Taz1 Binding to a Fission Yeast Model Telomere

FORMATION OF TELOMERIC LOOPS AND HIGHER ORDER STRUCTURES*

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Similar to its human homologues TRF1 and TRF2, fission yeast Taz1 protein is a component of telomeric chromatin regulating proper telomere maintenance. As mammalian TRF1 and TRF2 proteins have been shown to directly bind telomeric DNA to form protein arrays and looped structures, termed t-loops, the ability of Taz1p to act on fission yeast telomeric DNA in similar ways was examined using purified protein and model DNA templates. When incubated with Taz1p, model telomeres containing 3′-single-stranded telomeric overhangs formed t-loops at a frequency approaching 13%. Termini with blunt ends and non-telomeric overhangs were deficient in t-loop formation. In addition, we observed arrays of multiple Taz1p molecules bound to the telomeric regions, resembling the pattern of TRF1 binding. The presence of t-loops larger than the telomeric tract, a high frequency of end-bound DNAs and a donut shape of the Taz1p complex suggest that Taz1p binds the 3′ overhang then extrudes a loop that grows in size as the donut slides along the duplex DNA. Based on these in vitro results we discuss possible general implications for fission yeast telomere dynamics.

Telomeres, the DNA-protein complexes at the ends of linear chromosomes, stabilize the termini and protect them from end-to-end fusion. With a few exceptions, telomeres of most eukaryotic cells consist of an array of short repeats rich in guanines on the strand running 5′ to 3′ toward the chromosome end. Another general feature of nuclear telomeres is the presence of a 3′ overhang of the G-rich strand at the termini (reviewed in Ref. 1). It has been suggested that telomeres exist in at least two different states (2), they “open” to allow telomerase to access the end of the telomeric DNA and then “close” to protect chromosome ends from unwanted recombination and mask them from the double strand break repair systems. Changes from open to closed states would be mediated by specific protein components of the telomeric chromatin (3, 4).

Telomeric loops (t-loops) formed by an inversion of the 3′ single-stranded (ss) overhang of mammalian telomeres into the duplex telomeric region (5) were suggested to hide the natural end of the chromosome and presumably reflect the closed state of the telomere. T-loops were subsequently found at the termini of micronuclear chromosomes of Oxytricha nova (6), at the telomeres of Trypanosoma brucei (7) and Pismum sativum (8), as well as at the ends of linear mitochondrial DNA of the yeast Candida parapsilosis (9), suggesting that they may represent an evolutionary ancient means of telomere maintenance (10). Recently Nikitina and Woodcock (11) demonstrated that mouse and chicken telomeres could be visualized in a t-loop structure following gentle isolation of the telomeres in a chromatinized state.

In the original study of mammalian t-loops it was found that the formation of loops is mediated by TRF2 protein (5). TRF2 and its homologue TRF1 were identified as proteins that bind double-stranded (ds) telomeric DNA as homodimers (12–14). Multiprotein complexes built around TRF1 and TRF2 have recently been identified with the TRF1 complex being implicated in telomere length regulation and the TRF2 complex in telomere protection (4, 15). The TRF-associated proteins include Tin2 (16), tankyrase (17), hRap1 (18), Werner and Bloom syndrome helicases (19), DNA polymerase β (20), and Pot1 and Pp1 (21).

Although TRF1 and TRF2 exhibit a high degree of homology, their role in telomere dynamics seems to be different, probably because of the differences at their N termini (22). In contrast to TRF2, TRF1 is unable to promote t-loop formation in vitro. Whereas TRF1 forms filamentous structures on telomeric repeat arrays and promotes parallel pairing of telomeric tracts in vitro (23), TRF2 induces t-loop formation and binds preferentially to the ss/ds junction at the 3′ telomeric overhang (24). A 5′-TTAGGG-3′ overhang of at least six nucleotides is required for loop formation. Termini with 5′ overhangs or blunt-ended ends are deficient in loop formation.

