Published online 15 November 2006 *Nucleic Acids Research, 2006, Vol. 34, No. 21* ***6327–6336***
doi:10.1093/nar/gkl786

The telomerase-recruitment domain of the telomere binding protein Cdc13 is regulated by Mec1p/Tel1p-dependent phosphorylation

Shun-Fu Tseng[1], Jing-Jer Lin[2] and Shu-Chun Teng[1,3,*]

[1]Department of Microbiology, College of Medicine, National Taiwan University, Taipei, Taiwan, [2]Institute of Biopharmaceutical Science, National Yang-Ming University, Taipei, Taiwan and [3]Institute of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan

Received August 23, 2006; Revised and Accepted September 28, 2006

ABSTRACT

The DNA damage-responsive protein kinases ATM and ATR phosphorylate SQ/TQ motifs that lie in clusters in most of their *in vivo* targets. Budding yeast Cdc13p contains two clusters of SQ/TQ motifs, suggesting that it might be a target of Mec1p/Tel1p (yeast ATR/ATM). Here we demonstrated that the telomerase recruitment domain of Cdc13p is phosphorylated by Mec1p and Tel1p. Gel analysis showed that Cdc13p contains a Mec1/Tel1-dependent post-translational modification. Using an immunoprecipitate (IP)-kinase assay, we showed that Mec1p phosphorylates Cdc13p on serine 225, 249, 255 and 306, and Tel1p phosphorylates Cdc13p on serine 225, 249 and 255 *in vitro*. Phenotypic analysis *in vivo* revealed that the mutations in the Cdc13p SQ motifs phosphorylated by Mec1p and Tel1p caused multiple telomere and growth defects. In addition, normal telomere length and growth could be restored by expressing a Cdc13–Est1p hybrid protein. These results demonstrate the telomerase recruitment domain of Cdc13p as an important new telomere-specific target of Mec1p/Tel1p.

INTRODUCTION

Telomeres are dynamic DNA–protein complexes that protect the ends of linear chromosomes, prevent detrimental chromosome rearrangements and defend against genomic instability and the associated risk of cancer (1–3). Telomeres, consisting of tandem repeats of short G-rich sequences, are synthesized by the enzyme telomerase (4,5). The catalytic core of telomerase is composed of a reverse transcriptase and an RNA subunit. The reverse transcriptase utilizes the RNA component as a template to add the G-rich repeats onto the 3′ ends of the chromosome (4–6). In most human somatic cells, telomerase activity is absent, and telomeres are gradually shortened with successive cell divisions due to incomplete replication, which eventually causes replicative senescence. Once telomeres become sufficiently short, they are thought to lose the ability to protect the ends of the chromosomes from being recognized as broken ends, and being subjected to nuclease digestion and active recombinational repair. Continuous telomere shortening in human fibroblasts leads to chromosome fusions, crisis and apoptosis (7). Very few human cells can bypass the crisis either through telomerase reactivation or through an alternative recombination pathway for telomere lengthening (8–10).

In budding yeast *Saccharomyces cerevisiae*, several genes encoding components of the telomerase enzyme have been identified and mutations in these genes cause a gradual loss of telomere length (11,12). *EST2* and *TLC1* encode the reverse transcriptase catalytic protein subunit and the templating RNA, respectively (12–14). In addition, the protein encoded by *EST1* is associated with the RNA component of telomerase and is bound to single-stranded telomeric DNA *in vitro* (15–18). Other accessory factors, such as Cdc13p, are required for the *in vivo* action of telomerase. Cdc13p is a single-strand telomere binding protein (19–23). Studies of different alleles of *CDC13* revealed that Cdc13p is involved in both telomere protection and telomerase recruitment (21,24–26). A mutation allele of *cdc13*, *cdc13-2* (a Glu252→Lys change), causes a gradual loss of telomere length (21) whereas a truncated form of *cdc13*, *cdc13-5* (an N-terminal amino acids 1–694 fragment of Cdc13p), has long telomeres (25). Genetic and physical interactions were also observed between *CDC13* and *EST1* (21,24,27). Therefore, Cdc13p may cooperate with Est1p to recruit telomerase to telomeres or activate telomere-bound telomerase for its replication (22,28).

In eukaryotes, the maintenance of genome integrity relies on a set of surveillance systems, called checkpoints. These checkpoints are responsible for proper detection and repair of DNA damage caused by environmental stresses or irregularities

*To whom correspondence should be addressed. Tel: +886 2 2312 3456, ext. 8282; Fax: +886 2 23915293; Email: scteng@ha.mc.ntu.edu.tw

during DNA metabolisms. Damage and replication defects are recognized by the putative protein complex containing protein kinases such as human ATM and ATR (ATM-related), and fission yeast *Schizosaccharomyces pombe* Rad3 (29). *S.cerevisiae MEC1* and *TEL1* are homologous to the human ATR and ATM, respectively (30). Mec1p and Tel1p function upstream in the checkpoint pathway by phosphorylation of serine and threonine residues in the context of SQ or TQ motifs (31). Mec1p and Tel1p are important regulators of a group of proteins that detect, signal and repair DNA damage (32,33). Interestingly, the loss of Mec1p results in a modest decrease in telomere length, while telomeres are very short but stable in a *tel1* strain. Cells lacking both *TEL1* and *MEC1* have very short telomeres and undergo senescence similar to that observed in telomerase-deficient cells (34,35). Furthermore, telomerase activation requires the ATM kinase functions in humans, *S.cerevisiae* and *S.pombe* (35–39). These results suggest that proteins involved in DNA damage checkpoints may play a role in telomere replication.

We previously showed that *CDC13*, *MEC1* and *TEL1* are all involved in telomere–telomere recombination (40). In this study, we demonstrate that Cdc13p is an *in vivo* target of Mec1/Tel1 kinases. The telomerase recruitment domain of Cdc13p is a direct substrate for the Mec1/Tel1 kinase as evident by the immunoprecipitate (IP)-kinase assay. Phosphorylation sites on Cdc13p were identified and the telomeric phenotypes of yeast strains carrying mutations on the phosphorylation sites were examined.

MATERIALS AND METHODS

Strains, plasmids, yeast and telomere experiments

All the yeast operations were performed by standard methods (41). Yeast strains used in the study were derivatives of YPH501 (*MAT***a**/*MAT*α*A ura3-52*/*ura3-52 lys2-801 amber*/*lys2-801 amber ade2-101 ochre*/*ade2-101 ochre trp1*α*Δ63*/*trp1*α*Δ63 his3*α*Δ200*/*his3*α*Δ200 leu2-*α*Δ1*/*leu2-*α*Δ1*). The yeast strains carrying *mec1*, *sml1*, *tel1*, *cdc13* and *CDC13-Myc*$_9$ were described previously (15,23,40). YEp24*CDC13-URA3* was constructed as previously described (26). pRS314*CDC13*, pRS304*cdc13* and pRS304*cdc13*-Myc$_9$ were obtained from Dr Virginia Zakian (23,27). The 1 kb SacII fragment from pRS304*cdc13*-Myc$_9$ was inserted into the SacII-digested pRS314*CDC13* to make pRS314*CDC13*-Myc$_9$. Point mutations were introduced into the *CDC13* using QuikChange site-directed mutagenesis (Stratagene). To generate chromosomal *cdc13* mutants, pRS304*cdc13* mutants were XhoI-digested and transformed into *CDC13* strains. pVT1091 (p*CDC13-EST1*) plasmid (28) was kindly provided by Dr Victoria Lundblad. To generate pVL1091 mutant plasmids, the 1.4 kb BamHI–StuI fragments in pRS314*CDC13*-AA or pRS314*CDC13*-EE mutants were cloned into the BamHI–StuI-digested pVT1091. We carried out cellular senescence assays as described (40). All primer sequences for PCR and mutagenesis are available upon request. Telomere blot analysis and serial liquid dilutions were performed as described previously (42,43). Cultures were inoculated directly from the FOA plate into 10 ml of liquid YEPD medium and allowed to grow to stationary phase on a 30°C roller drum. The cultures were diluted repeatedly 1:10 000 into fresh medium for 48 or 72 h for six times. Data shown here are representatives of three or more experiments from independent colonies.

