Telomere-binding and Stn1p-interacting activities are required for the essential function of Saccharomyces cerevisiae Cdc13p

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ABSTRACT

Yeast Saccharomyces cerevisiae Cdc13p is the telomere-binding protein that protects telomeres and regulates telomere length. It is documented that Cdc13p binds specifically to single-stranded TG1–3 telomeric DNA sequences and interacts with Stn1p. To localize the region for single-stranded TG1–3 DNA binding, Cdc13p mutants were constructed by deletion mutagenesis and assayed for their binding activity. Based on in vitro electrophoretic mobility shift assay, a 243-amino-acid fragment of Cdc13p (amino acids 451–693) was sufficient to bind single-stranded TG1–3 with specificity similar to that of the native protein. Consistent with the in vitro observation, in vivo one-hybrid analysis also indicated that this region of Cdc13p was sufficient to localize itself to telomeres. However, the telomere-binding region of Cdc13p (amino acids 451–693) was not capable of complementing the growth defects of cdc13 mutants. Instead, a region comprising the Stn1p-interacting and telomere-binding region of Cdc13p (amino acids 252–924) complemented the growth defects of cdc13 mutants. These results suggest that binding to telomeres by Cdc13p is not sufficient to account for the cell viability, interaction with Stn1p is also required. Taken together, we have defined the telomere-binding domain of Cdc13p and showed that both binding to telomeres and Stn1p by Cdc13p are required to maintain cell growth.

INTRODUCTION

Telomeres are the specialized structure at the very ends of eukaryotic chromosomes. Telomeres are essential for the maintenance of chromosome integrity (1,2). They prevent end-to-end fusion of chromosomes, protect chromosome from degradation by nucleases and facilitate the complete replication of chromosomes. They also suppress the expression of nearby genes, a phenomenon known as telomere position effect (TPE) (3). Telomeres might also position chromosomes within the nucleus (4). In most of the cloned telomeric sequences, telomeres are composed of short tandem repeated sequences with the strand running in a 5′ to 3′ direction toward the guanine (G)-rich ends (1). The sequence and the number of repeats vary considerably in different species. For example, the telomere sequences of yeast Saccharomyces cerevisiae are ∼250–300 bp of TG1–3/C1–3, whereas those of human are ∼10 kb TTAGGG/CCCTAA repeats (1,2). Besides the double-stranded telomeric DNA repeats, telomeres in all of the organisms that have been analyzed, also contain a G-rich single-stranded tail (5–7). In S.cerevisiae, a >30 base single-stranded TG1–3 tail was detected in late S phase of the cell cycle (8). This single-stranded tail was postulated as an intermediate during telomere replication (8,9).

Protein factors that interact with telomeres participate in telomere functions (10). Factors that bind to the double-stranded telomeric DNA sequences have been identified from many organisms (11–16). These proteins are essential for the maintenance of telomere functions and cell viability (11,12,16). For example, in S.cerevisiae, Rap1p has been shown to bind double-stranded telomeric DNA (11,17,18). Mutations on Rap1p affect the length of telomere, TPE, localization of the telomere within the nucleolus, telomere recombination and cell viability (19–21). Moreover, protein factors bound to the single-stranded telomeric DNA were identified in several organisms (22–30). Among these protein factors, Oxytricha telomere-binding protein is well characterized. The protein is heterodimeric and is composed of α and β subunit (31–33). The α subunit is a single-stranded DNA-binding protein that binds to the G4T4 single-stranded end of the telomere. Although the β subunit is not directly involved in binding, it is required for terminus-specific binding. In the yeast genome, there is no homologue of the Oxytricha α and β-like binding proteins. Instead, Cdc13p binds to single-stranded TG1–3 sequences in vitro and interacts with telomeres in vivo (24,25,34). It is believed that, in yeast, Cdc13p is the functional equivalent of Oxytricha α and β binding proteins despite there being no sequence similarity between Oxytricha telomere-binding proteins and yeast Cdc13p (J.-J.Lin, unpublished observation). Furthermore, Cdc13p dissociates from single-stranded TG1–3 DNA at 300 mM NaCl (J.J.Lin and V.A.Zakian, unpublished result) whereas the binding of Oxytricha proteins to G4T4 DNA remains associated even at 2 M NaCl (33). Thus, Cdc13p might represent a class of single-stranded telomere-binding protein that is different from the Oxytricha protein.
**MATERIALS AND METHODS**