Saccharomyces cerevisiae with its powerful genetic and molecular biologic tools would seem to offer a valuable system for the detailed analysis of t-loop formation. Indirect genetic evidence suggests that telomeres in S. cerevisiae appear to form fold-back structures thus emphasizing telomere looping as a common theme in telomere architecture (25–28). However, it seems unlikely that the budding yeast telomeres form “true” t-loops. First, telomeric sequences in S. cerevisiae are very heterogeneous (C2–3ACA1–6/T1–6GTG2–3) (29, 30), which would decrease the probability that the 3′ telomeric overhang could invade into the double-stranded telomeric region based on base complementarity. In addition, the budding yeast genome does not contain a gene encoding a TRF-like telomeric protein. Rap1 protein contains its own Myb domain and seems to fulfill functions as ds, double-stranded; Ni-NTA, nickel-nitrilotriacetic acid; nt, nucleotide(s).
cells. Deletion of taz1 telomeric chromatin (41). Telomeric repeats thus emphasizing its role in inheritance of telomeres even in the absence of which is essential for cell cycle progression at 20 °C, a temperature not dependent on homologous recombination (42). Taz1p is also involved in Ku-dependent nonhomologous recombination and are type under stress-free conditions, higher levels of homology with hTRF2 (37). Homologous to hTRF1, whereas other regions of Taz1p show Myb domain and TRF homology domain of Taz1p are more strongly conserved with hTRF1 and hTRF2 is consistent with the observation that the myb domain and TRF homology domain of Taz1p are more homologous to hTRF1, whereas other regions of Taz1p show higher levels of homology with hTRF2 (37).

Disruption of the taz1 gene in S. pombe is not lethal and taz1 haploids grow vegetatively at the same rate as wild-type cells. Deletion of taz1 causes a roughly 10-fold increase in telomere length (35). However, despite the changes in telomere length, taz1 cells did not exhibit a decrease in viability after extensive subculturing (35). In contrast to vegetative growth, sexual reproduction of taz1 cells is aberrant because of defective meiosis (35). Detailed analysis of the role of Taz1p in meiotic division revealed that it is necessary for telomere aggregation adjacent to the spindle pole body during meiotic prophase. In the absence of Taz1p, telomere clustering at the spindle pole bodies is disrupted, meiotic recombination is reduced (3–10-fold), and both spore viability and the ability of zygotes to re-enter mitosis is impaired (38–40).

In addition to its role in meiosis, Taz1p is required for the repression of telomere-adjacent gene expression, most likely by establishing or maintaining a telosome structure (35). This hypothesis is supported by the observation that mating of taz1 and taz1 cells results in diploids, in which the telomeres remain longer than normal for many generations, although the telomere position effect is quickly restored. Importantly, Taz1p remains associated with subtelomeres even in the absence of telomeric repeats thus emphasizing its role in inheritance of telomeric chromatin (41).

Although the mitotic growth of taz1 cells is similar to wild-type under stress-free conditions, taz1 cells exhibit lethal telomere fusions when subjected to nitrogen starvation inducing an uncommitted G1 state (42). The fusions are mediated by the Ku-dependent nonhomologous end joining pathway and are not dependent on homologous recombination (42). Taz1p is also essential for cell cycle progression at 20 °C, a temperature at which taz1 mutants exhibit a G2/M checkpoint delay, chromosome missegregation, and double-strand DNA breaks (43). Thus, analogous to mammalian TRF2 protein, Taz1p helps provide a telomeric cap that prevents Ku from recognizing telomeres as double-strand breaks.

**Experimental Procedures**

**Enzymes and Oligonucleotides—** Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase, calf intestinal phosphatase, and the Klenow fragment of *Escherichia coli* DNA polymerase I were from New England Biolabs (Beverly, MA) and were used according to the manufacturer's instructions. [γ-32P]ATP (3000 Ci/mm) was from ICN Biomedicals (Irvine, CA). Oligonucleotides were purchased from MWG Biotech Inc. (Greensboro, NC) or Invitrogen (Carlsbad, CA) and either contained a 5′ phosphate, or were phosphorylated by T4 polynucleotide kinase and unincorporated nucleotides were removed using a QiAquick nucleotide removal kit (Qiagen).