Immunoprecipitation, gel electrophoresis and western blot analysis

To detect Cdc13p, cells were grown in YPD broth. Whole cell proteins were extracted and resolved by SDS–PAGE as described previously (44). Cdc13 protein was detected with an anti-Myc antibody (Santa Cruz Biotechnology). Immunoprecipitation of Cdc13-Myc was done with agarose-conjugated 9E10 monoclonal antibodies (sc-40; Santa Cruz Biotechnology). For 2D gel electrophoresis separations, immunoprecipitated proteins were denatured in 2D sample buffer (7 M Urea, 2 M Urea, 4% CHAPS, 1% DTT and 2% IPG buffer). Proteins were separated by isoelectric focusing using the IPGphor apparatus (Amersham Biosciences) and active in-gel rehydration as described on immobilized linear pH gradient (IPG, pH 4–7) strips (Amersham Biosciences). Isoelectric focusing was performed for a total of 30 000 Vh, starting at 200 V and gradually raising the voltage to 5000 V. The IPG strip was then equilibrated for 15 min in 50 mM Tris (pH 8.8), 6 M urea, 30% glycerol, 1% SDS and 1% DTT, and for 20 min in 50 mM Tris, 6 M urea, 30% glycerol, 1% SDS and 5% iodoacetamide before loading on to SDS–polyacrylamide slab gels (5%) for separation according to molecular mass.

Phosphatase treatment of immune complexes of Cdc13p

Agarose-bound Cdc13p immunocomplexes were additionally washed twice with basic phosphatase buffer (50 mM Tris–HCl, pH 7.5, 5 mM DTT and 2 mM $MnCl_2$) and then incubated with 100 units of calf intestinal phosphatase (New England Biolabs) for 30 min at 30°C. The reaction was stopped by addition of ice-cold basic kinase buffer containing 10 mM Na_3VO_4 and 10 mM NaF. Phosphatase-treated immune complexes were washed twice with basic kinase buffer and used in kinase assays as described below.

Recombinant protein purification

Plasmid pGEX-4T*CDC13*(129–332) was constructed by ligating a HindIII–Klenow fragment containing amino acids 129–332 of Cdc13 from pRS314*CDC13* into BamHI–Klenow treated pGEX-4T (Amersham/Pharmercia). Plasmid pGEX-4T*CDC13*(570–676) was constructed by ligating a PCR product containing amino acids 570–676 of Cdc13 from pRS314*CDC13* into BamHI–Klenow and XhoI treated pGEX-4T. Plasmid pET28c*CDC13*(185–332) was constructed by ligating a BamHI–Klenow and NotI treated fragment containing amino acids 185–332 of Cdc13p from pGEX-4T*CDC13*(129–332) into pET28a (Novagen) with the same sites. Plasmid pGEX-4T*CDC13*(185–332) was constructed by ligating a NcoI–Klenow and XhoI-treated fragment containing amino acids 185–332 of Cdc13p from pET28c*CDC13*(129–332) into BamHI–Klenow and XhoI-treated pGEX-4T. Plasmid pGEX-4T*CDC13*(276–332) was constructed by self-ligating the BamHI–NsiI–Klenow fragment of pGEX-4T*CDC13*(185–332) backbone. Plasmid pGEX-4T*CDC13*(185–275) was constructed by self-ligating

the NsiI–XhoI–Klenow fragment of pGEX-4T*CDC13*(185–332) backbone. Recombinant proteins in *E.coli* were overexpressed and purified as described by the manufacturer (Amersham Biosciences). Glutathione–agarose was used in our association assays. The bound GST-fusion proteins were detected by Coomassie blue staining.

Immunoprecipitation-kinase analysis

Log phase cells were lysed and immunoprecipitated by incubation with anti-HA antibody and protein A beads. The IP-kinase assay was performed as described (45). Strains KSC1333 (*MEC1-HA sml1::HIS3*), KSC1752 [*mec1-HA(KD) sml1::HIS3*], LSS93 (*HA-TEL1*) and LSS233 [*HA-Tel1(KD)*](45,46) were kindly provided by Drs Katsunori Sugimoto and Akira Matsuura.

In vivo telomerase assay

In vivo telomerase assay was performed as described previously (47). UCC5706 strain was kindly provided by Dr Daniel Gottschling. *CDC13*, *cdc13*(AA) and *cdc13*(EE) were generated by one-step chromosomal insertion using XhoI-digested pRS304*CDC13*, pRS304*cdc13*-AA and pRS304*cdc13*-EE.

Two-hybrid assay

The two-hybrid assay was performed essentially as described (48). To construct pACT-*POL1*, a 1.5 kb DNA fragment encoding amino acids 47–560 of Pol1p (48) was PCR amplified. The PCR product was cloned into pGEMTeasy (Promega). The BamHI/SalI-digested *POL1* from pGEMTeasy-*POL1* was inserted into the BamHI/XhoI-digested pACT2. To construct the full-length *CDC13* and *cdc13*-AA in pAS2, the NcoI/SalI fragment of *CDC13* from pTHA-*CDC13* (20) was inserted into the NcoI/SalI-digested pAS2. Point mutations were introduced into the *CDC13* as described above. Plasmids were transformed into yeast strain Y190 (48). Transformed clones were selected on SC-Leu, SC-Trp plates and confirmed for interaction on SC-Leu, SC-Trp, SC-His plates with 3-AT. Fresh transformed colonies of 5–10 were mixed and spotted in 10-fold serial dilutions onto plates. Plates were kept at 30°C until colonies formed. Liquid lacZ assays were done as described in Miller (49) and Guarente (50).

RESULTS

MEC1/TEL1-dependent Cdc13p phosphorylation *in vivo*

In order to further understand the biological regulation of Cdc13p, we first investigated whether Cdc13p could be phosphorylated *in vivo*. Wild-type yeast cells containing chromosomal Myc-tagged Cdc13p were subjected to western blot analysis to detect potential phosphorylation status. As telomere length was normal in cells expressing this Myc-tagged protein (data not shown), this tagged gene was able to provide the telomere functions of Cdc13p. Western blot analysis showed that levels of Cdc13-Myc were equivalent in the wild-type, *mec1* and *tel1*strains, but slightly decreased in the *mec1 tel1* strain (Figure 1A). The migration of Cdc13p bands in gel was slightly faster in the *mec1 tel1* background, suggesting that Cdc13p might be

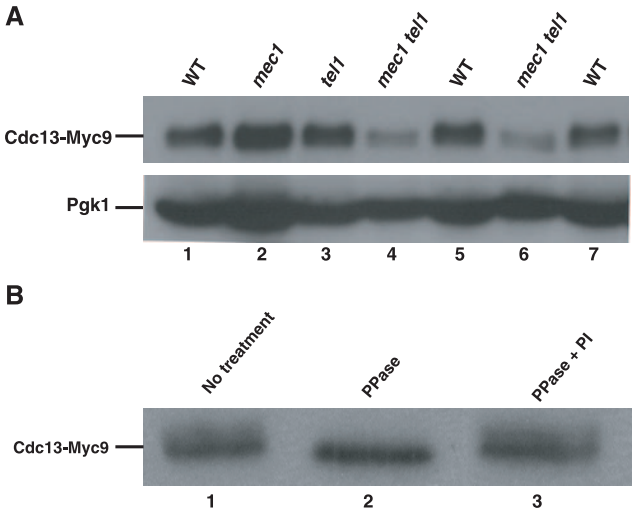

Figure 1. *MEC1/TEL1*-dependent phosphorylation of Cdc13p *in vivo*. (**A**) Cdc13p phosphorylation was analyzed by western blot analysis. Lysates from different strains were TCA precipitated for western blot analysis to detect Cdc13p by a Myc antibody. Pgk1p is the loading control. (**B**) Phosphatase treatment of wild-type Cdc13p. Immunoprecipitated Cdc13p was incubated in PPase buffer, PPase buffer with PPase, or PPase buffer containing PPase and PPase inhibitors (PI). Western blot analysis was performed as in (A).

phosphorylated in a *MEC1/TEL1*-dependent manner *in vivo* (Figure 1A). To test whether Cdc13p is phosphorylated *in vivo*, we treated the Myc-antibody precipitated Cdc13p with phosphatase (PPase). When Cdc13p was treated with calf intestinal PPase, the smear bands of Cdc13p diminished (Figure 1B). PPase converted the electrophoretic behavior of Cdc13p from broad fuzzy bands to a sharp single band with greater mobility. When lysates were treated with PPase in the presence of the PPase inhibitor, Cdc13p migrated to a similar position of the untreated protein (Figure 1B, lane 3). Because Cdc13p from the *mec1 tel1* strain still migrated as doublets (Figure 1A, lane 4) and the gel mobility of Cdc13p in the *mec1 tel1* strain did not migrate to the same position as that of PPase-treated wild-type Cdc13p (Figure 1B, lane 2 and data not shown), Cdc13p might be phosphorylated in a *MEC1/TEL1*-dependent as well as a *MEC1/TEL1*-independent manner *in vivo*.

