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An Arabidopsis Splicing RNP Variant STEP1 Regulates Telomere Length Homeostasis by Restricting Access of Nuclease and Telomerase

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Telomere is an essential DNA-protein complex composed of repetitive DNA and binding proteins to protect the chromosomal ends in eukaryotes. Telomere length is regulated by a specialized RNA-dependent DNA polymerase, telomerase and associated proteins. We show here a potential role of STEP1 that was previously isolated by affinity chromatography in controlling telomere length. While STEP1 requires both RNA-binding domains for telomere binding and subsequent DNA protection, it requires only one RBD to interact with telomerase. The differential telomerase inhibitory activity depending on STEP1 concentrations may suggest that STEP1 contributes to controlling telomere length homeostasis, likely by limiting the accessibility of nuclease or telomerase to telomeric DNA.

INTRODUCTION

Upon DNA replication during cell division, one strand of chromosomal DNA, the lagging strand, is always incompletely synthesized. This phenomenon is known as the 'end-replication problem', and leads to the generation of a single-stranded 3′ overhang. The resulting single-stranded DNA at the chromosomal ends is then degraded by nucleases, which causes the gradual shortening of chromosomes in somatic cells following every cell division (Djojosubroto et al., 2003; Lingner et al., 1995; Shore and Bianchi, 2009). Eukaryotic cells however have invented a machinery called telomere to protect their chromosomal ends from being recognized as DNA breaks and attacked by nucleases or from chromosome-chromosome fusions (Greider, 1996; Shore and Bianchi, 2009). Telomere is a specialized structure consisting of repetitive DNA sequences and associated proteins. Although the telomere length is primarily regulated by telomerase, an RNA-dependent DNA polymerase, telomere-binding proteins are also critical for regulating its structure and stability (Bianchi and Shore, 2008; Greider, 1996).

Telomere-binding proteins are largely divided into two groups, double-stranded and single-stranded telomeric DNA binding proteins. TRF1 and TRF2 are the best-studied human proteins that are known to bind to double stranded telomeres. These proteins bind telomeric DNA through the myb domain and contribute to the formation of a specific telomere structure, t-loop. They can also modulate telomerase activity via direct interaction or by changing the telomere structure (van Steensel and de Lange, 1997; van Steensel et al., 1998). Proteins belonging to the other group bind single strand telomeric DNA. Two well-known motifs required for binding to single-stranded telomeric DNA are the oligosaccharide/oligonucleotide-binding (OB) fold and the RNA-binding domain (RBD). POT1 interacts with single strand telomere through the OB folds and contributes to controlling telomere length and protecting the chromosomal ends (Baumann and Cech, 2001). Interestingly, almost all heterogeneous nuclear ribonucleoproteins (hnRNPs) that are involved in monitoring transcription have been found to bind single strand telomeric DNA through the RBDs (Ford et al., 2002). hnRNP A1 and its smaller splicing variant UP1 interact with both single-stranded telomeric DNA and the telomerase RNA component, thereby facilitating telomere elongation (Fiset and Chabot, 2001; LaBranche et al., 1998). A *Caenorhabditis elegans* hnRNP, HRP-1, has also been shown to play a positive role in regulation of telomere length (Joeng et al., 2004).

Proteins that bind double strand telomere in plants also possess the myb domain. The rice protein RTBP and the tobacco protein NgTRF1 bind telomeric DNA through their myb domains and contribute to maintenance of the telomere length and genome stability (Hong et al., 2007; Yang et al., 2003; 2004; Yu et al., 2000). Although Arabidopsis also has two POT1-like proteins containing the OB fold, their binding to telmeric DNA is not yet clear. However, these proteins interact with telomerase and control the telomere length homeostasis in Arabidopsis (Surovtseva et al., 2007). We have previously identified single strand telomere-binding proteins by affinity chromatography and mass spectrometry using Arabidopsis nuclear extracts (Kwon and Chung, 2004). WHY1, which was previously known to be involved in plant defense responses, binds single strand telomeric DNA, interacts with telomerase and negatively regulates telomerase activity and telomere length *in vivo* (Yoo et al., 2007). An UP1-like protein has also been retrieved from affinity chromatography. STEP1, a splicing variant of CP31 (Cheng et al., 1994; Ohta et al., 1995) that is a chloroplast RNA-editing

