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

Shun-Fu Tseng¹, Jing-Jer Lin² and Shu-Chun Teng¹,3,*

¹Department of Microbiology, College of Medicine, National Taiwan University, Taipei, Taiwan, ²Institute of Biopharmaceutical Science, National Yang-Ming University, Taipei, Taiwan and ³Institute 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.
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 MECl and TEL1 are homologous to the human ATM 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 MECl 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 MEC1 and S.pombe Tel1p function upstream in the checkpoint pathway by phosphorylation of serine and threonine residues on Cdc13p to be 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 (MATa/MATa ura3-52/ura3-52 lys2-801 amber/lys2-801 amber ade2-101 ochre/ade2-101 ochre trp1α/Δ63/ trp1α/Δ63 his3α/Δ200/his3α/Δ200 leu2-αl/leu2-αl). The yeast strains carrying mec1, sml1, tel1, cdc13 and CDC13-Myc9 were described previously (15,23,40). YEp24CDC13-URA3 was constructed as previously described (26). pRS314CDC13, pRS304cde13 and pRS304cde13-Myc9 were obtained from Dr Virginia Zakian (23,27). The 1 kb SacII fragment from pRS304cde13-Myc9 was inserted into the SacII-digested pRS314CDC13 to make pRS314CDC13-Myc9. Point mutations were introduced into the CDC13 using QuickChange site-directed mutagenesis (Stratagene). To generate chromosomal cde13 mutants, pRS034cde13 mutants were Xhol-digested and transformed into CDC13 strains, pVT1091 (pCDC13-EST1) plasmid (28) was kindly provided by Dr Victoria Lundblad. To generate pVL1091 mutant plasmids, the 1.4 kb BamHI–StuI fragments in pRS314CDC13-AA or pRS314CDC13-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 Biotecnology). Immunoprecipitation of Cdc13-Myc was done with agarose-conjugated 9E10 monoclonal antibodies (sc-40, Santa Cruz Biotecnology). 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 MnCl2) 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 Na3VO4 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-4TCDC13(129–332) was constructed by ligating a HindIII–Klenow fragment containing amino acids 129–332 of Cdc13 from pRS314CDC13 into BamHI–Klenow treated pGEX-4T (Amersham/Pharmacia). Plasmid pGEX-4TCDC13(570–676) was constructed by ligating a PCR product containing amino acids 570–676 of Cdc13 from pRS314CDC13 into BamHI–Klenow and XhoI treated pGEX-4T. Plasmid pET28ccCDC13(185–324) was constructed by ligating a BamHI–Klenow and NotI treated fragment containing amino acids 185–332 of Cdc13 from pGEX-4TCDC13(129–332) into pET28a (Novagen) with the same sites. Plasmid pGEX-4TCDC13(185–324) was constructed by ligating a NcoI–Klenow and Xhol-treated fragment containing amino acids 185–332 of Cdc13 from pET28ccCDC13(129–332) into BamHI–Klenow and XhoI-treated pGEX-4T. Plasmid pGEX-4TCDC13(185–324) was constructed by self-ligating the BamHI–NsiI–Klenow fragment of pGEX-4TCDC13(185–332) backbone. Plasmid pGEX-4TCDC13(185–275) was constructed by self-ligating
the NsiI–XhoI–Klenow fragment of pGEX-4TCDC13 (185–332) backbone. Recombinant proteins in E.coli were over-expressed 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**

**RESULTS**

MEC1/TEL1-dependent phosphorylation of Cdc13p 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, mecl and tell strains, but slightly decreased in the mecl tell strain (Figure 1A). The migration of Cdc13p bands in gel was slightly faster in the mecl tell background, suggesting that Cdc13p might be 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 mecl tell strain still migrated as doublets (Figure 1A, lane 4) and the gel mobility of Cdc13p in the mecl tell 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 mecl tell 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
for its ability to phosphorylate Cdc13p. Mec1p and Tel1p immunoprecipitated Mec1 and Tel1 kinases were tested and presented as in (A).

Immunoprecipitated Mec1 and Tel1 kinases were tested performed and presented as in (A).

Cdc13p from untreated or PPase-treated wild-type cells. The assay was point shifts. (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 Cdc13p(185–275) subunit was completely abolished (Figure 3B). Consistent with the previous results (Figure 3B), the Cdc13p(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).