Since Cdc13-Myc is a relatively big protein (118 kDa) and 1D gel electrophoresis was hard to clearly resolve the difference of modifications in Cdc13p, to gain further insight into the phosphorylations of Cdc13p by Mec1/Tel kinases, we separated these modified Cdc13p by using 2D gel electrophoresis. Different forms of Cdc13p were resolved by isoelectric focusing (pH 4–7 linear gradient) followed by SDS–PAGE and then detected by western blotting using a Myc antibody. In addition to the predicted Cdc13p-Myc at pI 5.31 (spot 2 in Figure 2A), a variant form at pI 5.22 (spot 1) (assuming the linearity of the supplied pH gradient gels) was observed (Figure 2A). However, in the *mec1 tel1* strain, this spot disappeared (Figure 2A). PPase-treatment also made the spot 1 of Cdc13p of wild-type strain vanished (Figure 2B). These results suggest that the expression of a variant Cdc13p was contributed by Mec1/Tel1 kinases-mediated phosphorylation. Owing to the limited availability

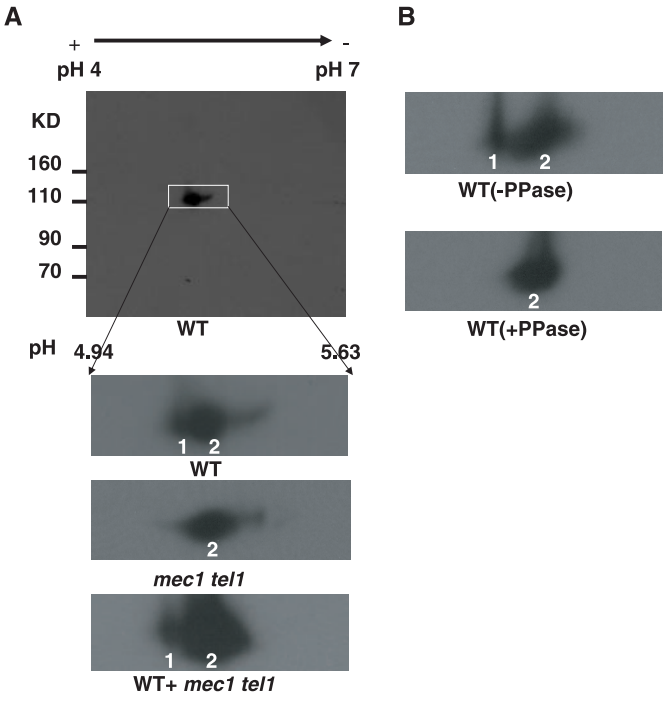

Figure 2. 2D gel electrophoresis analysis of Cdc13p. (**A**) Western blot analysis of the 2D gel electrophoresis pattern of Cdc13p from wild-type or *mec1 tel1* cells. Immunoprecipitated Cdc13p was subjected to 2D gel electrophoresis and western blot analysis. The membrane was detected using a Myc antibody. Area of Cdc13p was enlarged at the bottom. As a merged control, equal amount of precipitated Cdc13p from wild-type and *mec1 tel1* cells were combined prior to protein separation. The different Cdc13p forms were numbered according to their position relative to the scale of isoelectric point shifts. (**B**) Western blot analysis of the 2D gel electrophoresis pattern of Cdc13p from untreated or PPase-treated wild-type cells. The assay was performed and presented as in (A).

of commercial strips for isoelectric focusing gel, we could not further improve the separation between spot 1 and 2 in Figure 2. Therefore, we could not rule out the possibility that there are other variant(s) of Cdc13p between spot 1 and 2 contributed by *MEC1*/*TEL1*-dependent as well as *MEC1*/*TEL1*-independent regulation.

The telomerase recruitment domain of Cdc13p is phosphorylated by Mec1/Tel1 immunoprecipitates

The simplest explanation for the *MEC1*/*TEL1*-dependent Cdc13p phosphorylation is that Cdc13p is directly phosphorylated by Mec1p/Tel1p. The ATM/ATR family of kinases has a preference for phosphorylation of serine in SQ motifs or threonine in TQ motifs (31). Cdc13p contains 10 such motifs (Figure 3A). Moreover, these types of S/TQ motifs are usually located in clusters (31,51). Two such clusters in Cdc13p were found: one in the telomerase recruitment domain (RD) (S225, S249, S255 and S306) and the other in the DNA binding domain (DBD) (S611, S643 and S652) (Figure 3A). As the first step in determining whether Cdc13p is a substrate of Mec1p/Tel1p, we tested whether Mec1p/Tel1p could phosphorylate Cdc13p *in vitro*. Immunoprecipitated Mec1 and Tel1 kinases were tested for its ability to phosphorylate Cdc13p. Mec1p and Tel1p immunoprecipitates were incubated under *in vitro* kinase conditions in the presence of [γ-^{32}P]ATP and recombinant GST-Cdc13(129–332) or GST-Cdc13(570–676) containing the telomerase recruitment or DNA binding domain, respectively. The reactions were subjected to SDS–PAGE and autoradiography (Figure 3A). The phosphorylated GST-Cdc13(129–332) subunit was observed, while neither GST alone (data not shown) nor the GST-Cdc13(570–676) protein showed detectable phosphorylation by Mec1p or Tel1p. To confirm that Mec1 kinase activity was indeed required for Cdc13 phosphorylation, assays were conducted using extracts isolated from a *mec1* mutant strain. Strain KSC1752 [*mec1-HA*(*KD*)] expresses an HA-tagged Mec1-kd protein with two amino acid changes in its catalytic domain (52) (Figure 3A). Based on assays using equivalent amounts of protein, inactivation of Mec1 kinase activity completely eliminated Cdc13p phosphorylation. Similar results were observed by the Tel1 kinase dead protein (52) (Figure 3A). These results demonstrate that Mec1/Tel1 kinases phosphorylate the telomerase recruitment domain of Cdc13p.

S225, S249, S255 and S306 are the *in vitro* phosphorylation sites of Cdc13p

In order to locate the sites of phosphorylation on Cdc13p, GST was fused to the amino acids 185–275 and 276–332 of the telomerase recruitment domain of Cdc13p, independently. These two recombinant GST-fusion proteins were then purified and used as substrates in IP-kinase assays. As shown in Figure 3B, both GST-fusion proteins were labeled by Mec1p, but only the 185–275 fragment of Cdc13p was phosphorylated by Tel1p. There are three SQ sites (residue 225, 249 and 255) within the 185–275 fragment of Cdc13p and one SQ site (residue 306) within the 276–332 fragment of Cdc13p. To determine the exact phosphorylation sites, we purified mutant GST-Cdc13p containing single, double or triple serine (S) to alanine (A) mutations at these sites. These substrates were then used in the IP-kinase assay. Compared to wild-type Cdc13p, phosphorylation of the triple mutant Cdc13(185–275) subunit was completely abolished (Figure 3B). Consistent with the previous results (Figure 3B), the Cdc13(276–332), but not the Cdc13(276–332)S306A, was phosphorylated by Mec1p (Figure 2B). These results indicate that the phosphorylation sites of Cdc13p by Mec1p are located at amino acids 225, 249, 255 and 306, and the phosphorylation sites of Cdc13p by Tel1p are located at amino acids 225, 249 and 255. Therefore, we conclude that S225, S249, S255 and S306 are the major phosphorylation sites of Cdc13p by Mec1/Tel1 kinases (Figure 3C).