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protein, contains no transit peptide but binds single-stranded telomeric DNA via both RBDs (Kwon and Chung, 2004). Its exclusive localization to nuclei and *in vitro* activity to inhibit telomerase activity suggest that STEP1 also plays a role in telomere homeostasis through interaction with the single stranded 3′ overhang and/or telomerase in plants. We here present that STEP1 interacts via RBD1 with the Arabidopsis telomerase but inhibits *in vitro* telomerase activity mainly by limiting telomerase accessibility. Requirement of both RBDs for binding single strand telomeric DNA and for subsequent DNA protection from nuclease attack, but requirement of only RBD1 for interacting with telomerase suggest that unlike UP1 STEP1 may differentially regulate the length of telomere. Retained interaction between STEP1 and human telomerase even in the presence of RNase indicates that STEP1 is a component of whole telomerase complex but it interacts with telomerase peptide but not with the RNA template or with other structural component of the telomerase complex.

MATERIALS AND METHODS

Plant growth

Arabidopsis thaliana (Col-0) plants were grown on MS-agar plates after seed sterilization for one week. Total proteins containing the telomerase activity were extracted as described previously (Kwon and Chung, 2004).

Telomere DNA protection assay

Recombinant proteins fused to GST or not were purified from *Escherichia coli* (BL21) as previously described (Kwon and Chung 2004). For nuclease protection assays, purified proteins were incubated with 5′ end-labeled $(TTTAGGG)_4$ for 10 min in binding buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 1 mM DTT, 50 mM NaCl and 5% glycerol] containing 0.5 μg of poly (dI-dC) and 0.5 μg of nonspecific single-stranded oligonucleotide, and DNase I (0.02 μg ml^{-1}) was added. After adding 0.5 M EDTA to terminate the DNA digestion, the ethanol-precipitated DNA was separated on a 10% acrylamide gel and then detected by autoradiography.

Co-precipitation assay of Arabidopsis telomerase

To co-immunoprecipitate the telomerase, anti-STEP1 antibody, which was generated and purified using RBD2, was mixed with plant extracts, retrieved by absorbing Protein A-Sepharose, and then analyzed by telomere-repeat amplification protocol (TRAP) assays as described previously (Kwon and Chung, 2004). To pull-down telomerase, glutathione Sepharose 4B-absorbed GST-fused recombinant proteins were mixed with plant extracts, sedimented by centrifugation and then analyzed by TRAP assays.

Co-precipitation assay of human telomerase

To assess the ability of STEP1 to inhibit human telomerase activity, the telomerase-enriched extracts were prepared from HeLa cells as described previously (Kim et al., 2003). To transiently express STEP1-V5 and hTERT-HA, H1299 cells were transfected using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. Cells were then lysed by incubation in lysis buffer [10 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$, 50 mM KCl, 0.01% Nonidet P-40, 20% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and 2 mM DTT], and the cell debris was removed by centrifugation. For immunoprecipitation, the lysates were cleared with protein A-Sepharose and then incubated with anti-V5 or anti-HA antibody coupled to protein A-Sepharose. After extensive washing, the precipitates were subject to immunoblot using anti-HA or anti-V5 antibody. To examine the RNase effect, the lysates were treated with RNase A before immunoprecipitation.