**Construction of S. pombe Model Telomere DNA—** A plasmid containing a block of fission yeast consensus telomere repeats 5′-GGTTACA-3′ flanked by asymmetric restriction sites for BbsI and BamHI was constructed using the approach of Stansel et al. (24). Several cycles of telomeric track expansion resulted in a plasmid (pLT500) containing 74 telomeric repeats (516 bp). All plasmid constructs were propagated in *Escherichia coli* Sure2 cells (Stratagene, La Jolla, CA).

**Ligation of Oligonucleotide Tails to pLT500—** The plasmid pLT500 was digested with BbsI and BamHI and dephosphorylated using calf intestinal phosphatase. Ligation reaction mixtures contained 3 μg of the linearized plasmid, a 5-fold molar excess of oligonucleotide, and 400 units of T4 DNA ligase in 50 μL Tris-HCl, pH 7.5, 10 μM MgCl2, 10 mM dithiothreitol, 1 mM ATP, 25 μg/ml bovine serum albumin, and T4 DNA ligase (400 units). The free oligonucleotides were removed using a QiAquick PCR purification kit (Qiagen).

**Purification of Recombinant Taz1p-His (Further Taz1p)—** The bacterial strain overproducing Taz1p with an N-terminal His tag (pQE30 vector, Qiagen) and protocol for its purification on Ni-NTA Superflow agarose were provided by Dr. Julia P. Cooper (Cancer Research Institute, London, United Kingdom). For some preparations, Talon™ Metal affinity resin (Clontech, Palo Alto, CA) was employed instead of Ni-NTA. Typically, a protein extract from a 1-liter culture was loaded on 1 ml of the beads and the bound proteins were eluted stepwise with increasing concentrations (50–250 mM) of imidazole. The Taz1p containing fractions were dialyzed using a Slide-A-Lyzer cassette (Pierce) against 1 liter of 20 mM HEPES-NaOH, pH 7.5, 20% glycerol, 100 mM NaCl, 1 μg/ml leupeptin and stored at −20 °C. The fractions eluted from the nickel column contained two major bands migrating at apparent molecular weights of 90 and 70 kDa based on SDS-PAGE on a 10% gel (45). The two forms of Taz1p were separated by reloading the sample on the Ni-NTA column followed by elution with a gradient of 50–250 mM imidazole.

**Mass Spectrometry—** The precise molecular weights of Taz1p and a C-terminal truncated form were determined by Dr. Christoph Borchert in the University of North Carolina Michael Hooker Proteomics Core Facility using an Electrospray Ionization Quadrupole Time-of-flight Mass Spectrometer following in-gel tryptic digestion of the proteins (46).

**Immunoblot Analysis—** Proteins were separated by SDS-PAGE and electrotransferred to a nitrocellulose filter (BA85, Schleicher & Schuell) in a buffer containing 25 mM Tris, 192 mM glycine, pH 8.3, 20% meth-

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anol for 60 min at 250 mA. Probing with the anti-penta(His) antibodies (Qiagen) and subsequent chemiluminescent assay using the BM Chemiluminescence Western blotting Kit (Roche Diagnostics) were performed according to the corresponding manufacturer’s instructions.

