that required the long-patch BER pathway in vivo.

TRF2 promotes Pol β strand displacement DNA synthesis on a telomeric D-loop substrate. To determine whether the stimulation of Pol β DNA strand displacement synthesis by TRF2 was unique to BEB substrates, we tested another telomeric substrate that requires strand displacement synthesis for primer extension. Telomeres can form lasso-like structures in which the 3’ telomeric tail invades the telomeric duplex region forming a large t-loop that is stabilized by an intratelomeric D-loop. Fotiadou et al. (27) proposed that Pol β may function with TRF2 in remodeling the telomeric t-loop/D-loop structures, perhaps by extending the 3’ invading tail. We constructed a model telomeric D-loop in vitro, which consisted of a bubble with two 33-bp duplex arms and a 33-bp melted region in which an invading strand that mimics the 3’ telomeric tail was hybridized (ref. 17; Fig. 10). Pol β initiated DNA synthesis at the 3’ OH of the invading strand and increased the percent of primers extended in a dose-dependent manner (Fig. 10, lanes 2-5). The 3’ tail was extended up to 3 nt. More importantly, TRF2 addition increased the percent of primers extended, as well as the product lengths, at each concentration tested (Fig. 10, compare lanes 2-5 with lanes 6-9). Thus, TRF2 promotes Pol β strand displacement DNA synthesis on two distinctly different telomeric substrates.

Discussion

Relationships between proteins that function in DNA double-strand break repair and telomeric proteins are well documented in the literature. Such studies indicate roles for double-strand break repair proteins in telomere maintenance and conversely support potential roles for TRF2 in general double-strand break repair (reviewed in ref. 11). Here we report that interactions between telomeric proteins and repair factors extend to proteins involved in dealing with DNA base damage. A screen for TRF2
interactions with proteins involved in BER of oxidative damage confirmed interactions with Pol β and WRN and identified FEN-1 as a novel binding partner. Human Pol β and FEN-1 interact with TRF2 both in vivo by immunoprecipitation and in vitro by binding assays with purified proteins. TRF2 stimulated Pol β DNA synthesis on a variety of substrates including telomeric and nontelomeric primer/template and BER substrates and telomeric D-loops. These findings strongly support a role for TRF2 in common DNA metabolic pathway(s) with Pol β, as well as other BER factors.

Mouse TRF2 was previously detected in the Pol β immunoprecipitates from murine cells overexpressing Pol β (27). Here we showed that endogenous human Pol β was in TRF2 immunoprecipitates from HeLa whole-cell extracts (Fig. 1A), thereby extending the relevance of this interaction to human cells and to endogenous protein levels. Yeast two-hybrid data show that the Pol β 31-kDa domain binds the ΔBTRF2 fragment, which lacks the TRF2 NH2 terminus (27). Our experiments with TRF2 fragments revealed that the same ΔBTRF2 fragment precipitated endogenous Pol β from HeLa extracts (Fig. 2). In addition, we observed binding to fragments containing only the NH2-terminal or the COOH-terminal domain, which were not tested previously. These were also the primary binding sites for FEN-1 (Fig. 2). Given that the COOH-terminal domain contains the telomeric DNA binding domain, Pol β and/or FEN-1 binding to TRF2 may potentially interfere with TRF2 binding to telomeric DNA. Consistent with this, Pol β overexpressed in mouse cells colocalized with TRF2 outside of the telomeres and induced telomere dysfunction (27), indicating potential TRF2 displacement from telomeres.