**Strains**

*Escherichia coli* strain DH5α was used as host for plasmid construction and propagation, and strain BL21(DE3)pLysS for Cdcl3p (451–693) purification. Yeast strain YPH499 [MATα ura3-52 lys2-801 ade2-101 trp1ΔΔ3 his3Δ200 leu2Δ1 (38)] with URA3 and ADE2 placed near the telomere of chromosomes VII-L and V-R (YPH499UTAT), respectively, was used as the host for analyzing TPE. Yeast strains, His-Tel and HIS-Int-CA, used for one-hybrid analysis were kindly provided by V. A. Zakian [Princeton University (34)]. Strain 2758-8-4b (MATα cdc13-1 his7 leu2-3, 112 ura3-52 trp1-289, provided by L. Hartwell, University of Washington) was used as the host for complementation tests.

**Plasmid construction**

Deletion mutants of Cdcl3p (Fig. 1A) were generated by fusion of various regions of CDC13 with the glutathione S-transferase (GST) gene of pGEX plasmids (PharMacia). The 4.4-kb Small-HpaI fragment containing full length CDC13 from pTHA-CDC13 (25) was ligated to the Small-digested pGEX-3X to generate pGST-CDC13. Plasmid pGST-CDC13 was digested with BglII and self-ligated to generate pGST-CDC13(1–600), which deleted the BglII–BglII fragment within CDC13. Plasmid pGST-CDC13(451–924) was constructed by ligating the 2.9-kb BamHI–HpaI fragment containing half of CDC13 to BamHI- and Small-digested pGEX-3X. Plasmid pGST-CDC13(451–924) was digested with either PvuII or BglII, and self-ligated to generate pGST-CDC13(451–871) and pGST-CDC13(451–600), respectively. Plasmid pGST-CDC13(451–693) was constructed by digesting pGST-CDC13(451–924) with SalI and NruI, treating with T4 DNA polymerase and self-ligation. A 1.4-kb EcoRI–SalI fragment of pTHA-CDC13 was ligated to EcoRI- and SalI-digested pGEX-4T-1 to generate pGST-CDC13(510–924). To construct pGST-CDC13(601–856), a 0.8-kb BglII–BglII fragment from pTHA-CDC13 was inserted into BamHI-digested pGEX-1. The NruI–SalI fragment of pGST-CDC13(601–856) was deleted to generate pGST-CDC13(601–693). Expression of these fusion proteins was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) and confirmed by Western blotting analysis using antibody against GST (data not shown).

Plasmid pET6H-CDC13(451–693), which was used to purify Cdc13(451–693)p, was constructed by inserting the NcoI–NruI-digested pGST-CDC13(451–693) into NcoI–SalI-digested pET6H (a gift from C.-H. Hs., National Marine University, Taiwan). The resulting plasmid was used to express 6x His-tagged Cdc13(451–693)p under the control of the T7 promoter.

To construct a plasmid for one-hybrid analysis, the DNA fragment encoding Cdc13(451–693)p was amplified by PCR using pTHA-CDC13 as the template and with primers ML11 (5'-CCGCCTCGAGATCTGATGGGCAATGC-3') and ML12 (5'-CCGCCTCGAGATGAGAACCGTTTCT-3'). The 0.7-kb PCR product was subcloned into Small-digested pUCl8 to generate pUC-CDC13(451–693). The 0.7-kb DNA fragment from XhoI-digested pUC-CDC13(451–693) was then ligated with the XhoI-digested pJG4-5 (39) or pRF4-6NL (34) to generate pJG-CDC13(451–693) or pRF-CDC13(451–693), respectively.