**Results**

Production of Full-length and C-terminal Truncated Taz1p—The fractions eluted from the nickel column contained two major bands (Fig. 1A, lane 1). Mass spectrometry analysis (“Experimental Procedures”) revealed that the higher molecular weight protein corresponds to full-length Taz1 (with the hexahistidine tag), whereas the lower molecular weight species corresponds to Taz1p truncated at its C terminus (Taz1pΔC). This truncation is likely because of premature transcription termination, as coupled transcription/translation of the Taz1 gene from a PCR template yielded the same two forms and the proportion of the truncated form did not increase with time, arguing against a possibility that it is a result of specific proteolytic cleavage of the full-length Taz1p (data not shown). The truncation eliminates 107 amino acids including almost the entire Myb DNA-binding domain that is analogous to a dominant negative mutant of TRF2 (Fig. 1B). It would be of interest to assess a possible physiological role of regulation of Taz1p activity by producing the two forms individually in *S. pombe*.

Subjecting the mixture of Taz1p and Taz1pΔC to gel filtration on Superose 6 did not result in separation of the two forms (Fig. 1C). However, we observed that the protein eluted from the column together with apoferritin (440 kDa), indicating that in its native state Taz1p forms a trimer of dimers (3 × (2 × 75) = 450; see also the section “Taz1p Forms Oligomeric Donut Structures”). As the presence of Taz1pΔC in the multimeric complex may change its DNA binding properties, we developed conditions for enrichment of either of the Taz1p forms (Fig. 1A, lanes 2 and 3; see “Experimental Procedures”). Testing both forms by gel-retardation assays using a DNA template carrying 18 fission yeast telomeric repeats demonstrated that in contrast to the full-length Taz1p, Taz1pΔC is unable to form a complex with the probe (Fig. 1D).

**Generation of Model Telomere**—To directly visualize Taz1p interactions with telomeric DNA in *vitro*, a model DNA containing a fixed number of 5′-GGTTACA-3′ repeats was created by exposure of cloning (see “Experimental Procedures” and Ref. 24). The final plasmid construct (pLT500) carries 74 telomeric repeats (518 bp), which is about twice as long as the telomeric tract on the fission yeast chromosomes (Fig. 2). Although the strategy also yielded a plasmid with 38 repeats (266 bp) where the length of the array is more similar to that of natural telomeres, pLT500 was chosen for electron microscopic (EM) analysis because longer terminal loops would be more easily visualized by EM. As described for the human model telomere (24), 3′ terminated ss oligonucleotide tails with different lengths and sequence can be easily ligated to the end of the model DNA. Using *E. coli* SSB protein as a marker for the presence of a ssDNA tail, EM analysis revealed that >90% of the model telomere DNA molecules contained as DNA ends.

T-loop Formation on a Model Telomere Mediated by Taz1p—The standard template used for this study contains a 49-nucleotide (nt) 3′ overhang, (GGTTACA)7, ligated to the terminus of the model telomere DNA. This substrate was incubated for 20 min with Taz1p using conditions optimized by EM (“Experimental Procedures”). The presence of NaCl (~100 mM) was required to prevent aggregation of the protein. Concentrations of nonionic detergents such as Triton X-100 even as low as 0.025% dramatically reduced the amount of protein bound. Importantly, inclusion of 10 mM 2-mercaptoethanol into the reaction increases the proportion of molecules with Taz1p bound within the telomeric tract and decreases nonspecific binding.

As seen by EM the DNA was present in a variety of forms. First, a fraction of the linear DNA molecules were present with no protein bound (not shown) and the amount of Taz1p added was adjusted so that this ranged between 10 and 35% of the total. At a ratio of Taz1p to DNA of 1 μg of protein: 1 μg of DNA, the most common DNA species consisted of a model telomere with a protein particle located at the very end of the DNA (Fig. 3A, upper left corner). Although Taz1p was identified as a protein with affinity to the ds telomeric DNA of *S. pombe*, a high fraction of the molecules inspected by EM had Taz1p bound at the very end of the model telomere as opposed to internally along the telomeric tract. These results support a hypothesis that Taz1p prefers a ss/ds telomeric junction over the ds region of the telomere and that this preference might be important for a physiological role(s) of Taz1p, including t-loop formation.