Our data provide functional significance for the physical interaction between TRF2 and Pol β. TRF2 consistently and repeatedly stimulated Pol β DNA synthesis on a variety of DNA substrates, including nontelomeric ones (Figs. 5–10). In contrast, TRF2 failed to stimulate the bacterial polymerase Klenow, suggesting the stimulation is specific to Pol β. WRN, FEN-1, and PARP1 proteins also promote Pol β DNA synthesis; however, the mechanisms are distinct from that of TRF2. First, these proteins specifically enhance Pol β strand displacement DNA synthesis; thus, stimulation was only detected in the presence of a downstream oligonucleotide and not with primer/template substrates (37). In contrast, TRF2 stimulated Pol β DNA synthesis on substrates with a downstream oligonucleotide (BER and D-loop; Figs. 8–10) as well as on those without (primer/template; Figs. 5–7).
Second, WRN and FEN-1 stimulate Pol λ by converting the substrate and by removing obstacles to DNA synthesis. WRN unwinds the duplex region downstream from the nick, thus removing the barrier to Pol λ DNA synthesis (37). We have not detected any strand displacement activity by TRF2 in standard helicase assays with forked duplexes or D-loops (16, 17). FEN-1 promotes Pol λ indirectly by stimulating FEN-1 activity (40). Because TRF2 lacks nuclease and helicase activities, the stimulation of Pol λ must occur via a novel mechanism. Substantial stimulation was observed even on substrates that lacked telomeric sequence, indicating that TRF2 binding to the substrate is not required for optimal stimulation. Thus, TRF2 may modulate Pol λ activity via a direct protein-protein interaction with the enzyme.

Numerous BER and DNA replication proteins interact with both Pol λ and FEN-1, including APE1, PCNA, WRN, PARP1, DNA ligase I, XRCC1, and p53 (reviewed in ref. 24). Here we report that TRF2 is among these proteins. The novel finding that TRF2 interacts with FEN-1 in cellular extracts and in vitro (Figs. 1–Figs. 3) lends further support to a role for TRF2 in a BER protein network. Among these proteins, WRN and APE1 stimulate both Pol λ (37, 43) and FEN-1 (44, 45). Whereas TRF2 stimulated Pol λ, we did not observe any obvious modulation of FEN-1 activity by TRF2, at least under the conditions tested. However, it is noteworthy that a 100-fold molar excess of TRF2 did not interfere with FEN-1 cleavage of a telomeric substrate that TRF2 could bind to (Fig. 4 B). This is consistent with our previous finding that TRF2 does not interfere with APE1 activity on a telomeric substrate (26). Therefore, these data collectively support a role for TRF2 in facilitating BER, in contrast to previous reports which indicate that TRF2 inhibits double-strand break repair at the telomeres (21, 22).

What might be the physiologic role(s) for TRF2 interactions with BER proteins? One possibility is that TRF2 may up-regulate BER at the telomeric ends by interacting with key BER factors such as Pol λ and FEN-1. An accumulation of lesions and mutations in the telomeres may contribute to telomere dysfunction and, consequently, genomic instability. Numerous studies have reported increased telomere erosion/loss in human primary fibroblasts in response to mild chronic oxidative stress induced by a variety of factors (25, 30, 46–48). Oxidizing and alkylating agents induce a high density of single-strand breaks in telomeric

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**Figure 9.** TRF2 stimulation of Pol λ on telomeric BER substrates. A, a schematic of the 39-bp DNA substrate containing an 8-oxo-guanine at position 17 within two tandem telomeric repeats is shown both before and after treatment with OGG1 and APE1. B, the substrate was pretreated with OGG1 (128 nmol/L) for 20 minutes at 37°C. The pretreated DNA (100 nmol/L) was incubated with 3.4 ng/μL APE1 for 25 minutes at 37°C together with increasing Pol λ concentrations (0.62, 1.2, 2.5, and 5 nmol/L; lanes 2-5 and 6-9, respectively). The reactions in lanes 6 to 9 also contained increasing TRF2 concentrations (3.7, 7.5, 15, and 30 nmol/L, respectively). C, the OGG1-pretreated DNA substrate was incubated with 3.4 ng/μL APE1 in the presence (+) or absence (−) of Pol λ (1.2 nmol/L), TRF2 (7.5 nmol/L), or FEN-1 (30 nmol/L) as indicated. ▲, heat-inactivated control. D, quantitation of unreacted substrate (0), short-patch (1), and long-patch (2-7) BER intermediates. E, quantitation of individual long-patch BER intermediates. Reaction products were calculated as a function of total radioactivity as described in Materials and Methods for reactions containing 1.2 nmol/L Pol λ alone (solid columns) or together with 7.5 nmol/L TRF2 (hatched columns). Columns, mean of three independent experiments; bars, SD.
The telomeric D-loop substrate (25 nmol/L) was incubated with increasing Pol β concentrations alone (0.62, 1.2, 2.5, and 5 nmol/L; lanes 2-5) or together with increasing TRF2 concentrations (3.7, 7.5, 15, and 30 nmol/L; lanes 6-9). The reactions were initiated by adding substrate and were incubated for 15 minutes at 37°C. The reaction products were run on a 20% denaturing gel and were visualized by a Phosphorimager.