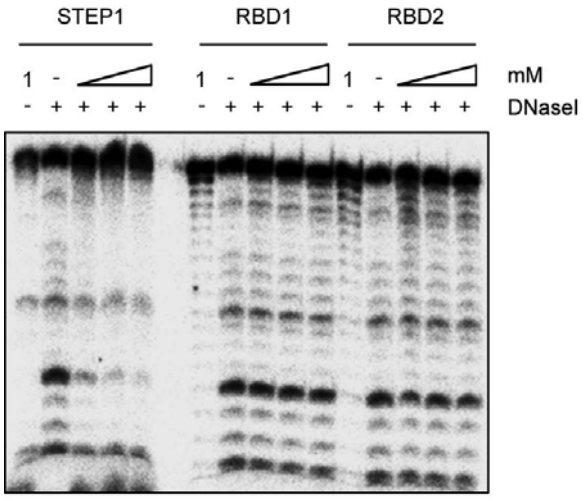

Fig. 1. Telomere protection requires both RBDs of STEP1. One nano gram of [^{32}P]-endlabeled single-stranded plant telomere repeats $(TTTAGGG)_4$ was incubated with increasing amounts of the indicated recombinant proteins (0, 0.1, and 0.5 mM). Subsequently, DNase I (50 μg ml^{-1}) was added as indicated and the nuclease reactions were terminated by adding 0.5 M EDTA. The resulting DNA materials were retrieved by ethanol precipitation, separated on an acrylamide gel and analyzed by autoradiography.

RESULTS AND DISCUSSION

One of the major roles of single stranded telomere-binding proteins is to protect the 3′ overhang from nuclease attack, which may be otherwise regarded as broken DNA to be repaired (Lingner et al., 1995). Since STEP1 directly and specifically binds to plant single-stranded telomeric DNA sequences (Kwon and Chung, 2004), we further examined its function in telomere protection. Single-stranded plant telomere sequence repeats $(TTTAGGG)_4$ were end-labeled with [γ-^{32}P]-ATP, incubated with various amounts of purified recombinant STEP1 and then subjected to DNA digestion with DNase I. As expected, the addition of STEP1 efficiently blocked the digestion of plant telomere repeats by DNase I (Fig. 1). STEP1 contains two RBDs that are both required to bind single strand telomeric DNA (Kwon and Chung, 2004). Therefore, we tested each RBD for the DNA-protecting activity. Unlike STEP1, RBD1 or RBD2 alone failed to protect single strand telomeric DNA from being digested by DNase I (Fig. 1). This indicates that the protection of single strand telomeric DNA *in vitro* depends on STEP1 binding to the telomeric DNA because each RBD is not able to bind to the single strand telomere alone.

UP1 is proposed to recruit telomerase at the chromosomal ends because it can simultaneously interact with single strand telomeric DNA and the telomerase RNA template called hTR (Fiset and Chabot, 2001). Similar features of STEP1 to UP1 prompted us to test whether STEP1 also interacts with telomerase in Arabidopsis. We first generated polyclonal anti-STEP1 antibody using RBD2. Anti-STEP1 antibody specifically detected RBD2-containing polypeptides, but not the N-terminal half (NT) and RBD1 of CP31 (Fig. 2A). To understand the interaction between STEP1 and plant telomerase, we tested whe-