The second major species consisted of model telomeres in which one end was folded back into a loop with a protein complex at the loop junction (t-loops) (Fig. 3). Measurement of the size of the loops revealed that a substantial number of loops exceeded the 500-bp length of the telomeric tract (data not shown). Whereas most of the internally bound Taz1p was...
within 500 bp of an end and thus presumably bound to the telomeric DNA, in some cases Taz1p was present further in from an end and thus along the plasmid sequences. Inspection of the sequence of the pBluescript vector revealed two cryptic Taz1p-binding sites (GGTTAC) starting at positions 44 (direct strand) and 2,708 (complementary strand), respectively. This may account for some of the Taz1p binding in the plasmid sequences as well as for the loops larger than 500 bp.

When Taz1p was allowed to bind to the model telomere DNA in the presence of 2-mercaptoethanol, in more than 50% of the molecules inspected, protein particles formed an array that decorated the telomeric segment (Fig. 4). The protein formed distinct spherical balls distributed along the repeat tracts in a manner indicative of little cooperativity. This binding pattern is similar to that of hTRF1 bound to human telomeric arrays (23). The Taz1p array-covered telomeres often formed a fold-back structure (Fig. 4, A and B) suggesting that TRF1-like binding does not affect the ability of Taz1p to mediate t-loop formation. This observation goes in line with a hypothesis that as the single TRF1/TRF2 homologue, fission yeast Taz1p fulfills the functions of both TRF1 and TRF2 in remodeling the telomere.

When the template with a 49-nt (GGTTACA)7 3’ overhang was incubated with Taz1p in 2-mercaptoethanol containing buffer and 1,500 DNAs were scored 33.8% had a Taz1p particle bound at one end, 13.3% 5.3% had one end folded back into a loop with Taz1p at the loop junction, 23.8% 0.3% had a Taz1p particle bound within the telomeric repeat tract, 23.3% 8.9% were protein-free and 5.9% 1.6% were scored as containing protein bound internally along the plasmid sequences (Table II).

To examine the dependence of Taz1p for its binding to different single-stranded tails on the model telomere, different oligonucleotides (Table I) were ligated to the BsmBI-linearized pLT500. Incubation of these model telomeres with Taz1p was carried out and the DNA-protein species were scored by EM

**FIG. 1.** Full-length and truncated versions of Taz1p. A, examples of fractions containing either both or just one of the Taz1p forms assayed by SDS-PAGE. Lane 1, mixture of Taz1p and Taz1pΔC; lane 2, purified Taz1pΔC; lane 3, purified Taz1p. B, diagram of the full-length Taz1p, Taz1pΔC, and TRF2 (adapted from Ref. 37). Note that Taz1pΔC lacks almost the entire Myb-like domain responsible for DNA binding. TRFH, TRF homology domain. C, a mixture of Taz1p and Taz1pΔC was subjected to fast protein liquid chromatography gel filtration chromatography on Superose 6 ("Experimental Procedures"). The fractions were assayed for the presence of Taz1p by immunoblot using anti-penta(His) antibodies. Positions of the corresponding molecular weight standards are indicated by arrows. D, gel retardation assay for the full-length Taz1p and Taz1pΔC using the linearized plasmid containing 18 fission yeast double-stranded telomeric repeats as a probe ("Experimental Procedures"). Lane 1, negative control; lanes 2–5, Taz1pΔC (250, 750, 1250, and 2500 ng, respectively); lanes 6–9, full-length Taz1p (250, 750, 1250, and 2500 ng, respectively).
**Fig. 3.** Taz1p mediates t-loop formation at the model telomere. Taz1p was incubated with the model telomere DNA and the resulting DNA-protein complexes were prepared for EM by adsorption to thin carbon foils, dehydration, and rotary shadowcasting with tungsten ("Experimental Procedures"). Shown are examples of t-loops and one end-bound molecule (upper left corner in panel A). Shown in reverse contrast, the bar is equivalent to 1000 bp.