Phenotypic analysis of *cdc13-S* mutants

To identify the potential cellular functions for Mec1/Tel1-mediated phosphorylation of Cdc13p, we tested whether mutations in these phosphorylation sites would affect the growth and replication of telomeres. In order to examine these phenotypes, a strain STY1077 was created in which the chromosomal *CDC13* gene was deleted and was complemented by *CDC13* on a single-copy *URA3* plasmid (Figure 4A). STY1077 was transformed with either

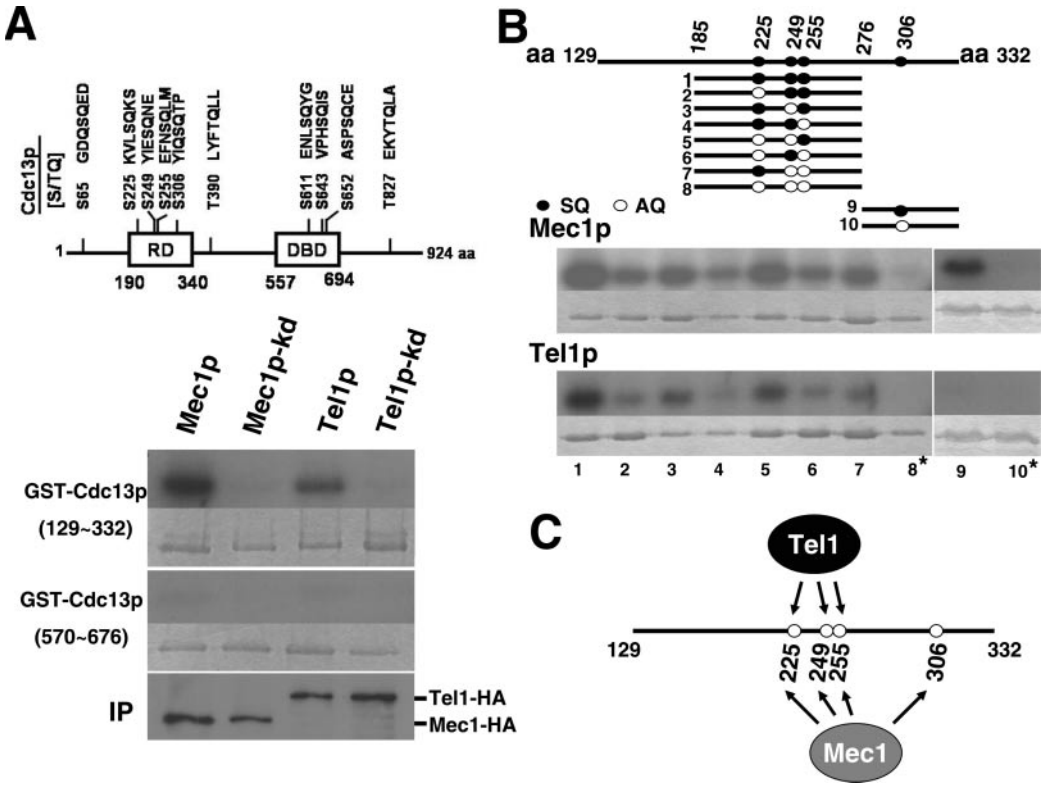

Figure 3. Cdc13p is directly phosphorylated by Mec1p and Tel1p *in vitro*. (**A**) Phosphorylation of the telomerase recruitment domain of Cdc13p. Schematic diagram of Cdc13p illustrates its domain structure and potential S/T phosphorylation sites. GST-fusion proteins to the telomerase recruitment (RD) and DNA binding (DBD) domains of Cdc13p were purified. Strains KSC1333 (*MEC1-HA sml1*::*HIS3*), KSC1752 (*mec1-HA*(*KD*) *sml1*::*HIS3*), LSS93 (*HA-TEL1*) and LSS233 (*HA-Tel1*(*KD*)) were lysed, and extracts were immunoprecipitated with anti-HA antibodies. Immunoprecipitated kinases were analyzed by SDS–PAGE and western blotted with anti-HA antibody (shown at the bottom). IP-kinase assay was performed with GST-Cdc13p(185–332) or GST-Cdc13p(570–676) and [γ-^{32}P]ATP. Proteins were resolved by SDS–PAGE and the phosphorylated proteins were detected by autoradiography (shown on top). The kinase reactions were also Coomassie stained (shown below) to confirm that lanes were equally loaded. (**B**) Mec1p/Tel1p phosphorylation sites in the telomerase recruitment domain of Cdc13p. The kinase assay was performed as in (A) except that purified GST–Cdc13p(185–275) or GST–Cdc13p(276–332) was included in the kinase reaction. IP-kinase assays were performed as in (A) using strains KSC1333 (*MEC1-HA sml1*::*HIS3*) and LSS93 (*HA-TEL1*). Schematic diagram of Cdc13p illustrates the positions of four SQ motifs in the telomerase recruitment domain. Amino acid positions are numbered at the top. Recombinant wild-type and mutant GST–Cdc13p containing the S to A mutations were used as substrates in the Mec1-HA kinase assay (left) and Tel1-HA kinase assay (right). Asterisks indicate the complete abolishment of phosphorylation. (**C**) Summary of the Mec1p and Tel1p phosphorylation sites in the Cdc13p telomerase recruitment domain between amino acids 129 and 332.

CDC13 or different *cdc13-S* mutant genes on low-copy plasmids. Cells carrying *cdc13-S* mutants were obtained following selection against the *CDC13* plasmid by growth on 5-FOA. As shown in Figure 4B, single mutations from S to alanine (A), aspartic acid (D) and glutamic acid (E) at 249 and 255 all caused telomere shortenings, indicating that these two residues are important for telomere maintenance. Moreover, while single mutations S225A and S306A caused no observable telomere and growth defects, single mutations S249A and S255A maintained short telomeres stably after several streakings (Figure 4B and data not shown) and double mutations S249/255AA resulted in gradual telomere shortening and cellular senescence (Figures 5 and 6). The S225/249/255AAA triple mutant grew poorly even in the first restreak (Figure 6) and the S225/249/255/306AAAA mutant was lethal on the tetradissection plates (data not shown). Interestingly, this cellular senescence of S249/255AA mutant was not observed in mutation from S to D (S249/255DD) or E (S249/255EE) (Figure 6). Short telomeres were stably maintained in these double mutations S249/255DD and S249/255EE strains (Figure 5 and data not shown), suggesting that modified acidic charge residues may partially mimic phosphorylation status and suppress the phenotype. The expression pattern of Cdc13p (Supplementary Figure S1) and the amount of single-stranded telomeres (Supplementary Figure S2) of individual mutant in *S.cerevisiae* are quite similar to those of wild-type, suggesting that these phenotypic alterations may not be related to protein stability and telomere binding ability of Cdc13p, respectively. Altogether, these results highlight the significance of these phosphorylation sites for the telomere replication by the telomerase.

S249 and S255 of Cdc13p are required for the efficient *in vivo* action of telomerase

To determine whether these phosphorylations are essential for the action of telomerase *in vivo*, we tested the sudden telomere elongation by the *in vivo* telomerase assay developed by Diede and Gottschling (47). Strains were arrested in M phase by nocadazole treatment, and then subjected to induction of *HO* expression. In contrast to wild-type cells