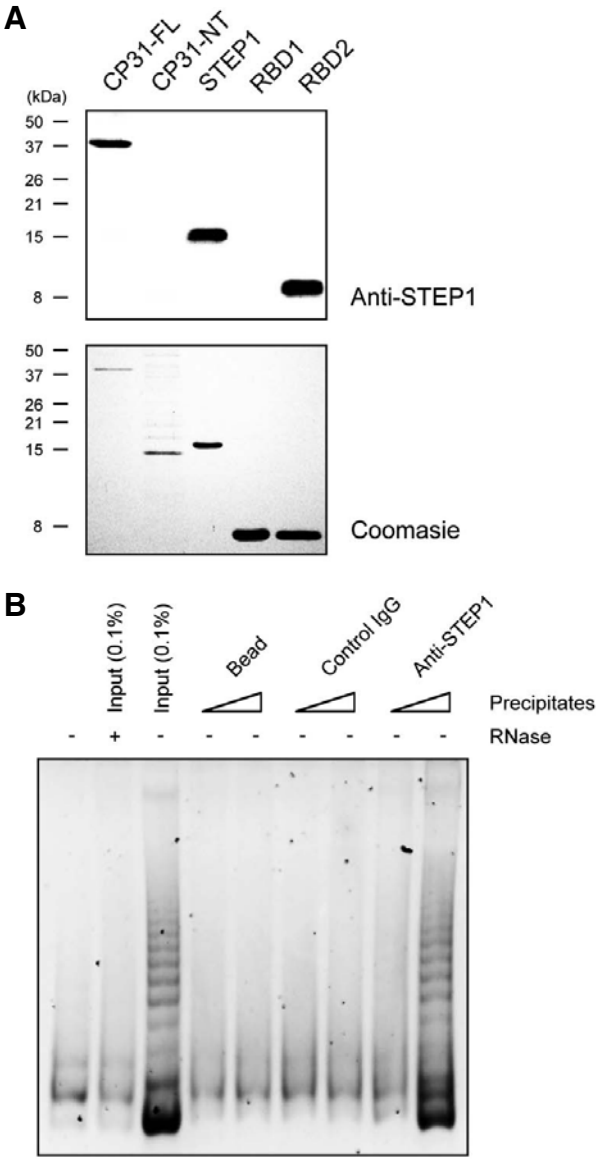

Fig. 2. STEP1 interacts with telomerase *in planta*. (A) Specificity of the raised anti-STEP1 antibody using RBD2. The indicated polypeptides devoid of GST were bacterially expressed and purified. To test the specificity of anti-STEP1 antibody, the indicated proteins were separated on an acrylamide gel and subjected to immunoblot analysis (upper panel). The purity of proteins used was visualized by Coomaise staining (lower panel). CP31-FL, full-length of CP31; CP31-NT, N-terminal half of CP31 (Kwon and Chung, 2004). (B) Anti-STEP1 antibody precipitated plant telomerase. Plant extracts containing telomerase activity (lanes 2 and 3) incubated with the indicated antibodies. After extensive washing, the resulting immunocomplexes (2 and 10 μl, respectively) resuspended in 50 μl of extraction buffer were subjected to TRAP assays. The specificity of the telomerase activity in plant extracts was evaluated based on the disappearance of telomere-lengthening activity by RNase A addition (lane 2). As a control, Protein A-Sepharose alone and rabbit IgG were used for the co-immunoprecipitation (lanes 4-7).

ther anti-STEP1 antibody can co-immunoprecipitate telomerase activity together with STEP1. For this, we incubated protein extracts from 7 day-old Arabidopsis seedlings in which the telomerase activity is detected by telomere-repeat amplification protocol (TRAP) assays (Fig. 2B), with anti-STEP1 antibody, and sedimented the immunocomplex with Protein A-Sepharose. We then examined the presence of telomerase by *in vitro* TRAP assays. We observed the telomere-elongating activity in the immunoprecipitates of anti-STEP1 antibody (Fig. 2B). However, Protein A-agarose alone and the control rabbit IgG failed to co-precipitate the telomerase activity (Fig. 2B). Taken together, these findings suggest that STEP1, at least CP31, physically interacts with plant telomerase.

Although STEP1 interacts with plant telomerase, it is unclear whether they directly interact with each other or if STEP1 indirectly interacts with telomerase by binding to the telomerase RNA template. However, the RNA template equivalent to hTR has not yet been identified in plants. Since we previously showed that STEP1 can also inhibit the activity of human telomerase (Fig. 3A), we therefore heterologously tested the RNA-dependence on STEP1 interactions with telomerase using human culture cell system. We transfected and expressed V5-tagged STEP1 (STEP1-V5) and/or the HA-tagged human telomerase catalytic subunit (hTERT-HA) in the human lung carcinoma cell line, H1299. hTERT-HA was previously shown to be successfully reconstituted as a functional telomerase in Saos-2 cells in which telomerase activity is rarely detectable (Kim et al., 2005). Immunoblot analysis using the corresponding antibodies revealed that both tagged proteins are well-expressed in transfected H1299 cells (Fig. 3B). Next, cell extracts containing either protein or both were used to perform co-immunoprecipitation (co-IP) using anti-V5 or anti-HA antibody. We detected the presence of hTERT-HA in the STEP1-V5 immunoprecipitates (Fig. 3B), indicating that STEP1 physically interacts with human telomerase as in plants. However, we failed to detect the presence of STEP1-V5 in the hTERT-HA immunoprecipitates (data not shown). This suggests that in the telomerase complex including hTERT-HA and STEP1-V5, the V5 epitope of STEP1-V5 might not be accessible to used anti-V5 antibody. To understand the role of hTR in mediating the interactions between STEP1 and hTERT, we again performed the same co-IP reactions using anti-V5 antibody in the presence or absence of RNase. We detected the hTERT-HA in the immunoprecipitates, regardless of the presence of RNase (Fig. 3C), implying that unlike the animal counterpart UP1, the STEP1 interactions with human telomerase are not mediated by the telomerase RNA component, but directly or via mediation by another accessory protein comprising the telomerase complex. However, the expected high sequence divergence between human and plant telomerase RNA components still opens a possibility that STEP1 may directly interact with plant telomerase RNA subunit.