**Fig. 4.** Multiple binding of Taz1p along the telomeric tract. Incubation of Taz1p with the model telomere in a buffer containing 10 mM 2-mercaptoethanol resulted in loops covered with protein (A), loops with multiple protein particles at the junction (B), and linear chains of Taz1p covering the length of the telomeric tract including the end (C), or without end binding (D). Samples were prepared for EM as described in the legend to Fig. 3, and are shown in reverse contrast. The bar is equivalent to 1000 bp.
using the criteria as described above (Table II). From these results it is clear that Taz1p requires a ss telomeric overhang for t-loop formation. In contrast to a telomeric overhang containing 7 repeats (49 nt) and 11 repeats (77 nt), respectively, a telomeric overhang containing two repeats (14 nt) and non-telomeric overhangs were unable to promote t-loop formation. In addition, in contrast to the full-length Taz1p, Taz1p/H9004C exhibited very poor binding to the DNA template and only a background level of t-loops was observed (data not shown).

**Taz1p Forms Oligomeric Donut Structures**—Inspection of the Taz1 complexes that formed the t-loops frequently showed a single, large discrete particle at the junction of the loop and the tail (Fig. 3, A, D, F, and G) and often these particles appeared to have a donut-like appearance. A panel of such examples is shown at higher magnification in Fig. 5 (A–D). Indeed inspection of the Taz1 protein free of DNA on the EM substrate revealed many of these donut-like particles and their size was much larger than what would be expected from the known mass of a Taz1p homodimer (150 kDa). Furthermore, when the Taz1p/H9004C preparation was used, long filamentous structures were present that were absent in preparations with the full-length Taz1p (data not shown). To examine the protein by a more gentle preparative method that does not employ chemical fixation or exposure to organic solvents, preparations of Taz1p and Taz1p/H9004C were prepared by glycerol spray/low voltage EM (“Experimental Procedures”). Examination of the samples at 16 kV revealed oligomeric donut-like particles in the Taz1p sample (Fig. 5, E–G). These donuts were not present

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**TABLE I**

**Oligonucleotides**

SpTEL_2, SpTEL_7, and SpTEL_11 contain 2, 7, and 11 consensus telomeric repeats, respectively; SpTEL_R contains a 49-nt random sequence; SpTEL_2R contains 2 telomeric repeats followed by 35-nt random sequence; Non-tel was adopted from Ref. 35; HIS3 was adopted from Ref. 50.

| Name       | Sequence (5′→3′)                          |
|------------|------------------------------------------|
| SpTEL_2    | tacaGGTTACAAGGTTACA                      |
| SpTEL_7    | tacaGGTTACAAGGTTACA                      |
| SpTEL_11   | tacaGGTTACAAGGTTACA                      |
| SpTEL_R    | tacaGGTTACAAGGTTACA                      |
| SpTEL_2R   | tacaGGTTACAAGGTTACA                      |
| Non-tel    | tacaGGTTACAAGGTTACA                      |
| HIS3       | tacaGGTTACAAGGTTACA                      |

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**TABLE II**

**Effect of the single-stranded overhang on the number of t-loops and the frequency and location of Taz1p binding**

The percentages of the corresponding species of molecules are expressed as a fraction of the total molecules. The numbers in parentheses represent the standard deviations from one experiment to another (n = number of inspected molecules per corresponding terminal structure).

