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Formation of C-terminally truncated version of the Taz1 protein employs cleavage-box structure in mRNA

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Abstract

When expressed in various hosts the *taz1*⁺ gene encoding the fission yeast telomere-binding protein produces two forms of polypeptides: full-length (Taz1p) and truncated (Taz1pΔC) version lacking almost entire Myb-domain. Whereas Taz1p binds telomeric DNA *in vitro*, Taz1pΔC forms long filaments unable of DNA binding. The formation of Taz1pΔC is a result of neither site-specific proteolysis, nor premature termination of transcription. *In silico* analysis of the *taz1*⁺ RNA transcript revealed a stem-loop structure at the site of cleavage (cleavage box; CB). In order to explore whether it possesses inherent destabilizing effects, we cloned CB sequence into the open reading frame (ORF) of glutathione-S-transferase (GST) and observed that when expressed in *Escherichia coli* the engineered gene produced two forms of the reporter protein. The formation of the truncated version of GST was abolished, when CB was replaced with recoded sequence containing synonymous codons thus indicating that the truncation is based on structural properties of *taz1*⁺ mRNA.

Keywords

telomere; fission yeast; RNA processing; Taz1; stem-loop

Introduction

The DNA-protein complexes at the ends of linear chromosomes (telomeres), stabilize the termini by enabling cells to distinguish the natural chromosome ends from DNA breaks and by repressing DNA repair reactions. The specific telomeric structure is also crucial for proper replication and maintenance of the chromosome termini [1,2]. Key role in these processes is played by a set of proteins that specifically recognize and bind to the telomeric DNA along with proteins that directly interact with these telomeric DNA binding factors. At mammalian and fission yeast telomeres this core complex consists of several proteins and is termed shelterin [3-5].

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In mammalian shelterin complex three of the subunits bind directly to double-stranded (ds) DNA (TRF1, TRF2) or single-stranded (ss) DNA (POT1), whereas the remaining subunits (RAP1, TIN2, TPP1) are attached to the complex via mutual protein-protein interactions. Central role is played by the TIN2 protein that connects TRF1, TRF2, RAP1 and TPP1 [3]. TRF2 and its homologue TRF1 were first identified as proteins capable of specific binding to telomeric dsDNA as homodimers [6-8]. Apart from shelterin proteins, TRF1 and TRF2 are known to interact with number of different proteins including tankyrase [9], Werner and Bloom syndrome helicases [10], SNM2/Apollo nuclease [11] and the major DNA damage sensor at telomeres and dsDNA breaks, the MRN complex [12].

Although TRF1 and TRF2 are paralogues, their roles in telomere dynamics seem to be different [13]. Both proteins have almost identical domain structure with only minor differences. Yet they do not form heterodimers and interact with different subset of proteins [2]. Unlike TRF2, TRF1 is unable to promote t-loop formation *in vitro* [14,15]. Instead, TRF1 forms filamentous structures on telomeric repeat arrays and promotes parallel pairing of telomeric tracts [16].

Similar to its human homologues TRF1 and TRF2, the fission yeast Taz1p (Telomere associated *Schizosaccharomyces pombe*) protein binds to telomeric double-stranded (ds) DNA and is a key component of telomeric chromatin regulating proper telomere maintenance [17-20]. Moreover, recent findings suggest that Taz1p is, like its mammalian counterparts, core of yeast shelterin [4]. Like the human homologues, it was shown to form multimers and promote formation of t-loops *in vitro* [21]. In addition, Taz1p plays a role in meiotic cell division, regulation of gene expression, DNA recombination and replication at telomeres [17-19, 22-26].