Since Cdc13p is a telomere-binding protein, the cellular localization of Cdc13p should be in the nucleus. To make certain that the truncated Cdc13p would be delivered into the nucleus, two oligonucleotides NLSw (5'-CATGGC-GACCTTCCGCTTCTTCTTGGGG-3') encoding the nuclear localization sequence (NLS) of SV40 large T antigen (39,40) were annealed and ligated with SpeI- and NcoI-digested pTHA-CDC13. The resulting plasmid, pTHA-NLS-CDC13, encoded a fusion protein with three HA-tag and NLS at the N-terminal of Cdc13p. Similarly, plasmid vector pTHA-NLS was constructed by inserting NLS sequences into pTHA (25). To construct plasmid pTHA-NLS-CDC13(451–924), a 1.6-kb DNA fragment encoding amino acids 451–924 of Cdc13p was PCR amplified with primers ML01 (5'-TGCCATGAGGGATCGTGGGGACAATGT-3') and CDC133 (5'-AATGCGACTGACTTCTTTTATTCC-3') using Vent DNA polymerase (New England Biolabs). The PCR product was digested with NcoI and SalI, and was inserted into NcoI- and SalI-digested pTHA-NLS. To generate pTHA-NLS-CDC13(451–924), plasmid...
pTHA-NLS-CDC13(451–924) was digested with NruI and SalI, blunted by T4 DNA polymerase and self-ligated. Plasmid pTHA-NLS-CDC13(1–252) was constructed by ligating the NcoI–EcoRI fragment (0.8 kb) encoding the N-terminal 252-amino-acid polypeptide, of pAS-CDC13(1–252) to NcoI- and EcoRI-digested pTHA-NLS. A 2-kb NcoI–SalI fragment of pAS-CDC13(252–924) was ligated with NcoI- and SalI-digested pTHA-NLS to generate pTHA-NLS-CDC13(252–924). Expression of full-length and truncated CDC13 was confirmed by western blotting analysis on the extracts prepared from yeast cells with anti-HA antibody 12CA5 (data not shown).

Two-hybrid plasmids were constructed by subcloning STN1 into pACT2 and several fragments of CDC13 into pAS2 (37,41). Plasmid pACT-STN1 was constructed by ligating a NcoI–BamHI 1.6-kb fragment containing full-length STN1 from pJG-STN1 (unpublished construct) into pACT2 with the same sites. To construct full-length CDC13 into pAS2, the NcoI–SalI fragment (3 kb) of pTHA-CDC13 (25) was ligated into the NcoI- and SalI-digested pAS2. Plasmid carrying the temperature-sensitive allele of CDC13, cdc13-1, was constructed by replacing the 1.4-kb fragment of pAS-CDC13 with the equivalent of pTHA-CDC13 (25). Plasmid pAS-CDC13(252–924) was constructed by first ligating the 1.4-kb EcoRI–SalI CDC13 fragment of pTHA-CDC13 into EcoRI- and SalI-digested pAS2-1 followed by inserting a 0.8-kb EcoRI–EcoRI fragment of CDC13 isolated from pTHA-CDC13 with EcoRI digestion.

**Preparation of E.coli extracts**

To prepare protein extracts from *E.coli*, a 10-ml culture of *E.coli* containing a Cdc13p-deletion construct was grown in LB medium with 50 µg/ml ampicillin at 30°C to OD_{600} = 0.6. At this point, IPTG was added to a final concentration of 1 mM and the cells were allowed to grow at 30°C for an additional 16 h before harvesting by centripugation. The pellets were resuspended in 0.5 ml buffer A (50 mM Tris–HCl pH 7.5, 1 mM EDTA, 1× protease inhibitor cocktail (Calbiochem) and 50 mM glucose) and were sonicated. Cell debris was removed by centrifugation in an Eppendorf microfuge for 10 min at 4°C, the supernatants were aliquoted and frozen in a dry ice-ethanol bath, and stored at −70°C. The concentration of protein in the *E.coli* extract was ~2–6 mg/ml as determined using a Bio-Rad protein assay kit with bovine serum albumin as a standard.

**Purification of 6× His-tagged Cdc13(451–693)p**

To purify 6× His-tagged Cdc13(451–693)p, a 500-ml culture of IPTG-induced *E.coli* harboring pET6H-CDC13(451–693) was collected by centrifugation. Cells were resuspended in 30 ml of GdHCl buffer (6 M guanidine–HCl, 0.1 M NaH_2PO_4, 0.01 M Tris pH 8.0), followed by gentle shaking for 1 h at 4°C. The suspension was then centrifuged at 13 000 g for 15 min at 4°C. The clear supernatant was collected and applied to a 5 ml Ni-NTA–agarose column (Qiagen) previously equilibrated with GdHCl buffer. The column was washed stepwise with 20 ml of GdHCl buffer containing 1 mM imidazole followed by 20 ml of GdHCl buffer containing 20 mM imidazole. The bound protein was eluted by 12 ml of GdHCl buffer containing 200 mM imidazole. To renature the purified Cdc13(451–693)p, protein eluted from the Ni-NTA–agarose column was diluted to ~50 µg/ml using GdHCl buffer and dialyzed against renaturation buffer (100 mM Tris pH 8.0, 2 mM EDTA, 2 mM DTT, 0.4 M L-arginine, 20% glycerol) at 4°C for 12 h.