| Terminal structure | total n | unbound | tip | <500 bp | >500 bp | t-loops |
|--------------------|---------|---------|-----|---------|---------|---------|
| (GGTTACA)_n        | 500     | 17.4    | 25.8| 50.8    | 4.6     | 1.4     |
| (GGTTACA)_n       | 1500    | 23.3 (8.9)| 33.8 (1.8)| 23.8 (0.3)| 5.9 (1.6)| 13.3 (5.3) |
| (GGTTACA)_n       | 500     | 3.4     | 37.8| 46.6    | 2.0     | 10.4    |
| (GGTTACA)_n       | 600     | 15.6 (3.4)| 23.3 (4.7)| 56.8 (1.1)| 2.1 (0.1)| 2.3 (2.4) |
| (GGTTACA)_n       | 500     | 18.2    | 32.6| 43.4    | 3.8     | 2.0     |
| (GGTTACA)_n       | 500     | 20.6    | 36.0| 37.8    | 4.8     | 0.8     |
| (GGTTACA)_n       | 600     | 9.6 (0.6)| 27.5 (0.7)| 59.2 (1.1)| 1.7 (1.0)| 2.0 (0)  |
| (GGTTACA)_n       | 500     | 13.2    | 23.4| 59.4    | 2.2     | 1.8     |
mammalian cells. Therefore, even though telomeres in a homologue of TRF2, a crucial player in t-loop formation, have been extremely instrumental in telomere biology (49), they lack of telomere remodeling is needed. Although budding yeast have a system that could provide a detailed understanding of this type of telomere maintenance, but also has exciting evolutionary implications (10). Information about the cellular components required for their formation is still rudimentary (24, 48). A simple model suggests not only its general importance for telomere maintenance and mechanism of formation may or may not be strictly analogous to t-loops in mammalian cells. On the other hand, the presence of a TRF-like protein, Taz1p, in S. pombe as well as similar “telomeric” phenotypes of its defective forms (35), make the fission yeast a potentially valuable model for studying t-loop dynamics.

In this study, model telomere DNAs and purified Taz1 protein were used to examine the features of Taz1p-telomere interactions. As in the case of TRF2, Taz1p induced t-loop formation when the model telomere contained a 3' overhang with consensus telomeric repeats. Similar to TRF2, efficient Taz1p-mediated t-loop formation depended on the presence of a ss telomeric overhang. Non-telomeric ss overhangs had a limited potential for promoting both looping and Taz1p binding to the tip of the model template. This is in agreement with the observation of Vassetzky et al. (50) that complexes of Taz1p and a telomeric probe can be disrupted by high molar ratios of single-stranded G-rich oligonucleotides. It was also recently demonstrated that Taz1p plays a role in a double-stranded DNA break repair (43). These properties of Taz1p may reflect its potential ability to directly bind and act on double-stranded DNA breaks, in particular if the DNA end contains an extruded single strand.

Taz1p exhibits amino acid sequence homology to both TRF1 and TRF2. Its apparent preference for ss/ds telomeric junctions and a role in mediating t-loop formation is reminiscent of the binding characteristics of TRF2. On the other hand, under some conditions, Taz1p is able to form protein arrays on the telomeric repeat DNA, thus resembling TRF1 binding to human telomere (23). It was suggested that TRF1 may be involved in the early steps of folding the telomeric DNA back on itself and/or may aid in compacting the telomeric chromatin (5, 24). As Taz1p is the only TRF-like protein in S. pombe, our results support its dual function (performed by TRF1 and TRF2 in mammalian cells) at fission yeast telomeres.

Glycerol spray/low voltage EM and gel filtration analysis showed that Taz1p exists in an oligomeric state in solution and both methods were in agreement that the major oligomeric

![Fig. 5. Taz1p forms donut-like oligomers. A–D, enlargements of Taz1p bound to telomeric sequences in molecules prepared for EM as described in the legend to Fig. 3. Examples are shown of donut-like Taz1p oligomers at the t-loop junction (A–C) and at the end of a telomeric tract (D). Taz1p in the absence of DNA was prepared without fixation by glycerol spray/low voltage EM and 4 examples of donut oligomers are shown (E–H). Examples are shown in reverse contrast. The bar is equivalent to 26 (A–D) and 20 nm (E–H).](image-url)
species is likely a hexamer of ~470 kDa. The EM results revealed the presence of a donut-like particle with a distinct 3-nm hole in the center. When the C terminus was truncated, Taz1pΔC, long protein filaments were abundant and their width was similar to the diameter of the donuts. Structurally rings and filaments are closely related as a filament can be composed of rings stacked one on top of the next, or by a transition between a closed flat ring and an open lock washer, the latter of which could assemble into a filamentous chain. In the case of Taz1pΔC, the truncation may facilitate this transition.