We observed that when produced in *Escherichia coli*, Taz1p forms two polypeptide species; full length Taz1p and Taz1p Δ C lacking the entire Myb-domain at the C-terminus (the dimerization domain is apparently not affected by the truncation) [21]. Interestingly, the gene encoding mouse telomeric protein TRF2 produces three splice variants, which all encode proteins lacking the DNA-binding Myb-domain potentially involved in delocalization of Rap1 from telomeres [27]. Importantly, Spink et al. [28], using Taz1p with green fluorescent protein (GFP) tag added to its N-terminus showed that similarly as we observed in *E. coli*, Taz1p may exist in two forms also in its native host *S. pombe*, although they are not a result of an alternative splicing since the *taz1*⁺ primary transcript does not contain an intron. With regard to the dimeric nature of Taz1p, the ability of Taz1p Δ C to form dimers and potential dominant negative effect of the truncated form on the full-length Taz1p, we investigated the possible causes of Taz1p truncation. We provide indirect evidence that the truncation does not occur after translation, but rather post-transcriptionally, most likely during translation. To find the possible regulation point of full-length and truncated *taz1*⁺ expression, we performed *in silico* analysis of the *taz1*⁺ RNA transcript that predicted presence of a region with relatively stable stem-loop structure (named cleavage box (CB)) at the site of truncation indicating that the truncation may take place either at transcriptional or post-transcriptional level. We studied the consequences of disruption of the predicted regulatory hairpin on Taz1p production by introducing point mutations into the hairpin stem and by replacing the whole CB region with recoded sequence. In order to explore whether the *taz1*⁺ hairpin structure possesses inherent destabilizing effect on proteosynthesis in general, we cloned CB sequence into the open reading frame ORF of glutathione-S-transferase (GST), expressed the engineered gene in *E. coli* and probed for potential multiple forms of the GST protein.

Material and Methods

RNA isolation

Total cellular RNA from cultures of *S. pombe* strains MR1 (*h-ura4-D18 leu1-32*) and 005 (*h-leu1-32*) grown to early stationary phase in YES medium (0.5% (w/v) yeast extract, 3% (w/v) glucose, supplemented with 0.0225% (w/v) adenine, histidine, leucine, uracil and lysine hydrochloride, respectively) was extracted as described in [29].

Northern blot analysis

The RNA samples were electrophoretically separated in 1% agarose/1.11% formaldehyde gel and capillary transferred overnight onto a Hybond N+ membrane (Amersham) with a Turboblotter Transfer System (Schleicher and Schuell) in DEPC treated 10x SSC (1.5 M NaCl, 0.15 M Na-citrate). RNA was fixed to the membrane by a combination of baking for 1 h at 80°C and UV (312 nm) for 1 min (each side). Membrane was prehybridized for 1 h at 42°C in a hybridization buffer (5x SSPE [0.75 M NaCl, 50 mM NaH₂PO₄, 5 mM EDTA-NaOH], 2x Denhardt's solution [0.04% (w/v) Ficoll 400, 0.04% (w/v) polyvinylpyrrolidone, 0.04% (w/v) BSA fraction V], 0.5% SDS, 50% formamide, in DEPC-treated H₂O) and subsequently hybridized under the same conditions with *taz1*⁺-specific probe prepared by PCR amplification of *taz1*⁺ ORF (using TAZ1-UP and TAZ1-DOWN primers) and labelled with [α^{32} P]-dCTP using Ready-To-Go DNA Labelling Beads (-dCTP) (Amersham). The membrane was rinsed once with washing buffer (2x SSC) for 5 min at room temperature and then twice with the same buffer for 15 min at 50°C. Membranes were exposed to Storage Phosphor Screen GP (KODAK) for 12–72 h. Signal detection was performed using the Personal Molecular Imager (BioRad).

RT-PCR analysis of *taz1*⁺ mRNA

Isolated RNA (~30 µg) was polyadenylated using Poly(A) Tailing Kit (Ambion) and subsequently cleaned using RNeasy kit (Qiagen). 1 µg of purified RNA was used in reverse transcription reaction with oligo-dT 3' RACE adapter using FirstChoice[®]RLM-RACE Kit (Ambion). 2 µl of the RT reaction were then used in PCR with 3' RACE Outer Primer (Ambion) and *taz1*⁺-specific primer (TAZ1-UP) derived from the start region of its ORF.

Preparation of plasmid constructs

Plasmid constructs carrying Taz1-N-His and Taz1-C-His were prepared by InFusion PCR cloning system (Clontech) using TAZ1_TALON_Sense and TAZ1_TALON_Anti primers (Table 1) and pEcoli-Nterm-6xHN and pEcoli-Cterm-6xHN (Clontech) as destination vectors.