**Electrophoretic mobility shift assay (EMSA)**

Oligonucleotide TG22 (5′-GGTTGGGTGGTGTTGGTGTTGGG-3′), TG10 (5′-GGGTTGGTGTTGGTGTTGG-3′), TG15 (5′-TGGTGGTTGGTGTTGGTGTTGG-3′), TG20 (5′-TGGTGGTTGGTGTTGGTGTTGG-3′), TG25 (5′-TGGTGGTTGGTGTTGGTGTTGG-3′), TG30 (5′-TGGTGGTTGGTGTTGGTGTTGG-3′) or TG35 (5′-TGGTGGTTGGTGTTGGTGTTGG-3′) was first 5′-end-labeled with [γ-32P]ATP (3000 mCi/mM, NEN) using T4 polynucleotide kinase (New England Biolabs) and subsequently purified from a 10% sequencing gel after electrophoresis. To perform the assays, cell extracts were mixed with 2.0 ng of 32P-labeled TG22 or TG15 DNA with a total volume of 15 µl containing 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM DTT and 1 µg of single stranded poly(dI–dC). The mixtures were allowed to incubate at room temperature for 10 min. Then, 3 µl of 80% glycerol was added and the mixtures were loaded on an 8% non-denaturing polyacrylamide gel, which was pre-run at 125 V for 10 min. Electrophoresis was carried out in TBE (89 mM Tris–borate/2 mM EDTA) at 125 V for 105 min. The gels were dried and autoradiographed. For competition analysis, 2.0 ng 32P-labeled TG15 was mixed with varying amounts of non-radioactive competitors before addition of the cell extracts. Binding activity was quantified with a PhosphorImager (Molecular Dynamics).

**One-hybrid analysis**

One-hybrid analysis was performed using the methods described by Bourns et al. (34). Plasmid pJG4-5, pJG-CDC13(451–693) or pRF-CDC13(451–693) was first transformed into yeast strains HIS-Tel and HIS-Int-CA, and selected on plates lacking tryptophan. To test for telomere-binding activity *in vivo*, cells were grown in liquid medium lacking tryptophan for ~16 h. Then, cells were spotted in 10-fold serial dilutions on yeast synthetic complete medium (YC) plates lacking tryptophan, or lacking histidine, or with 10, 20 or 40 mM of 3-amino-1,2,4-triazole (3-AT, Sigma, St Louis, MO). Plates were incubated at 30°C until the colonies could be observed. Because HIS-Tel cells carrying the B42 transcription-activation domain fused with the DNA-binding region of Cdc13p did not grow well on plates with galactose, all the experiments were performed in plates containing 1.5% galactose and 1% glucose. The HIS-Int-CA strain grew better in this condition than in plates containing 3% galactose.

**Complementation of cdc13Δ by cdc13 fragments using plasmid loss experiments**

Plasmid loss experiments were carried out to test if binding of single-stranded TG1 to and/or STN1 interacting activity of Cdc13p is sufficient to complement the lack of viability caused by the cdc13Δ mutation. Briefly, plasmid YEP24-CDC13 [abbreviation of plasmid YEP24-CDC13-161-4 (35)] was transformed into a diploid strain YJL401-UTAT [CDC13/cdc13Δ::HIS3 (25)] carrying one allele of the cdc13 null mutation. The transformants were sporulated and subjected to tetrad analysis. Haploid strain YJL501 was selected from the spores that were Ura+(YEP24-CDC13), His+ (cdc13Δ::HIS3) and Ade+ (ADE2 near the telomere of chromosome V-R). Such a strain requires a plasmid carrying CDC13 (YEP24-CDC13) for its
viability. Subsequently, plasmid pTHA-NLS, pTHA-NLS-CDC13, pTHA-NLS-CDC13(1–252), pTHA-NLS-CDC13(252–924), pTHA-NLS-CDC13(451–924) or pTHA-NLS-CDC13(451–693) was separately transformed into YJL501. The resulting transformants were spotted onto plates containing 0.5 mg/ml 5-fluoroorotic acid (5-FOA) and incubated at 30°C until colonies formed.

Two-hybrid analysis
Plasmids pACT2 and pACT-STN1 were transformed separately into yeast strain Y190. The resulting strains were then transformed with plasmids pAS2 or pAS2 containing CDC13 fragments. The HIS3 reporter system was also used to evaluate the interaction between Cdc13p and Stn1p. In the assays, 5–10 fresh transformed colonies from each transformation were mixed and spotted in 10-fold serial dilutions onto YC plates lacking histidine without or with 25 mM 3-AT. Plates were kept at 25 or 30°C until colonies formed.