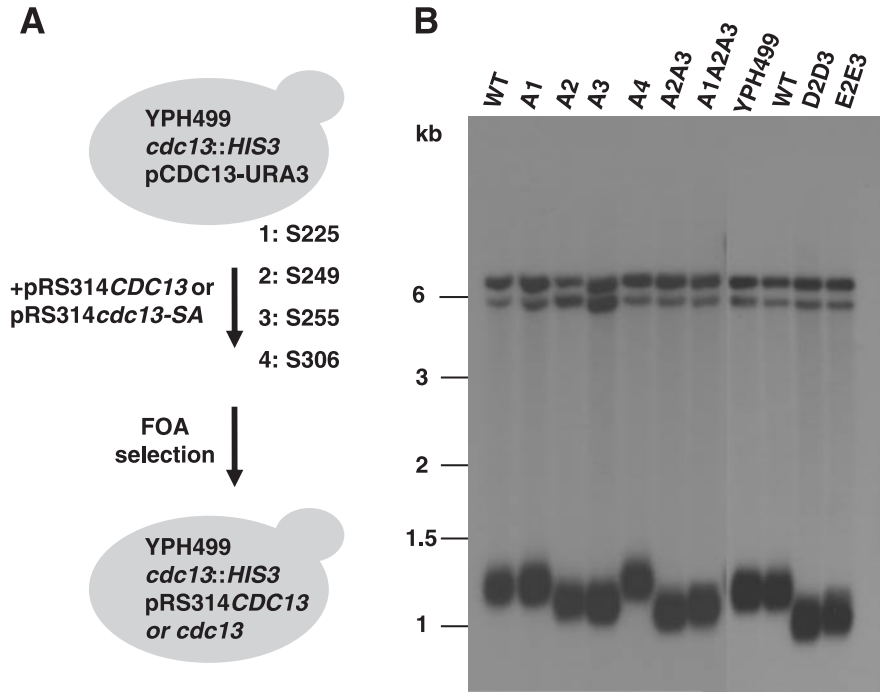

Figure 4. Telomere analysis of *cdc13-S* mutants. (**A**) A *cdc13* strain was constructed that also carried a *CDC13 URA3* plasmid. Wild-type or *cdc13* mutants on a *CEN TRP1* plasmid were introduced into this strain. Since the drug 5-fluoro orotic acid (5-FOA) kills cells expressing Ura3p, only cells that lost the *CDC13 URA3* plasmid will grow on FOA plates. (**B**) Mec1p/Tel1p phosphorylation sites were mutated from S to alanine (A), aspartic acid (D) and glutamic acid (E). The telomere lengths were monitored in these *cdc13*-S mutant strains. DNA from each strain was digested with XhoI, separated in a 1% agarose gel, transferred to a nylon membrane, and hybridized with a Y′ probe. DNA from the wild-type YPH499 was loaded as a control. Collected yeast cells have lost the *CDC13 URA3* plasmid for at least 50 generations.

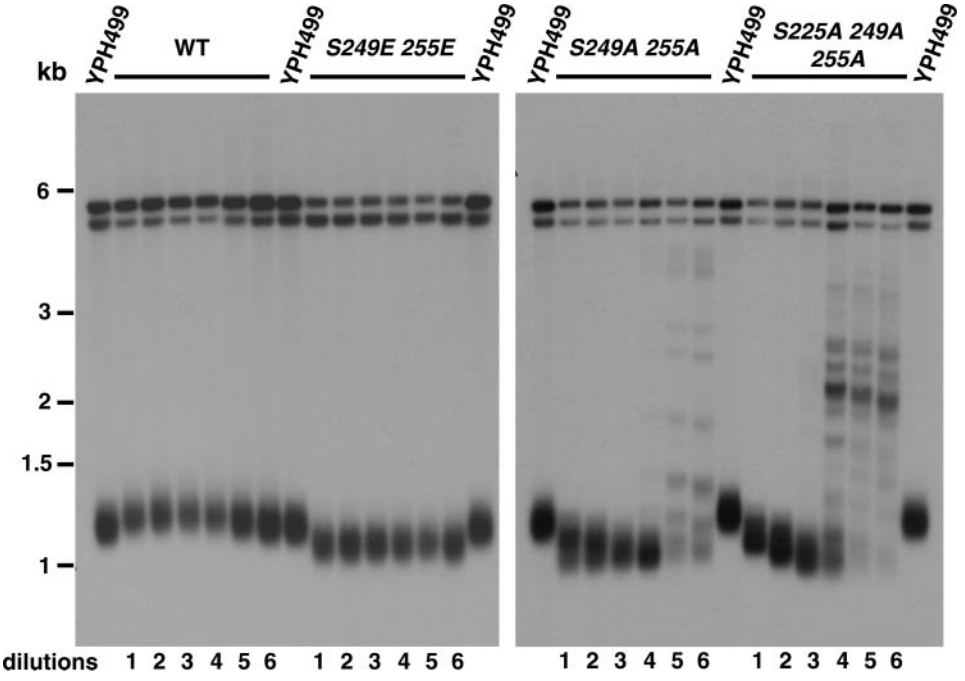

Figure 5. Telomere analysis of *cdc13-S* mutants following cell divisions. Assays were conducted as described in Figure 4. The telomere lengths were monitored in these *cdc13-SA* and *cdc13-SE* mutant strains. Genomic DNA from serial liquid dilutions was digested with XhoI, separated in a 1% agarose gel, transferred to a nylon membrane, and hybridized with a Y′ probe. DNA from the wild-type YPH499 was loaded as a control.

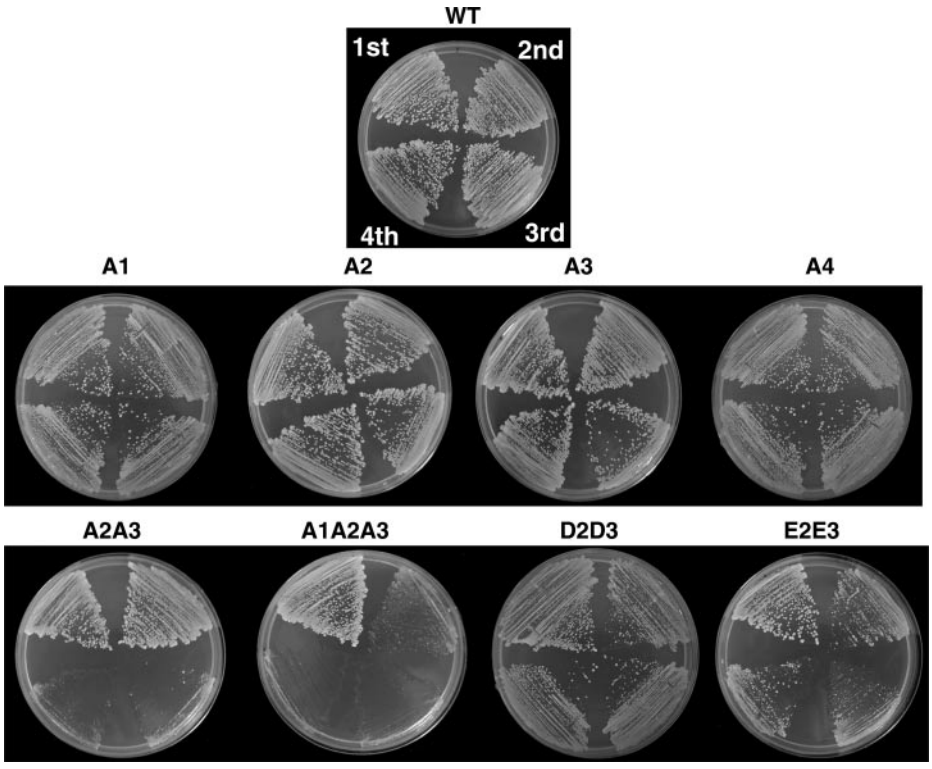

Figure 6. Growth of *cdc13-S* mutants. Spores of each strain were obtained from tetradissection. Each strain was repeatedly streaked on solid YEPD plates and grown for 3 days at 30°C. Cells from the first four restreaks are shown. It was noticeable that *cdc13AA* and *cdc13AAA* spores senesced fast, and S249/*255DD* or *EE* mutations suppressed this phenotype. This suppression was maintained at additional restreaks (data not shown).

which showed telomere elongations at the HO cut site, the TG_{1-3}/HO end was not elongated in S249/255AA and S249/255EE cells (Figure 7). These results indicate that phosphorylation of Cdc13p at amino acids 249 and 255 are required for the proper *in vivo* addition of telomeres.

Telomere lengthening by targeted Est1p occurs efficiently in the absence of the Mec1/Tel1 phosphorylation sites of Cdc13p

We demonstrated that *cdc13* phosphorylation mutants exhibited a strong defect in the telomere length regulation. Because these phosphorylation sites exist in telomerase recruit domain, we hypothesized that the recruitment of telomerase was blocked due to a reduced protein–protein interaction between Cdc13p and Est1p. We speculated that the *cdc13* mutation would be suppressed if telomeric binding of Est1p was forced by expression of a hybrid protein consisting of Cdc13p and Est1p. To test this, we examined the consequences of fusing Cdc13p to Est1p in telomere maintenance. We transformed wild-type and *cdc13* cells with the plasmid p*CDC13–EST1* (expressing the Cdc13–Est1 fusion) (28). Efficient telomere lengthening occurred in the *cdc13* mutant (>600 bp) (Figure 8), and the growth defect of *cdc13* mutant cells was suppressed by the transformation of p*CDC13-EST1* or p*cdc13-EST1*-S249/255AA (data not shown). This phenotype was observed in every clone that we analyzed, although in the p*cdc13-EST1*-S249/255AA containing cells (Figure 7, lane 6) the extent of telomere elongation was slightly shorter than that in the p*CDC13-EST1* containing cells (Figure 7, lane 5). These results confirmed that the telomere length defect associated with the phosphorylation mutation of Cdc13p can be complemented by targeting Est1p onto the telomere.