Site-specific mutagenesis of *taz1*⁺ was done by overlap-extension PCR using pairs of primers TAZ1-TOPO FOR+TAZ1-MM4 REV and TAZ1-MM3 FOR+TAZ1-DOWN and final amplicon was gained using TAZ1-TOPO FOR+TAZ1-DOWN (Table 1). Amplified fragments of *taz1*⁺ and *taz1*⁺ containing two substitutional mutations (*TAZ1-2MM*) were cloned into pENTR-TEV-D-TOPO vector using TOPO[®] Cloning System (Invitrogen) and recombined into pDEST17 vector using GATEWAY[™] Cloning System (Invitrogen) creating pHIS-TAZ1 and pHIS-TAZ1-2MM.

To prepare GST with inserted *taz1*⁺-CB and its recoded version, phosphorylated single-stranded oligonucleotides (TAZ1-CB Sense+TAZ1-CB Antisense and TAZ1-CB-REC Sense+TAZ1-CB-REC Antisense) (Table 1) containing 99 nt sequence of *taz1*⁺-CB or *taz1*⁺-CB-REC were reassociated and ligated into *MscI* digested pGEX-2T vector (GE Healthcare).

The recoded version of *taz1*⁺ ORF was constructed as follows. First, left and right parts of recoded *taz1*⁺ were amplified using TAZ1_TALON_Sense and TAZ1-Rec-Left-Anti-P (left part of the recoded *taz1*⁺) and TAZ1-Rec-Right-Sense-P and TAZ1_TALON_Anti (right part

of the recoded *taz1*⁺ (Table 1). The resulting PCR products were ligated and the ligation mixture was used for the second PCR using TAZ1_TALON_Sense+TAZ1_TALON_Anti as primers. The resulting product was cloned into linearized pEcoli-C-term vector using Direct PCR cloning kit (Clontech) yielding the plasmid TR1.1. Next, pENTR-TEV-D-TOPO containing *taz1*⁺ was recombined into pDEST15 using GATEWAY™ Cloning System (Invitrogen) creating pGST-TAZ1. Subsequently, the *KpnI-StuI* fragment containing part of *taz1*⁺ with recoded CB was excised from the TR.1.1 and cloned into pGST-TAZ1 or pHIS-TAZ1 linearized with the same restriction endonucleases yielding pGST-TAZ1-REC and pHIS-TAZ1-REC.

In vitro transcription/translation of *taz1*⁺

taz1⁺ coding sequence was amplified using the primers TAZ1_TNT_5' and TAZ1_TNT_3' (Table 1), pHIS-TAZ1 as a template and *Taq* DNA polymerase (Invitrogen) at 50°C as annealing temperature. The resulting DNA fragment was purified employing DNA Clean and Concentration kit (ZymoResearch) and added to the *in vitro* transcription/translation (TNT) reaction mix (Promega) containing [³⁵S] methionine (GE Healthcare). The resulting protein products were separated by 10% SDS-PAGE, gel was fixed for 10 min in 30% (v/v) methanol, 10% (v/v) acetic acid, dried and exposed to the KODAK X-OMAT film.

Immunodetection

Protein lysates were prepared from bacterial cultures expressing either recombinant His- or GST-tagged Taz1p, its mutated versions and GST modified with *taz1*⁺-CB or *taz1*⁺-CB-REC insertions. Proteins were separated by 10 or 14% SDS-PAGE, electrotransferred to a nitrocellulose membrane (GE Healthcare) and analyzed with anti-penta-His (Qiagen) or anti-GST (Sigma) primary antibodies (diluted as recommended by the suppliers).

Miscellaneous

Taz1-N-His and Taz1-C-His were purified as described by Tomaska et al. (2004) using Talon™ Metal affinity resin (Clontech). The standard DNA manipulations were performed as described by Sambrook and Russell (2001) or as suggested by the manufacturers. *In silico* predictions of the *taz1*⁺ mRNA secondary structure was predicted by using the MFOLD (version 2.3) program based on energy minimization using the server at <http://mfold.bioinfo.rpi.edu/> (Zuker, 2003).