A donut-like shape of the Taz1p oligomer may be a clue to an explanation of the puzzling observations of t-loops whose sizes exceeded the length of the telomeric tract present on the model telomere. One possible scenario would be that after binding of Taz1p to the ss/ds telomeric junction and formation of the t-loop, Taz1p mediates sliding of the 3’ tail through the template DNA molecule, thus leading to the generation of loops exceeding 500 bp in size. Sliding over the entire linear molecule would then result in the tip-bound DNAs being indistinguishable from the molecules that originated from a simple binding of Taz1p to the ss/ds junction. To test this scenario(267,512),(682,530), we placed a biotin-streptavidin block at the non-telomeric end of the plasmid and then added Taz1p. Although in some samples more than 10% circles were observed, there was a large variation between individual experiments (data not shown). Thus further work is needed to test the sliding model.

In our hands, a maximum of 10–15% of the input DNAs, as scored by EM, were assembled into t-loops by Taz1p. These values are close to those observed for TRF2-mediated t-loop formation (24). The explanations presented previously for why these values did not approach 100% (24) apply here as well. In particular, the 10–15% value likely represents equilibrium between formation and release of the loops, and other factor(s) not included in the reconstitution assay may be required for efficient t-loop formation. In addition, possible sliding of the 3’ overhang along the duplex may lead to a higher proportion of tip-bound molecules and underscoring the fraction of t-loops. At this point it is difficult to speculate what would drive these energy-dependent processes (similarly to TRF2, no requirement for nucleotide hydrolysis was observed) and what would be a physiological role of the Taz1p-dependent sliding. It was observed that fission yeast telomeres adopt a discrete non-nucleosomal chromatin structure and that this structure is disrupted in taz1Δ cells (35). A non-nucleosomal structure at the telomeres together with the sliding capacity of the Taz1p along the telomeric tract might be important for an establishment of t-loops. The original loop may be formed by invasion of the 3’ overhang anywhere within the telomeric tract by mechanisms described in Stansel et al. (24). Formation of this metastable complex may be followed by its sliding through the duplex until it is stalled by the first nucleosome marking a telomeric/subtelomeric junction (in the reconstituted system lacking any barrier, the complex slides all the way through the linear DNA). Within the cell, the maximal t-loop size would thus correspond to the length of the telomeric repeat array. This would argue that although the sizes of t-loops observed in the reconstituted system exceeded 500 bp, their in vitro sizes would be in agreement with the length of the telomeric tract. This may help the cell to monitor the length of its telomeres in addition to a one-dimensional “ruler” (e.g. analogous to that of a Rap1p-dependent counting machinery in S. cerevisiae) (31) also by its two-dimensional counterpart. Considering structural and functional similarities between Taz1p and TRF2 this model could also apply to higher eukaryotes. The correct adjustment of t-loops may be critical for the ability of the cell to sense and respond appropriately to the changes in telomere states (2, 51).

The results of our in vitro experiments do not unequivocally prove the existence of t-loops on S. pombe telomeres in vivo. The size of fission yeast telomeric tracts (<500 bp in S. pombe versus >5 kb in mammalian cells) and a low abundance of telomeres (6 telomeres per fission yeast cell versus 92 telomeres per human diploid cell) makes it difficult to quantitatively isolate S. pombe telomeric fragments and examine them for a presence of t-loops in vivo as we did in mammalian cells (5). In addition, t-loop formation may involve the participation of other proteins, such as Ku, Mre11 complex, spRap1, or Pot1, which will be included in our future experiments aimed at reconstitution of the fission yeast telosome. Nonetheless, the work described here provides a first step toward understanding telomere remodeling in fission yeast that, in addition to its evolutionary implications, may prove relevant in deciphering telomeric structure in higher eukaryotes.

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