RESULTS
Cdc13(451–693)p bound to single-stranded TG_{1–3} DNA in vitro

To identify the telomeric DNA-interacting region in Cdc13p, plasmids suitable to express GST fusions with various fragments of Cdc13p were constructed (Fig. 1A). Using ^3^P-labeled 22-base TG_{1–3} oligonucleotides (TG22) as substrate, the DNA-binding activity of these truncated Cdc13p was determined by EMSA. By comparing the gel-shift pattern with that of the vector alone, extra single-stranded TG_{1–3}-binding activity was observed in *E. coli* extracts expressing Cdc13p fragments containing amino acids 451–924, 451–871 or 451–693 (Fig. 1B). Among these fusion proteins, Cdc13(451–693)p was the shortest single-stranded TG_{1–3} DNA-binding fragment of Cdc13p. Western blotting analysis using anti-GST antibody confirmed that these truncated Cdc13p polypeptides were indeed expressed, albeit degradation of the full-length and some of these truncated forms of Cdc13p was also observed (data not shown). Therefore, while it is not clear if the fragment can be shortened further, it was evident that the single-stranded TG_{1–3}-binding activity of Cdc13p is located within the fragment comprising residues 451–693.

We also have expressed a 6x His-tagged-Cdc13(451–693)p in *E. coli* and purified this tagged protein using Ni-NTA–agarose (Fig. 2A). Using ^3^P-labeled single-stranded TG_{1–3} oligonucleotides as substrate, the DNA-binding ability of this recombinant polypeptide was determined by EMSA. The result shown in Figure 2B further demonstrated that this 6x His-tagged Cdc13(451–693)p is capable of forming complexes with the single-stranded TG_{1–3}. Evidently, the single-stranded TG_{1–3} binding activity of Cdc13p was located within amino acids 451–693. Results shown in Figure 2B also indicate that the length of DNA substrate affected the electrophoretic mobility of the Cdc13(451–693)p–DNA complex. Interestingly, a second migration band was apparent on TG30 or TG35 but not on TG25; the identity of this second migration band is uncertain (Fig. 2B, lanes 19–24 and 25–30). Under our assay condition, Cdc13(451–693)p bound TG15 with an apparent binding constant of 120 nM.

Cdc13(451–693)p specifically bound to single-stranded TG_{1–3} DNA

To evaluate the selectivity of the Cdc13(451–693)p binding activity, purified protein was mixed with ^3^P-labeled single-stranded TG_{1–3} and various amounts of unlabeled nucleic acid competitors before being subjected to EMSA analysis. As shown in Figure 3, unlabeled TG15 competed efficiently with ^3^P-labeled TG15. The binding was reduced by ∼50% when the competitor was present at equal concentrations (Fig. 3A, lanes 3–5 and B). On the other hand, vertebrate (T_{4}G_{4}) and *Tetrahymena* (T_{2}G_{3}) telomeric DNA did not compete for the binding activity of Cdc13(451–693)p to TG15 (Fig. 3A, lanes 6–14). Total yeast RNA, single-stranded C_{1–3}A DNA or duplex TG_{1–3}/C_{1–3}A DNA did not compete for Cdc13(451–693)p binding either (Fig. 3A, lanes 15–17, and...
data not shown). This result indicated that Cdc13(451–693)p bound specifically to single-strand TG1–3 telomeric DNA. With the exception of vertebrate telomeric DNA, which partially competed away the binding of Cdc13p to TG22 (25), Cdc13(451–693)p bound specifically to single-stranded TG1–3 telomeric DNA similarly to Cdc13p.