Based on the results obtained from the heterologous system, it is likely that STEP1 interacts with plant telomerase not via the RNA template component (Fig. 3C). Hence, we examined the binding region of STEP1 to interact with plant telomerase. We bacterially expressed and purified recombinant glutathione-*S*-transferase (GST)-fused STEP1 (GST-STEP1), GST-RBD1 or GST-RBD2. After absorbed to glutathione-Sepharose 4B, we mixed the GST-STEP1, GST-RBD1 or GST-RBD2 with the telomerase activity-containing plant extracts mentioned above. Similar to the results obtained from co-IP (Figs. 2B and 3B), the full-length STEP1 co-precipitated the plant telomerase as shown by the *in vitro* telomere-lengthening activity (Fig. 4). While GST-RBD1 was co-sedimented with plant telomerase, GST-RBD2 failed to retrieve the telomere-lengthening activity from the plant extracts (Fig. 4). These findings indicate that STEP1

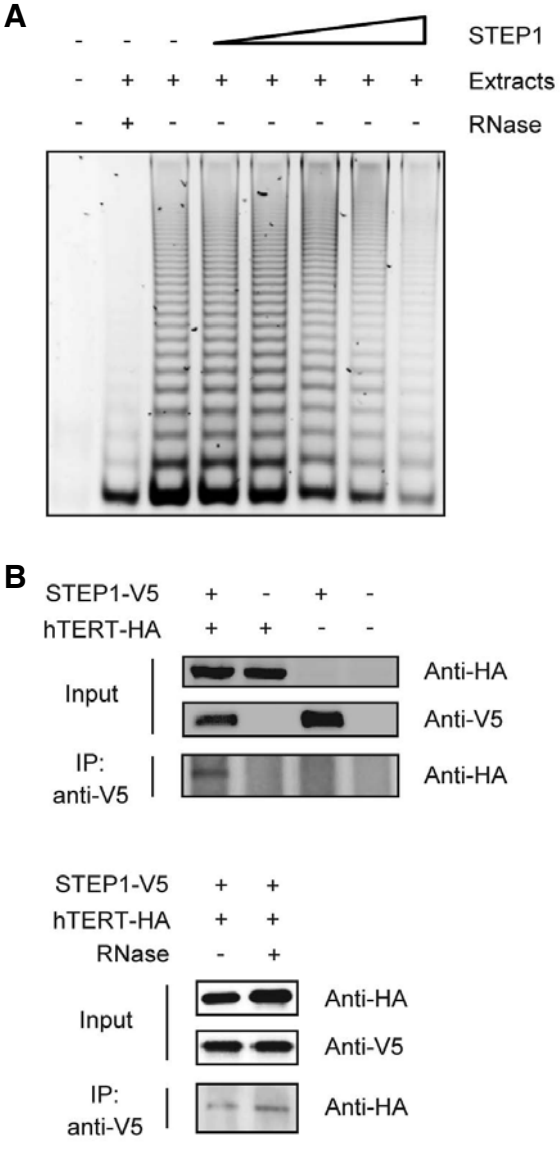

Fig. 3. STEP1 interacts with human telomerase not via the RNA template hTR. (A) At high concentrations, STEP1 inhibits the telomere-lengthening activity of human telomerase. Partially purified human telomerase extracted as previously described (Kim et al., 2003) was incubated with purified recombinant STEP1 (1, 5, 10, 20, and 30 pmole, respectively) and subjected to the TRAP assays. (B) STEP1 interacts with the human telomerase catalytic subunit (hTERT). STEP1-V5 and/or hTERT-HA were transiently expressed as indicated in the human lung carcinoma cell line, H1299. Cell extracts were subsequently incubated with anti-V5 antibody and Protein A-Sepharose, and the resulting immunoprecipitates were subject to immunoblot with anti-HA antibody. (C) The interactions between STEP1-V5 and hTERT-HA are not mediated by hTR. STEP1-V5 and hTERT-HA were transiently co-expressed in H1299 cells and cell extracts were subjected to co-immunoprecipitation assays as in (B). To remove hTR from the telomerase complex, cell extracts were incubated with RNase A prior to the immunoprecipitation.

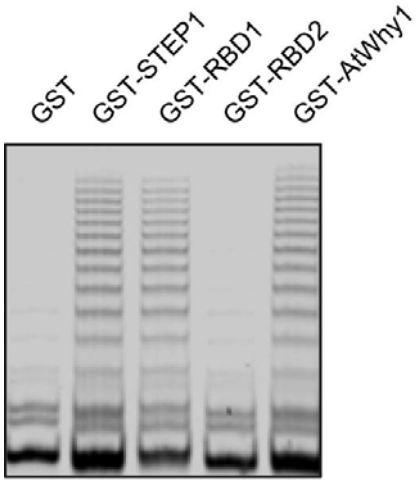

Fig. 4. STEP1 interacts with plant telomerase through its RBD1. Indicated glutathione-*S*-transferase (GST)-fused recombinant proteins were incubated with plant extracts containing telomerase activity, and semimented by glutathione Sepharose 4B. After extensive washing, the precipitates were subject to TRAP assays. GST alone or GST-AtWhy1 (Yoo et al., 2007) were used as a negative or a positive control, respectively.