Results and Discussion

Truncation of Taz1p probably occurs between transcription and translation

When producing Taz1p in *E. coli* as a fusion protein with N-terminally located oligo(His)₆ tag (Taz1-N-His), we noticed two major bands corresponding to full-length Taz1p and to Taz1p truncated at its C-terminus (Taz1pΔC) [21]. The truncation eliminates 107 amino acids including almost the entire DNA-binding Myb-domain that is analogous to a dominant negative mutant of TRF2 ([21]; Fig. 1A, B). Electron-microscopic analysis demonstrated that the truncated protein is unable to bind telomeric DNA and forms long worm-like filaments *in vitro* (Fig. 1C). It is possible that Taz1pΔC is able to titrate out the full-length Taz1p into the filaments and thus prevents its binding to DNA. To circumvent the problem of obtaining two forms of Taz1p, we constructed an expression vector, where the tag is placed at the C-terminus of the protein (Taz1-C-His). Affinity purification of such a fusion protein resulted in one predominant band corresponding to the full-length Taz1p (Fig. 1B).

The truncation of Taz1p probably does not occur by proteolytic cleavage. First, when purified full-length Taz1p (see above) was incubated with the bacterial extract, in spite of some

degradation, there was no accumulation of the shorter form (data not shown). Second, Taz1p did not exhibit intein-like properties, since incubation of the full-length Taz1p under various conditions did not result in generation of Taz1p Δ C. Furthermore, coupled transcription/translation of the *taz1*⁺ gene using purified RNA polymerase and reticulocyte lysate yielded two forms of Taz1p and proportion of the truncated form did not increase with time (Fig 2A) also excluding the possibility that the truncation is a result of a premature translation termination due to codon bias in *E. coli*. We also used Rosetta(DE3) strain (Novagen) expressing tRNAs underrepresented in *E. coli* and observed no effect on the formation of Taz1p Δ C (data not shown). We also tested, if the two forms of Taz1p are not a result of premature termination of transcription. However, Northern blot analysis (Fig. 2C) as well as RT-PCR analysis of total RNA isolated from *S. pombe* revealed only a single (full-length) RNA species (Fig. 2B). Finally, we addressed the question, if the purified Taz1p does not possess self-cleavage activity indicating a presence of an intein-like element. We incubated purified Taz1p under various conditions previously employed for different inteins. However, we did not observe any signs of self-cleavage activity of Taz1p (data not shown). All these results indicate that the truncation of Taz1p occurs posttranscriptionally, right before or during translation.

In silico analysis of the Taz1p cleavage box

According to the mass spectrometry analysis, the last aminoacid in Taz1p Δ C is Arg556 [21]. As our results point out that the truncation of Taz1p takes place at the level of RNA, we investigated a possibility that the nucleotide (nt) sequence adjacent to the 556th codon may acquire a secondary structure that may be the primary cause of the processing. Therefore we analyzed a region surrounding the truncation site and named it the cleavage box (CB). Employing an RNA secondary structure prediction utility ([30]; <http://www.bioinfo.rpi.edu/~zukerm/rna/>) we analyzed the CB for presence of stem loops. Indeed, several models predicted existence of stem-loops within the CB. Interestingly, one of the stem-loops in the thermodynamically most plausible model contained the 556th codon within the hairpin region (Fig. 3A).

To test the hypothesis that this stem-loop may be involved in the formation of Taz1p Δ C, we introduced two silent mutations into the hairpin stem expecting disruption of the stem-loop structure (Fig. 3B). However, when we tested the effect of the mutations on bacterially expressed Taz1p, we did not observe any reduction in proportion of the two forms (Fig. 3C).

Taz1p cleavage box destabilizes glutathione-S-transferase

As directed mutagenesis of two selected nucleotides did not abolish truncation of Taz1p, we decided to recode the entire CB taking advantage of genetic code degeneracy. *In silico* analysis of the resulting nt sequence (CB-REC) demonstrated that the region around the 556th codon is not a part of any stable secondary structure (Fig. 4A). In addition, to perform the analysis of CB and CB-REC, we introduced both sequences into the ORF encoding bacterial GST. Our aim was to see, if (i) introduction of CB will destabilize GST synthesis leading to generation of two forms of the modified protein and (ii) replacement of CB with CB-REC will eliminate the truncated form of the modified GST. Indeed, addition of the CB to GST ORF resulted in appearance of second, truncated, form of the modified GST protein. Size of the truncated form (~12 kDa) corresponded to the molecular weight predicted for a truncated version of the modified GST with Arg86 being the last amino acid (Fig. 4B, GST+CB). The wild-type GST formed only one polypeptide species (Fig. 4B, GST). Importantly, when instead of CB, CB-REC was introduced into the GST coding sequence, only the full length of the modified GST protein was observed (Fig. 4B, GST+CB-REC). These results not only support the hypothesis that introduction of the CB can lead to truncation of a heterologous protein, but also again