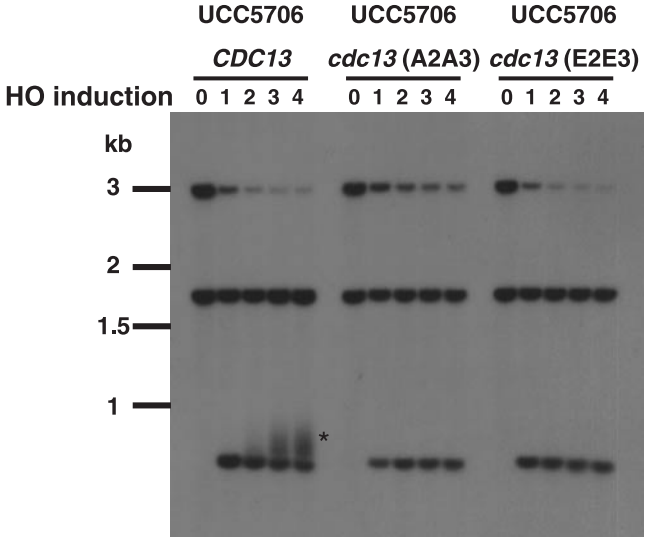

Figure 7. Lack of the *in vivo* telomere addition in *cdc13*-SA mutants. UCC5706 cells were introduced with wild-type or *cdc13-S* mutants. Resulting strains were arrested using nocodazole. When cells were arrested, galactose was added to induce HO endonuclease expression. DNA was isolated after 0, 1, 2, 3 and 4 h, and digested with SpeI and analyzed with a fragment that recognizes sequence distal to the HO recognition site. Telomere addition (*) was observed in the wild-type, but not in the *cdc13*AA or EE strain.

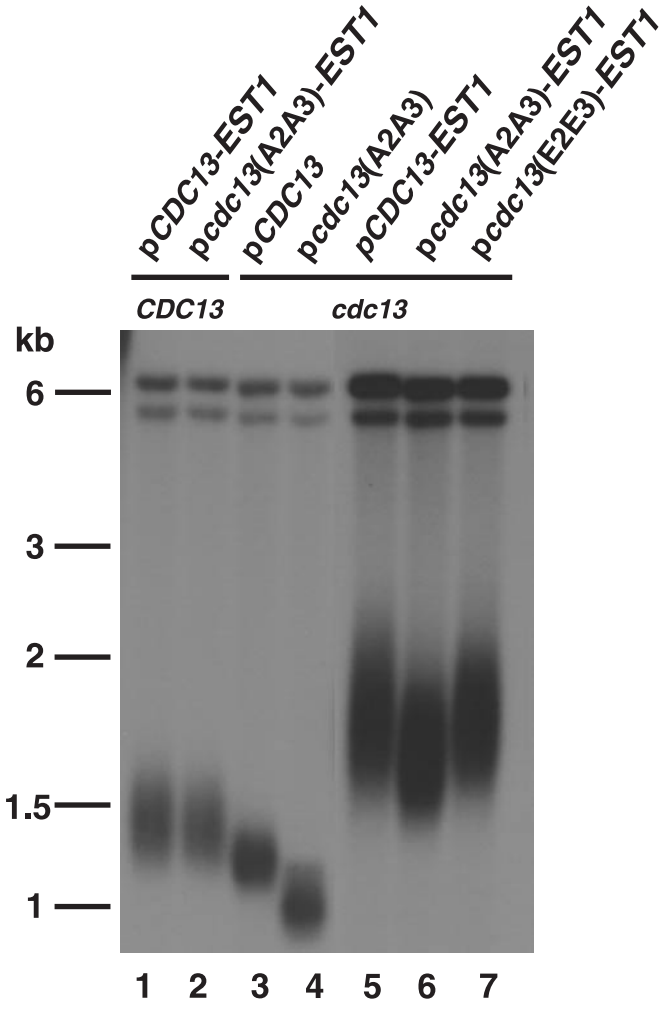

Figure 8. Telomere lengthening by targeted Est1p in *cdc13* cells. Telomere length was examined in strains of the indicated genotypes carrying a *CDC13-EST1* (lanes 1 and 5), *cdc13*AA-*EST1* (lanes 2 and 6), *cdc13*EE-*EST1* (lane 7), *CDC13* (lane 3) or *cdc13*AA (lane 4) plasmid. These strains were obtained as described in Figure 3. DNA was prepared at ~60 cell divisions after the loss of the *CDC13 URA3* plasmid. DNA from each strain was digested with XhoI, separated in a 1% agarose gel, transferred on to a nylon membrane, and hybridized with a Y′ probe.

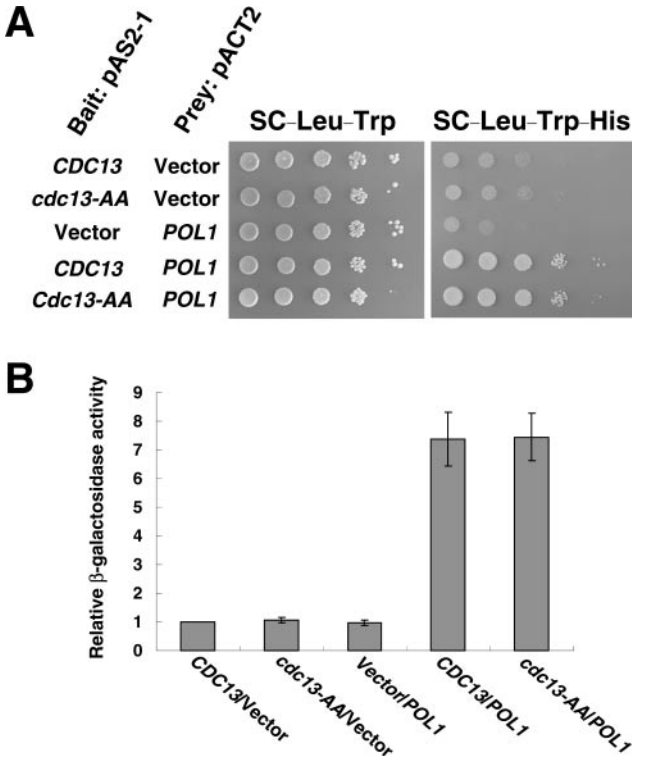

Figure 9. Interaction of Cdc13p-AA with Pol1p. (**A**) Yeast cells Y190/pAS2-1, Y190/pAS2-1-*CDC13* or Y190/pAS2-1-*cdc13-AA* carrying plasmid pACT2 or pACT2-*POL1* were grown on SC medium lacking leucine and tryptophan at 30°C. Cells were then analyzed by their ability to grow in the absence of histidine. Ten-fold serial dilution of cells were spotted on glucose plates either without leucine and tryptophan or without leucine, tryptophan and histidine and incubated at 30°C until colonies formed. Photographs of the plates are shown. (**B**) Extracts were produced from strains expressing both bait and prey vectors as indicated below. Data are the average of three independent β-galactosidase measurements. Error bars, SD.

Mutations at the Mec1p/Tel1p phosphorylation sites do not affect the Cdc13p–Pol1p interaction

It is possible that the phosphorylation site mutants might be simply improperly folded or otherwise perturb Cdc13p functions. To demonstrate that some activity of these *cdc13* mutants is preserved, we tested the Cdc13p–Pol1p interaction in *cdc13*-AA mutants. Cdc13p was shown to interact with Pol1p, the catalytic subunit of DNA polymerase α (27,48). Disruption of the activity of the lagging strand DNA replication machinery leads to unregulated elongation of the G-rich strand, accompanied by an impaired ability to regulate synthesis of the C-rich strand. (53–55). These data suggested that DNA polymerase α and Cdc13p play an important role in the coordination of telomerase action and the replication of the C-rich strand at chromosome ends. We demonstrated that Cdc13p interacted with Pol1p in a two-hybrid assay (Figure 9). Moreover, mutations in the Mec1p/Tel1p phosphorylation sites did not cause a loss of interaction. These results indicate that the phosphorylation mutations of Cdc13p may not perturb the global folding of Cdc13p.