Figure 10. TRF2 enhances Pol β extension of the 3’ tail of a telomeric D-loop. The telomeric D-loop substrate (25 nmol/L) was incubated with increasing Pol β concentrations alone (0.62, 1.2, 2.5, and 5 nmol/L; lanes 6-9) or together with increasing TRF2 concentrations (3.7, 7.5, 15, and 30 nmol/L; lanes 2-5). The reactions were initiated by adding substrate and were incubated for 15 minutes at 37°C. The reaction products were run on a 20% denaturing gel and were visualized by a Phosphorimager.

DNA (29), and we recently reported that 8-oxo-guanine and abasic lesions disrupt binding by TRF1 and TRF2 to telomeric DNA substrates (26). These studies indicate that repair of modified bases in the telomeres is likely to be critical for the optimal association of telomeric proteins and formation of the protective cap. Conversely, TRF2 may also participate in BER and cellular responses to base damage in genomic regions outside the telomeres. Consistent with this, TRF2 rapidly localizes to sites in the nuclei that were targeted with UVA light (13). Because this treatment induces double-strand breaks, TRF2 was proposed to localize to sites of double-strand breaks. However, UVA also induces base oxidation (30, 49), which may have also occurred in this system. Furthermore, TRF2 and Pol β were reported to colocalize in nuclear foci outside the telomeres (27). Our finding that TRF2 stimulates Pol β on nontelomeric BER substrates further supports the notion that TRF2 may cooperate with BER proteins in genomic regions outside of the telomeres.

Another nonmutually exclusive possibility is that TRF2 may function with BER proteins in remodeling telomeric ends to facilitate DNA replication and/or repair processes. Telomerase-independent models for telomere extension have been proposed whereby a polymerase engages the 3’ OH of the invading strand of the telomeric D-loop and performs strand displacement DNA synthesis (reviewed in ref. 50). Consistent with this, we found that TRF2 stimulated Pol β extension of the 3’ telomeric tail of a telomeric D-loop in vitro (Fig. 10). Such a cooperative event may prime the 3’ tail for a “hand off” to a more processive polymerase, similar to what has been proposed for long-patch BER (51). FEN-1, Pol β, and TRF2 interact physically and functionally with the WRN RecQ helicase, which dissociates model telomeric ends in vitro (17). These enzymes may cooperate in pathways at telomeric ends during repair and/or replication.

In summary, our data support the interaction of TRF2 with a network of key proteins involved in the repair of DNA base damage or BER. TRF2 stimulates Pol β DNA synthesis on a variety of telomeric and nontelomeric DNA substrates, including BER substrates and a telomeric D-loop. These findings have implications for the potential roles of TRF2 in cooperation with BER factors in repair pathways at the telomeres and, possibly, in general repair. Given the critical roles for telomere function and DNA repair in maintaining genomic integrity, cooperation among the factors involved in these processes may be critical in preventing cancer development and progression.

Acknowledgments


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We thank Lale Davat for technical assistance and Dr. Nadja Souza-Pinto for critical reading of the manuscript.

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