demonstrate that the truncation is not a post-translational event (amino acid sequences of GST+CB and GST+CB-REC are identical), but occurs right before or during translation.

Finally, we wanted to test, if replacement of the CB sequence directly within *tazI*⁺ gene, similarly as in case of GST, would abolish production of the truncated version of the protein. We replaced the CB with CB-REC in two recombinant versions of Taz1p containing either oligo(His) or GST tag at their N-termini. However, in neither case we observed elimination of the Taz1pΔC form (data not shown). It seems likely that whereas ~100 nt used as the CB is sufficient for truncation of GST, in case of *tazI*⁺ longer sequence is required for effective production of multiple forms. The different response could be also influenced by the sequence context of particular ORF. In fact, when we placed CB into the coding sequence of another reporter protein, maltose binding protein (MBP), we observed only production of the full-length polypeptide (data not shown). It is therefore possible that full potential of the CB might also depend on the position of insertion (near the start codon in case of GST compared to end of ORF in case of MBP), where also the actual length of the nascent peptide during translation might play a stabilizing role.

We investigated which portions of *tazI*⁺ mRNA contribute to the production/generation of the two Taz1p forms. Probably the most robust approach would require financially demanding recoding of the entire *tazI*⁺ sequence using synonymous codons. These experiments may shed some light not only on the mechanism of the Taz1pΔC generation, but may also reveal novel means of Taz1p activity regulation in *S. pombe*. Recent studies of mammalian telomeric dsDNA binding factors have employed several dominant negative forms of telomeric proteins, especially TRF2, to effectively remove the protein from telomeres and study subsequent events [31]. Hence, the fact that Taz1p may exist in two forms in its native host [28], where one of the forms (Taz1pΔC) exerts dominant negative effect on the full-length Taz1p substantiates future studies of this phenomenon.

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Figure 1. Truncated version of Taz1p forms filaments *in vitro*

(A) When produced in *E. coli*, Taz1p is present in two forms, full-length (Taz1p) and truncated (Taz1p Δ C) lacking almost entire Myb-domain. (B) The two forms of Taz1p are purified simultaneously by Ni²⁺- or Co²⁺-agarose, when the oligo(His)₆ tag is placed at the N-terminus of the protein (Taz1-N-His). Placing the tag at the C-terminus results in purification of predominantly full-length Taz1p. (C) When inspected by electron microscopy, the Taz1p Δ C forms worm-like filaments unable to bind DNA.

Figure 2. Truncation of Taz1 occurs between transcription and translation

(A) Coupled *in vitro* transcription/translation using *taz1*⁺ coding sequence containing T7 promoter as a template results in formation of two forms of Taz1 protein, whose proportion does not change with time. The picture shows the autoradiograph of 10% SDS-PAGE with separated proteins after the reaction in the presence of [³⁵S]-methionine. (B) RT-PCR analysis of total RNA isolated from *S. pombe* MR1 revealed the presence of only full-length form of *taz1*⁺ mRNA (M, λ /PstI; lane 1, - RT control; lane 2, *taz1*⁺ cDNA). (C) A single (full-length) form of *taz1*⁺ mRNA was also seen after Northern blot analysis of total RNA isolated from two *S. pombe* strains (MR1 in lane 1 and 005 in lane 2).

Figure 3. In silico analysis of 100 bp region surrounding the cleavage site reveals the presence of a stem-loop structure containing the Arg556 codon

The secondary structure of CB was analyzed by the software Mfold [30]. (A) The Arg556 codon (full circle) is located within a loop of a hairpin structure (shown in rectangle) of the wild-type (WT) CB. (B) Two substitutions targeted to the Arg558 located in hairpin stem (dashed circle) lead to disruption of the putative stem loop structure (2MM). (C) *taz1*⁺ coding sequence has been modified by introducing two substitutions (2MM) and expressed as Taz1-N-His in *E. coli* without (-), or with (+) induction by IPTG. Taz1p has been analyzed by Western blot using anti-penta-His antibodies. Anti-Taz1p antibodies gave the same results (data not shown).