DISCUSSION

The telomerase recruitment function of Cdc13p was previously demonstrated by expression of a Cdc13p–Est1p or a Cdc13p–Est2p fusion protein which supplants the need for Est1p in telomere maintenance (28), Cdc13p–Est1p two-hybrid interaction and other biochemical criteria (24,27). Here we report that the telomerase recruitment domain of Cdc13p is regulated by checkpoint kinases Mec1 and Tel1. Blockage of the Mec1p/Tel1p-mediated phosphorylation in Cdc13p results in an obstacle of telomere lengthening and cell proliferation. Although it remains possible that these mutations may affect sites of phosphorylation rather than simply eliminating phosphorylation sites, our *in vitro* data prefer the idea that those sites are direct substrates of Mec1/Tel1 kinases. Interestingly, the Cdc13p S249/255EE mutant does not display senescence phenotype (Figure 6), suggesting that the negative charge residues in these glutamic acids may mimic the Mec1p/Tel1p-mediated phosphorylation. However, it still exhibits telomere

shortenings (Figures 4 and 5) and defect of *de novo* telomere addition (Figure 7) under our assay condition. One explanation for these results is that Cdc13–EEp might partially, but not completely, replace the function of Mec1p/Tel1p-mediated phosphorylation on Cdc13p.

It was shown previously that a *mec1 tel1* strain has an *est* phenotype: its telomeres progressively shorten, and *mec1 tel1* cells senesce (35). Furthermore, when the Cdc13–Est1p fusion protein was expressed in *mec1 tel1* cells, telomere length was even longer than in the wild-type strain (23), which bypass the cellular senescence phenotype of the *mec1 tel1* cells (23). Our results elucidate that the cellular senescence phenotype of the *mec1 tel1* cells was derived from the lack of phosphorylation of Cdc13p S249 and S255 at the telomerase recruitment domain by Mec1p/Tel1p. Once this telomerase recruitment function is provided by the fusion protein, *mec1 tel1* cells no longer senesce (23). Moreover, the reduction of Cdc13p level in *mec1 tel1* cells (Figure 1A) may also partly contribute to the senescent phenotype.

There is an intriguing correlation between single-strand DNA binding proteins and checkpoint signaling. For example, RPA was shown to be required for the 'adaptation' response in the presence of a double strand break (DSB) (56). Zou and Elledge (57) showed that human ATRIP and yeast Ddc2 interact directly with RPA-coated ssDNA. Here we investigated the functional regulation between the checkpoint sensor kinases and single-strand telomere binding protein Cdc13. This study was undertaken since a variety of experimental results have implicated Mec1p–Tel1p in checkpoint control in yeast telomere (23,35–37,46,58). Moreover, we found previously that Cdc13p and Mec1p–Tel1p are all involved in telomere–telomere recombination (40). The sites involved in the Mec1–Tel1-mediated regulation in Cdc13p are very close to previously determined Cdc^{EST} site of Cdc13 (24) further highlighting the importance of this regulation. The role of Mec1p/Tel1p in the regulation of Cdc13p at telomeres may be evolutionarily conserved, as their functional homologues are present in human cells.

The controversy as to when and where Mec1p and Tel1p act still exists. Theoretically, phosphorylation may also be possible to be involved in the modification of Cdc13p prior to loading to telomeres. We found that S306 is only phosphorylated by Mec1p *in vitro*. Whether various post-translational modifications occur at different cell cycle and whether this regulates the timing on the action of telomerase remain to be determined. In addition, we also observed some *MEC1*/*TEL1*-independent phosphorylation of Cdc13p. Whether these phosphorylations mediate other functions of Cdc13p and what kinase(s) contribute to *MEC1*/*TEL1*-independent phosphorylation are under investigation.

ACKNOWLEDGEMENTS

We thank Drs Zee-Fen Chang, Ming-Kai Chern, Kou-Juey Wu and Tsai-Kun Li for their discussions and critical comments. We also thank Dr Jean-San Chia, Mr Chi-Chong Lai and Ms Pin-An Chen for their excellent technical assistance, and Drs Victoria Lundblad, Daniel Gottschling, Virginia Zakian, Katsunori Sugimoto and Akira Matsuura for providing plasmids and strains. This work was supported by grants from the National Science Council (NSC95-3112-B-002-023 and NRPGM-95-3112-B-002-023) and National Health Research Institute of Taiwan (NHRI-EX94-9328SI) to S.C.T. Funding to pay the Open Access publication charges for this article was provided by National Health Research Institute of Taiwan.

Conflict of interest statement. None declared.

REFERENCES

1. Shay,J.W., Zou,Y., Hiyama,E. and Wright,W.E. (2001) Telomerase and cancer. *Hum. Mol. Genet*., **10**, 677–685.
2. McEachern,M.J., Krauskopf,A. and Blackburn,E.H. (2000) Telomeres and their control. *Annu. Rev. Genet*., **34**, 331–358.
3. Zakian,V.A. (1996) Telomere functions: lessons from yeast. *Trends Cell. Biol*., **6**, 29–33.
4. de Lange,T. (2002) Protection of mammalian telomeres. *Oncogene*, **21**, 532–540.
5. Greider,C.W. and Blackburn,E.H. (1985) Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell*, **43**, 405–413.
6. Vega,L.R., Mateyak,M.K. and Zakian,V.A. (2003) Getting to the end: telomerase access in yeast and humans. *Nature Rev. Mol. Cell Biol*., **4**, 948–959.
7. Artandi,S.E. and DePinho,R.A. (2000) A critical role for telomeres in suppressing and facilitating carcinogenesis. *Curr. Opin. Genet. Dev*., **10**, 39–46.
8. Bryan,T.M., Englezou,A., Dalla-Pozza,L., Dunham,M.A. and Reddel,R.R. (1997) Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nature Med*., **3**, 1271–1274.
9. Dunham,M.A., Neumann,A.A., Fasching,C.L. and Reddel,R.R. (2000) Telomere maintenance by recombination in human cells. *Nature Genet*., **26**, 447–450.
10. Reddel,R.R., Bryan,T.M., Colgin,L.M., Perrem,K.T. and Yeager,T.R. (2001) Alternative lengthening of telomeres in human cells. *Radiat. Res*., **155**, 194–200.
11. Lendvay,T.S., Morris,D.K., Sah,J., Balasubramanian,B. and Lundblad,V. (1996) Senescence mutants of *Saccharomyces cerevisiae* with a defect in telomere replication identify three additional EST genes. *Genetics*, **144**, 1399–1412.
12. Singer,M.S. and Gottschling,D.E. (1994) TLC1: template RNA component of Saccharomyces cerevisiae telomerase. *Science*, **266**, 404–409.
13. Lingner,J., Hughes,T.R., Shevchenko,A., Mann,M., Lundblad,V. and Cech,T.R. (1997) Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science*, **276**, 561–567.
14. Counter,C.M., Meyerson,M., Eaton,E.N. and Weinberg,R.A. (1997) The catalytic subunit of yeast telomerase. *Proc. Natl Acad. Sci. USA*, **94**, 9202–9207.
15. Lin,J.J. and Zakian,V.A. (1995) An *in vitro* assay for *Saccharomyces* telomerase requires EST1. *Cell*, **81**, 1127–1135.
16. Steiner,B.R., Hidaka,K. and Futcher,B. (1996) Association of the Est1 protein with telomerase activity in yeast. *Proc. Natl Acad. Sci. USA*, **93**, 2817–2821.
17. Virta-Pearlman,V., Morris,D.K. and Lundblad,V. (1996) Est1 has the properties of a single-stranded telomere end-binding protein. *Genes Dev*., **10**, 3094–3104.
18. Zhou,J., Hidaka,K. and Futcher,B. (2000) The Est1 subunit of yeast telomerase binds the Tlc1 telomerase RNA. *Mol. Cell. Biol*., **20**, 1947–1955.
19. Bourns,B.D., Alexander,M.K., Smith,A.M. and Zakian,V.A. (1998) Sir proteins, Rif proteins, and Cdc13p bind *Saccharomyces* telomeres *in vivo*. *Mol. Cell. Biol*., **18**, 5600–5608.
20. Lin,J.J. and Zakian,V.A. (1996) The *Saccharomyces* Cdc13 protein is a single-strand TG1-3 telomeric DNA-binding protein *in vitro* that affects telomere behavior *in vivo*. *Proc. Natl Acad. Sci. USA*, **93**, 13760–13765.
21. Nugent,C.I., Hughes,T.R., Lue,N.F. and Lundblad,V. (1996) Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. *Science*, **274**, 249–252.