**Cdc13(451–693)p bound telomere in vivo**

Previously, a one-hybrid system was developed to examine whether a protein interacts with telomeres in vivo (34). In that system, a promoter-defective allele of HIS3 is placed near the telomere of chromosome VII-L, HIS-Tel. The protein to be tested is fused to the *E. coli* B42 transcription-activation domain. When this fusion protein interacts with telomeres, it activates the expression of HIS3. Thus, expression of His3p can be used as a means to identify telomere-interacting protein. To verify whether Cdc13(451–693)p binds telomere in vivo, this fragment was fused with the B42 transcription-activation domain (Act-Cdc13-DB) and expressed in yeast HIS-Tel or HIS-Int-CA. The HIS-Int-CA strain carried internal HIS3 with C1–3A sequences at the 5' region (34). Dilutions of cells were spotted onto plates without histidine to evaluate the expression of HIS3. HIS-Tel cells carrying plasmid vector alone (Act) or Cdc13(451–693)p without the B42 transcription-activation domain (Cdc13-DB) cannot grow on plates lacking histidine (Fig. 4, left panel). However, cells carrying the B42 transcription-activation domain fused with the DNA-binding region of Cdc13p (Act-Cdc13-DB) grew on plates lacking histidine (Fig. 4, left panel). The levels of cell growth with 3-AT for the HIS-Int-CA strain carrying plasmid vector alone (Act), Cdc13-DB or Act-Cdc13-DB were similar, indicating that Cdc13(451–693)p would not bind internal TG1–3/C1–3A duplex DNA (Fig. 4, right panel). Thus, Cdc13(451–693)p is sufficient to position itself to telomeres. Taken together, both in vitro and in vivo evidence indicate that the DNA-binding domain of Cdc13p was located in amino acids 451–693.

**Cdc13(451–693)p was not sufficient to complement cdc13 mutations**

It has been known that Cdc13p is essential for cell viability. The next question that was considered in this study was whether...
binding of Cdc13p to telomere is sufficient to account for its essentiality. Here, plasmids expressing Cdc13p, Cdc13(1–252)p, Cdc13(252–924)p, Cdc13(451–924)p or Cdc13(451–693)p were constructed and transformed into yeast strain 2758-8-4b, a cdc13-1 mutant of CDC13 (cdc13-1). Yeast strain 2758-8-4b (cdc13-1) grows normally at 25°C and arrests at G2/M phase of the cell cycle at 30°C. Full-length CDC13 complemented the temperature-sensitive phenotype of cdc13-1 at 30 or 37°C (Fig. 5A). Cells expressing Cdc13(252–924)p grew at all three temperatures tested. However, cells expressing other fragments of Cdc13p did not complement the temperature-sensitive phenotype of cdc13-1 at 30 or 37°C (Fig. 5A). Since Cdc13(451–693)p could not complement the growth arrest caused by the cdc13-1 mutation, telomere-binding activity alone was therefore not sufficient to account for the essentiality of Cdc13p.

To test whether Cdc13(252–924)p complements the null allele of cdc13, a plasmid loss experiment was conducted. Here, the cdc13Δ::HIS3 strain YJL501 requires the CDC13-bearing plasmid (YPE24-CDC13, with URA3 marker) to grow. If a second plasmid introduced into the yeast expresses functional CDC13, YJL501 then no longer requires plasmid YEP24-CDC13 for viability. Growth on 5-FOA is used to monitor the loss of YEP24-CDC13. As shown in Figure 5B, 5-FOA-resistant cells were observed in YJL501 transformed with plasmid expressing Cdc13p. Similarly, 5-FOA-resistant cells were observed in YJL501 expressing Cdc13(252–924), although this rescue was ∼10- to 100-fold less efficient than that of Cdc13p. However, transformation of YJL501 with plasmid vector, pTHA-NLS, or plasmids expressing other fragments of CDC13 did not yield any 5-FOA-resistant cells (Fig. 5B). We have also analyzed the meiotic products from CDC13/cdc13Δ::HIS3 diploid cells harboring plasmid pTHA-NLS-CDC13 or pTHA-NLS-CDC13(252–924). Having analyzed ∼2000 spore products each from CDC13/cdc13Δ::HIS3 cells carrying plasmid pTHA-NLS-CDC13 (Leu2 marker) or pTHA-NLS-CDC13(252–924) (Leu2 marker), we obtained 465 and 69 His+ Leu+ haploid cells, respectively (data not shown). Thus, even though it remains to be tested whether this complementation was caused by overexpression of Cdc13(252–924)p, Cdc13(252–924)p was sufficient to complement cdc13-1 and cdc13Δ mutations.