Figure 4. Introduction of the CB into a heterologous protein leads to its truncation, which can be eliminated by CB recoding

(A) The CB was recoded (CB-REC) taking advantage of the synonymous codons. The resulting sequence has been analyzed by Mfold software [30] to see that the codons for Arg556 (full circle) and Arg558 (dashed circle) are not located in a stable secondary structure. (B) CB and CB-REC coding sequences were introduced into the ORF encoding GST resulting in a protein having molecular weight increased by 4 kDa. All three versions of the genes (GST, GST+CB and GST+CB-REC) were expressed in *E. coli* and the resulting protein extracts were analyzed by Western blot using anti-GST antibodies. The 12 kDa polypeptide corresponds to the N-terminus of the truncated protein.

Table 1

List of oligonucleotides

Name	Sequence (5'→3')	Application *
TAZ1_TNT_5'		1
TAZ1_TNT_3'		1
TAZ1_TALON_Sense	TAAGGCCTCTGTGACATAAGCGTGCAAAGTACAGAAACG	2,3
TAZ1_TALON_Anti	CAGAATTCGCAAGCTTAGATTGATAATTAACAAGCTCTTC	2,3
TAZ1-Rec-Left-Anti-P	CCTGTAGCCCTCGTACGGATTCCCCTGACTCCGTCTGGTCCCA CTCCTAGCAGCAGATCTTCAATGGACACTCTCATTG	3
TAZ1-Rec-Right-Sense-P	ACGAGACGGAAGTGGACTGACGAAGAGGAAAACGAATTATAT GAGATGATTCTCAGCATGGCTGTTGTTGGTCTAAAAAT	3
TAZ1-UP	ATAAGCGTGCAAAGTACAGAAACGA	4,5
TAZ1-DOWN	TTAAGATTGATAATTAACAAGCTCT	4, 6, 7
TAZ1-TOPO FOR	CACCATAAGCGTGCAAAGTACAG	6, 7
TAZ1-MM4 REV	CTGTCCATTTTCTTCTAGTTCGATATC	7
TAZ1-MM3 FOR	GATATCGAACTAGAAGAAAATGGACAG	7
TAZ1-CB Sense	CCCGTTCGGGTACACGCAGGTCGCAAGGAAACCCATATGAAGGA TATCGAACTCGTAGAAAATGGACAGATGAAGAGGAGAATGAGCT TTACGAAATGA	8
TAZ1-CB Antisense	TCATTTCTGTAAGCTCATTCTCCTCTTCATCTGTCCATTTTCTACGA GTTTCGATATCCTTCATATGGGTTTCCTTGCGACCTGCGTGTACCCGA ACGGG	8
TAZ1-CB-REC Sense	CCAGGAGTGGGACCAGACGGAGTCAGGGGAATCCGTACGAGGGCT ACAGGACGAGACGGAAGTGGACTGACGAAGAGGAAAACGAATTA TATGAGATGA	8
TAZ1-CB-REC Antisense	TCATCTCATATAATTCGTTTTCTCTTCGTCACTCCACTTCCGTCTCG TCCTGTAGCCCTCGTACGGATTCCCCTGACTCCGTCTGGTCCCACTC CTGG	8

* 1, PCR for *in vitro* transcription/translation experiment; 2, amplification of *tazI*⁺ ORF for cloning into pEcoli-Nterm vector (Clontech); 3, amplification of the recoded version of *tazI*⁺; 4, PCR amplification of *tazI*⁺ hybridization probe; 5 RT-PCR analysis of *tazI*⁺ mRNA; 6, amplification of *tazI*⁺ and *tazI*⁺-2MM ORF for cloning into pENTR-TEV-D-TOPO vector (Invitrogen); 7, PCR mutagenesis of *tazI*⁺; 8, insertion of *tazI*⁺-CB or *tazI*⁺-CB-REC into GST fragment of pGEX-2T vector