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-32P]ATP and recombinant GST-Cdc13p(129–332) or GST-Cdc13p(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-Cdc13p(129–332) subunit was observed, while neither GST alone (data not shown) nor the GST-Cdc13p(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 mecl mutant strain. Strain KSC1752 [mecl-HA(KD)] expresses an HA-tagged Mec1-kd protein with two amino acid changes in its catalytic domain (52) (Figure 3A). 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
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 S225/249/255/306AAA mutant was lethal on the tetradissection plates (data not shown). Interestingly, this cellular senescence of S249/255AAA 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 Dieder and Gottschling (47). Strains were arrested in M phase by nocodazole treatment, and then subjected to induction of HO expression. In contrast to wild-type cells

---

**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 smn1::HIS3), KSC1752 (mecl-HA(KD) smn1::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-32P]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 smn1::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.
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 Y0 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 Y0 probe. DNA from the wild-type YPH499 was loaded as a control.
which showed telomere elongations at the HO cut site, the TG1–3/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 pCDC13–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 pCDC13–EST1 or pcdc13–EST1-S249/255AA (data not shown). This phenotype was observed in every clone that we analyzed, although in the pcdc13–EST1-S249/255AA containing cells (Figure 7, lane 6) the extent of telomere elongation was slightly shorter than that in the pCDC13–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.
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 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 evolutionary 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 MECl/TELI-independent phosphorylation of Cdc13p. Whether these phosphorylations mediate other functions of Cdc13p and what kinase(s) contribute to MEC1/TELI-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 telomeric 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. Genetica, 146, 1399–1412.
12. Singer,M.S. and Gottschling,D.E. (1994) TELC1: 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 Butcher,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 Butcher,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, Rf 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 is 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. \textit{Science}, \textbf{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 \textit{Saccharomyces cerevisiae} telomeres. \textit{Curr. Biol.}, \textbf{11}, 1328–1335.

24. Pennock,E., Buckley,K. and Lundblad,V. (2001) Cdc13 delivers separate complexes to the telomere for end protection and replication. \textit{Cell}, \textbf{104}, 387–396.

25. Chandra,A., Hughes,T.R., Nugent,C.I. and Lundblad,V. (2001) Cdc13b both positively and negatively regulates telomere replication. \textit{Genes Dev.}, \textbf{15}, 404–414.

26. Garvik,B., Carson,M. and Hartwell,L.L. (1995) Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. \textit{Mol. Cell. Biol.}, \textbf{15}, 6128–6138.

27. Qi,H. and Zakian,V.A. (2000) The \textit{Saccharomyces} telomere-binding protein Cdc13p interacts with both the catalytic subunit of DNA polymerase alpha and the telomerase-associated est1 protein. \textit{Genes Dev.}, \textbf{14}, 1777–1788.

28. Evans,S.K. and Lundblad,V. (1999) Est1 and Cdc13 as comediators of telomerase access. \textit{Science}, \textbf{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. \textit{Annu. Rev. Genet.}, \textbf{36}, 617–656.

30. Abraham,R.T. (2001) Cell cycle checkpoint signaling through the ATM and ATR kinases. \textit{Genes Dev.}, \textbf{15}, 2177–2196.

31. Rouse,J. and Jackson,S.P. (2002) Lcd1p recruits Mec1p to DNA lesions and Mec1p, two ataxia-telangiectasia gene acts as a DNA damage signal leading to cell growth control. \textit{Nucleic Acids Res.}, \textbf{32}, 511–512.

32. Rouse,J. and Jackson,S.P. (2002) Lcd1p recruits Mec1p to DNA lesions and Mec1p, two ataxia-telangiectasia gene acts as a DNA damage signal leading to cell growth control. \textit{Nucleic Acids Res.}, \textbf{32}, 511–512.

33. Rouse,J. and Jackson,S.P. (2002) Lcd1p recruits Mec1p to DNA lesions and Mec1p, two ataxia-telangiectasia gene acts as a DNA damage signal leading to cell growth control. \textit{Nucleic Acids Res.}, \textbf{32}, 511–512.

34. Rouse,J. and Jackson,S.P. (2002) Lcd1p recruits Mec1p to DNA lesions and Mec1p, two ataxia-telangiectasia gene acts as a DNA damage signal leading to cell growth control. \textit{Nucleic Acids Res.}, \textbf{32}, 511–512.

35. Ritchie,K.B., Mallory,J.C. and Petes,T.D. (1999) Interactions of TLC1 and MRE11–RAD50–XR2 proteins regulate adaptation to G2/M arrest after DNA damage. \textit{Cell}, \textbf{104}, 387–396.