22. Taggart,A.K., Teng,S.C. and Zakian,V.A. (2002) Est1p as a cell cycle-regulated activator of telomere-bound telomerase. *Science*, **297**, 1023–1026.
23. Tsukamoto,Y., Taggart,A.K. and Zakian,V.A. (2001) The role of the Mre11–Rad50–Xrs2 complex in telomerase-mediated lengthening of *Saccharomyces cerevisiae* telomeres. *Curr. Biol.*, **11**, 1328–1335.
24. Pennock,E., Buckley,K. and Lundblad,V. (2001) Cdc13 delivers separate complexes to the telomere for end protection and replication. *Cell*, **104**, 387–396.
25. Chandra,A., Hughes,T.R., Nugent,C.I. and Lundblad,V. (2001) Cdc13 both positively and negatively regulates telomere replication. *Genes Dev.*, **15**, 404–414.
26. Garvik,B., Carson,M. and Hartwell,L. (1995) Single-stranded DNA arising at telomeres in *cdc13* mutants may constitute a specific signal for the *RAD9* checkpoint. *Mol. Cell. Biol.*, **15**, 6128–6138.
27. Qi,H. and Zakian,V.A. (2000) The *Saccharomyces* telomere-binding protein Cdc13p interacts with both the catalytic subunit of DNA polymerase alpha and the telomerase-associated est1 protein. *Genes Dev.*, **14**, 1777–1788.
28. Evans,S.K. and Lundblad,V. (1999) Est1 and Cdc13 as comediators of telomerase access. *Science*, **286**, 117–120.
29. Nyberg,K.A., Michelson,R.J., Putnam,C.W. and Weinert,T.A. (2002) Toward maintaining the genome: DNA damage and replication checkpoints. *Annu. Rev. Genet.*, **36**, 617–656.
30. Abraham,R.T. (2001) Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev.*, **15**, 2177–2196.
31. Kim,S.T., Lim,D.S., Canman,C.E. and Kastan,M.B. (1999) Substrate specificities and identification of putative substrates of ATM kinase family members. *J. Biol. Chem.*, **274**, 37538–37543.
32. Carr,A.M. (2002) DNA structure dependent checkpoints as regulators of DNA repair. *DNA Repair (Amst).*, **1**, 983–994.
33. Rouse,J. and Jackson,S.P. (2002) Lcd1p recruits Mec1p to DNA lesions *in vitro* and *in vivo*. *Mol. Cell*, **9**, 857–869.
34. Craven,R.J. and Petes,T.D. (1999) Dependence of the regulation of telomere length on the type of subtelomeric repeat in the yeast *Saccharomyces cerevisiae*. *Genetics*, **152**, 1531–1541.
35. Ritchie,K.B., Mallory,J.C. and Petes,T.D. (1999) Interactions of TLC1 (which encodes the RNA subunit of telomerase), TEL1, and MEC1 in regulating telomere length in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **19**, 6065–6075.
36. Chan,S.W., Chang,J., Prescott,J. and Blackburn,E.H. (2001) Altering telomere structure allows telomerase to act in yeast lacking ATM kinases. *Curr. Biol.*, **11**, 1240–1250.
37. Mallory,J.C. and Petes,T.D. (2000) Protein kinase activity of Tel1p and Mec1p, two *Saccharomyces cerevisiae* proteins related to the human ATM protein kinase. *Proc. Natl Acad. Sci. USA*, **97**, 13749–13754.
38. Naito,T., Matsuura,A. and Ishikawa,F. (1998) Circular chromosome formation in a fission yeast mutant defective in two ATM homologues. *Nature Genet.*, **20**, 203–206.
39. Vaziri,H. (1997) Critical telomere shortening regulated by the ataxia-telangiectasia gene acts as a DNA damage signal leading to activation of p53 protein and limited life-span of human diploid fibroblasts. A review. *Biochemie (Moscow)*, **62**, 1306–1310.
40. Tsai,Y.L., Tseng,S.F., Chang,S.H., Lin,C.C. and Teng,S.C. (2002) Involvement of replicative polymerases, Tel1p, Mec1p, Cdc13p, and the Ku complex in telomere-telomere recombination. *Mol. Cell. Biol.*, **22**, 5679–5687.
41. Rose,M.D., Winston,F. and Hieter,P. (1990) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
42. Teng,S.C., Chang,J., McCowan,B. and Zakian,V.A. (2000) Telomerase-independent lengthening of yeast telomeres occurs by an abrupt Rad50p-dependent, Rif-inhibited recombinational process. *Mol. Cell*, **6**, 947–952.
43. Teng,S.C. and Zakian,V.A. (1999) Telomere-telomere recombination is an efficient bypass pathway for telomere maintenance in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **19**, 8083–8093.
44. Tseng,S.F., Chang,C.Y., Wu,K.J. and Teng,S.C. (2005) Importin KPNA2 is required for proper nuclear localization and multiple functions of NBS1. *J. Biol. Chem.*, **280**, 39594–39600.
45. Naiki,T., Wakayama,T., Nakada,D., Matsumoto,K. and Sugimoto,K. (2004) Association of Rad9 with double-strand breaks through a Mec1-dependent mechanism. *Mol. Cell. Biol.*, **24**, 3277–3285.
46. Takata,H., Kanoh,Y., Gunge,N., Shirahige,K. and Matsuura,A. (2004) Reciprocal association of the budding yeast ATM-related proteins Tel1 and Mec1 with telomeres *in vivo*. *Mol. Cell*, **14**, 515–522.
47. Diede,S.J. and Gottschling,D.E. (1999) Telomerase-mediated telomere addition *in vivo* requires DNA primase and DNA polymerases alpha and delta. *Cell*, **99**, 723–733.
48. Hsu,C.L., Chen,Y.S., Tsai,S.Y., Tu,P.J., Wang,M.J. and Lin,J.J. (2004) Interaction of Saccharomyces Cdc13p with Pol1p, Imp4p, Sir4p and Zds2p is involved in telomere replication, telomere maintenance and cell growth control. *Nucleic Acids Res.*, **32**, 511–521.
49. Guarente,L. (1983) Yeast promoters and lacZ fusions designed to study expression of cloned genes in yeast. *Methods Enzymol.*, **101**, 181–191.
50. Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
51. O'Neill,T., Dwyer,A.J., Ziv,Y., Chan,D.W., Lees-Miller,S.P., Abraham,R.H., Lai,J.H., Hill,D., Shiloh,Y., Cantley,L.C. *et al.* (2000) Utilization of oriented peptide libraries to identify substrate motifs selected by ATM. *J. Biol. Chem.*, **275**, 22719–22727.
52. Mallory,J.C., Bashkirov,V.I., Trujillo,K.M., Solinger,J.A., Dominska,M., Sung,P., Heyer,W.D. and Petes,T.D. (2003) Amino acid changes in Xrs2p, Dun1p and Rfa2p that remove the preferred targets of the ATM family of protein kinases do not affect DNA repair or telomere length in *Saccharomyces cerevisiae*. *DNA Repair (Amst.)*, **2**, 1041–1064.
53. Adams Martin,A., Dionne,I., Wellinger,R.J. and Holm,C. (2000) The function of DNA polymerase alpha at telomeric G tails is important for telomere homeostasis. *Mol. Cell. Biol.*, **20**, 786–796.
54. Adams,A.K. and Holm,C. (1996) Specific DNA replication mutations affect telomere length in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **16**, 4614–4620.
55. Carson,M.J. and Hartwell,L. (1985) CDC17: an essential gene that prevents telomere elongation in yeast. *Cell*, **42**, 249–257.
56. Lee,S.E., Moore,J.K., Holmes,A., Umezu,K., Kolodner,R.D. and Haber,J.E. (1998) *Saccharomyces* Ku70, mre11/rad50 and RPA proteins regulate adaptation to G2/M arrest after DNA damage. *Cell*, **94**, 399–409.
57. Zou,L., Liu,D. and Elledge,S.J. (2003) Replication protein A-mediated recruitment and activation of Rad17 complexes. *Proc. Natl Acad. Sci. USA*, **100**, 13827–13832.
58. Craven,R.J., Greenwell,P.W., Dominska,M. and Petes,T.D. (2002) Regulation of genome stability by TEL1 and MEC1, yeast homologs of the mammalian ATM and ATR genes. *Genetics*, **161**, 493–507.