interacts with plant telomerase through its RBD1. In addition, these results suggest that the mode of STEP1 interaction with plant telomerase is distinct from its binding to single-stranded telomeric DNA since telomere DNA-binding of STEP1 requires both RBDs.

Although STEP1 is very similar to UP1, the splicing variant of hnRNP A1 (Kwon and Chung, 2004), the mode by which STEP1 interacts with plant telomerase is different from that of UP1. It has been reported that UP1 simultaneously interacts with both single-stranded telomeric DNA and hTR, the telomerase RNA template, suggesting that it plays a role in recruitment of telomerase to the telomere (Fiset and Chabot, 2001). The STEP1 interaction with telomerase, although heterologous, does not require the RNA template of human telomerase because their interactions were not affected by RNase that completely abolishes the telomere-lengthening activity in cultured cell extracts (Figs. 3A and 3C). This indicates that STEP1 interacts with telomerase not via binding to the telomerase RNA component, but directly or via mediation by another accessory protein comprising the telomerase complex. Interestingly, RBD1 alone is able to interact with plant telomerase (Fig. 4), which fails to bind single-stranded telomeric DNA and consequently fails to protect the telomere from digestion by nucleases (Fig. 1). This suggests that STEP1 protects single-stranded telomeric DNA by direct binding and likely by restricting the nuclease accessibility. In addition, the *in vitro* inhibition of telomerase activity by STEP1 is likely due to its direct binding to telomeric DNA, resulting in less accessibility of the telomerase to the substrate DNA because STEP1 and RBD1 binding to telomerase has little influence on *in vitro* telomere-lengthening activity (Fig. 4). Unfortunately, we failed to isolate any homozygous T-DNA insertion *cp31* lines (data not shown), indicating that *CP31* is an essential gene. However, our previous and current data suggest that STEP1 is an additional plant telomere-protecting protein that limits the access of nucleases and telomerase to the chromosomal end 3′ overhang. The interaction of STEP1 with telomerase also suggests that depending on the length of single strand telomere, STEP1 may differentially regulate the accessibility of telomerase, because at low concentra-

tions STEP1 addition has no effect on the telomere-lengthening activity of telomerase (Fig. 3A). Therefore, it is possible that STEP1 may control the telomere length during S phase by protecting it from nuclease attack and by allowing telomerase access when short, but by inhibiting when long enough.

ACKNOWLEDGMENTS

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