Cdc13(252–924)p was capable of interacting with Stn1p

Cdc13p was shown to interact with Stn1p. To test if this interaction is required for the essential function of Cdc13p, the interaction between Cdc13p and Stn1p was evaluated. Two-hybrid analysis was used previously to establish the interaction between Cdc13p and Stn1p (37). Here, we used the same approach to dissect the region within Cdc13p that interacts with Stn1p. Plasmids were constructed in which CDC13 or its fragments were fused to the DNA-binding domain of GAL4. These plasmids were transformed into yeast strain Y190 carrying a plasmid with STN1 fused to the activation domain of GAL4 (PACT-STN1) to analyze for their interaction. The ability to grow on medium lacking histidine was used as the criterion to evaluate the interaction between Stn1p and various truncated forms of Cdc13p. As shown in Figure 6,
under our assay conditions, His⁺ colonies were apparent at 25 or 30°C in Y190 harboring plasmids pAS-CDC13 and pACT-STN1. This result was consistent with the previous report that Cdc13p interacts with Stn1p (37). His⁺ colonies were also apparent at 25 or 30°C in Y190 harboring plasmids pAS-CDC13(252–924) and pACT-STN1 indicating that Cdc13(252–924)p was capable of interacting with Stn1p. We also examined if Cdc13-1p, with Pro371 being replaced by Ser (24,25), might interact with Stn1p. Interestingly, the HIS3 expression level in Y190/pACT2 or Y190/pACT-STN1 carrying plasmid pAS2 (vector), pAS-CDC13 (1–924), pAS-CDC13-1 (P371S) or pAS-CDC13(252–924) (252–924) were grown on YC medium without leucine and tryptophan for 16 h at 30°C. Ten-fold serial dilutions of yeast cells were spotted on plates without leucine and tryptophan (YC-Leu-Trp), or without leucine, tryptophan and histidine with the addition of 25 mM of 3-AT (YC-Leu-Trp-His+3-AT), and incubated at 25°C (top panel) or 30°C (bottom panel) until colonies formed. The photographs of the plates are shown.

To address the possibility that reduced interaction between Cdc13-1p and Stn1p was due to the reduced stability of Cdc13-1p, the Cdc13-1p level at non-permissive temperature was evaluated. Here, we applied an immunoblotting assay using polyclonal antibodies raised against Cdc13(1–252)p (T.-L.Pang and J.-J.Lin, unpublished data) to evaluate the cellular level of Cdc13p. As shown in Figure 7, under the condition that >90% of the cells had arrested at G2/M phase, the Cdc13-1p level at the non-permissive temperature (30°C) was similar to the level of Cdc13-1p at the permissive temperature (25°C). Our results suggested that reduced interaction between Cdc13-1p and Stn1p was not due to the reduced stability of Cdc13-1p.

DISCUSSION

Cdc13p binds specifically to the single-stranded TG₁₋₃ tail of yeast telomere. Here, we have delineated the regions of Cdc13p responsible for this interaction. The single-stranded TG₁₋₃-binding domain of Cdc13p is within amino acids 451–693, and binding of the single-stranded TG₁₋₃ is specific. However, binding to telomeres by Cdc13p is not sufficient to account for the essential function of Cdc13p. Judging from the results the C-terminal 673-amino-acid polypeptide was sufficient to complement the growth defect phenotype of several cdc13 mutants and interaction with Stn1p. Our results indicated that the Stn1p-interaction function and the telomere-binding activity of Cdc13p were essential for cell growth.

Upon proteolytic degradation of the Cdc13p–DNA complex, a fragment of Cdc13p that covers amino acids 557 to 690 was shown to associate with single-stranded TG₁₋₃ DNA (42). In this report, our strategy to identify the DNA-binding region of Cdc13p was the subcloning of restriction enzyme-digested CDC13 fragments followed by evaluation of the single-stranded TG₁₋₃-binding activities of these expressed fragments. Using this approach, the smallest fragment that still contained the single-stranded TG₁₋₃-binding activities of Cdc13p was within amino acids 451–693 (Fig. 1). Smaller fragments such as Cdc13(510–693), Cdc13(451–600) or Cdc13(601–693) did not show detectable single-stranded TG₁₋₃ binding activity. It is unclear whether Cdc13p covers amino acids 557 to 690, did not interact with single-stranded TG₁₋₃ DNA. Nevertheless, our identification of

Figure 6. Cdc13(251–924)p interacts with Stn1p. Yeast cells Y190/pACT2 or Y190/pACT-STN1 carrying plasmid pAS2 (vector), pAS-CDC13 (1–924), pAS-CDC13-1 (P371S) or pAS-CDC13(252–924) (252–924) were grown on YC medium without leucine and tryptophan for 16 h at 30°C. Ten-fold serial dilutions of yeast cells were spotted on plates without leucine and tryptophan (YC-Leu-Trp), or without leucine, tryptophan and histidine with the addition of 25 mM of 3-AT (YC-Leu-Trp-His+3-AT), and incubated at 25°C (top panel) or 30°C (bottom panel) until colonies formed. The photographs of the plates are shown.

Figure 7. Western blotting analysis of Cdc13-1p. Strain 2758-8-4b (cdc13-1) was grown at permissive temperature (25°C) and shifted to non-permissive temperature (30°C) for 2 h. Total cell extracts prepared from these cells were separated by 10% SDS–PAGE and subjected to immunoblotting analysis using polyclonal antibodies raised against Cdc13(1–252)p. Bound antibodies were visualized by chemiluminescence using an ECL kit (Amersham-Pharmacia). Molecular markers are indicated on the left.
Cdc13(451–693) as a stably expressed telomeric-binding domain of Cdc13p provides useful information for future understanding of how Cdc13p binds and modulates telomere function.

The identity of the second migration bands using TG30 or TG35 as DNA substrate is unclear (Fig. 2). The simplest explanation for the appearance of this second migration band would be that TG30 or TG35 provides enough space for two molecules of Cdc13(451–693) to bind. This result would also imply that the optimal binding site of Cdc13(451–693) on DNA was ∼13–15 bases, an estimation that is in reasonable agreement with the results of using a 34-kDa Cdc13 DBD by Huges et al. (42). However, this explanation is complicated by observations from several reports that telomeric DNA-binding proteins can also promote the formation of a G-quartet structure (43,44). It will be interesting to know whether Cdc13p promotes the formation of a G-quartet structure upon binding.

Contrary to the ciliate telomere-binding proteins, both Cdc13p and Cdc13(451–693) preferred low salt for binding (data not shown). Sequence analysis of this 243-amino-acid region did not provide information on which residues within this region are responsible for interacting with telomeres. Evidence was presented that single-stranded DNA-binding proteins interact with DNA via hydrophobic interactions between aromatic side chains of the protein and the DNA bases (45–47). For example, the single-stranded DNA-binding protein T4 gp32 utilizes residues Tyr84, 99, 106, 115, 137, 186 and Phe138 to form a hydrophobic pocket to interact with the bases of single-stranded DNA (46). In addition, the Oxytricha α and β complex uses a series of aromatic amino acids including Tyr130α, Tyr293α, Tyr134β, His292α, Phe109β and Tyr239α to interact with the extended bases of single-stranded T₂G₄ DNA (47). It is likely that aromatic residues within the 243 amino acids of Cdc13p play a role in the interaction with telomeres. Interestingly, this region indeed has a high content of aromatic residues. However, it remains to be determined which amino acid residues are responsible for this protein–DNA interaction.

Using the one-hybrid system, Bourns et al. (34) showed previously that the N-terminal 251 amino acids of Cdc13p interacted with telomere, but that amino acids 252–508 or 509–924 of Cdc13p failed to do so. Since the N-terminal 251 amino acids were sufficient to target Cdc13p to telomere, it was concluded that the telomere-binding domain was within this region of Cdc13p. In contrast to our study, they showed that the telomere-binding domain was mapped to within the 451–693 amino acids of Cdc13p both in vitro and in vivo (Figs 1 and 4). Given that the N-terminal 1–251 amino acids of Cdc13p did not bind to single-stranded TG₃,₄ DNA in vitro (Fig. 1), it would be interesting to know how this region of Cdc13p interacts with telomeres in vivo. Clearly, in vivo one-hybrid system detected not only proteins that interact directly with telomeric DNA but also those that interact indirectly (34). For example, Sir and Rif proteins interact with telomeres by their ability to bind Rap1p (48–50). One possible explanation is that this N-terminal 251-amino-acid fragment interacts indirectly with the telomere. Conceivably, protein factors that associated with telomeres including Rap1p, Rif proteins and Sir proteins, are potential candidates for recruiting the N-terminal 251-amino-acid polypeptide of Cdc13p to telomeres.

A Pro371 to Ser substitution caused the phenotype of Cdc13-1p (24,25). On the basis of gel mobility-shift assay, the cdc13-1 mutation did not affect the telomere-binding activity of Cdc13p [J.-J.Lin and V.A.Zakian, unpublished result (24,42)]. In our two-hybrid system, the interaction of Cdc13-1p with Stn1p was temperature dependent and the interaction was reduced at the non-permissive temperature (Fig. 6). This result indicated that interaction between Cdc13p and Stn1p is essential for cell survival. While we cannot rule out that another uncharacterized change in Cdc13-1p is responsible for the cell cycle arrest at the non-permissive temperature, relatively weak interaction between Cdc13-1p and Stn1p might indeed cause the accumulation of single-stranded G-rich DNA near telomeres (35) leading to this phenotype.

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