Specific Binding of Single-stranded Telomeric DNA by Cdc13p of Saccharomyces cerevisiae*

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Cdc13p is a single strand telomere-binding protein of Saccharomyces cerevisiae; its telomere-binding region is within amino acids 451–693, Cdc13(451–693)p. In this study, we used purified Cdc13p and Cdc13(451–693)p to characterize their telomere binding activity. We found that the binding specificity of single-stranded TG₁₋₃ DNA by these two proteins is similar. However, the affinity of Cdc13(451–693)p to DNA was slightly lower than that of Cdc13p. The binding of telomeric DNA by these two proteins was disrupted at NaCl concentrations higher than 0.3 M, indicating that electrostatic interaction contributed significantly to the binding process. Because both proteins bound to strand TG₁₋₃ DNA positioned at the 3′ end, the 5′ end, or in the middle of the oligonucleotide substrates, our results indicated that the location of TG₁₋₃ in single-stranded DNA does not appear to be important for Cdc13p binding. Moreover, using DNase I footprint analysis, the structure of the telomeric DNA complexes of Cdc13p and Cdc13(451–693)p was analyzed. The DNase I footprints of these two proteins to three different telomeric DNA substrates were virtually identical, indicating that the telomere contact region of Cdc13p is within Cdc13(451–693)p. Together, the binding properties of Cdc13p and its binding domain support the theory that the specific binding of Cdc13p to telomeres is an important feature of telomeres that regulate telomerase access and/or differentiate natural telomeres from broken ends.

Single-stranded guanosine-rich DNA tail is a common structural feature in most of the eukaryotic telomeres (1–7). For example, ciliated protozoa telomeres are extended to form a 12–16-base single-stranded G-tail (1, 3). In Saccharomyces cerevisiae, transient single-stranded TG₁₋₃ tails with lengths larger than 30 bases are detected late in the S phase (4). This presence of single-stranded G-tails was postulated as an intermediate for telomere replication (8). Single-stranded G-tails could form a DNA quadruplex in vitro known as the G-quartet (9, 10), although it remains to be determined whether such a structure indeed exists in cells.

Protein factors that bind to the single-stranded telomeric DNA have been identified in several organisms (11–19). Among these protein factors, Oxytricha telomere-binding protein has been well characterized. It is heterodimeric and is composed of an α and β subunit (20–22). The α subunit is a single-stranded DNA-binding protein that binds to the G₄T₄ single-stranded end of a telomere. Although the β subunit is not directly involved in binding, it is required for making the terminus-specific binding. Cdc13p is a single-stranded TG₁₋₃-binding protein that interacts with telomeres in S. cerevisiae (13, 14, 23). However, although Cdc13p in yeast is the functional equivalent of Oxytricha α- and β-binding proteins, it shares no sequence similarity with Oxytricha telomere-binding proteins (13, 14, 23).

The binding of Cdc13p to telomeric DNA is essential for its function in telomeres (24), and it appears to have multiple functions in cells. For example, Cdc13p is involved in cell cycle control since a temperature-sensitive allele of CDC13, cdc13-1, causes cell cycles to arrest in the G2/M phase at non-permissive temperatures (25). The binding of Cdc13p to telomeres might cause yeast cells to differentiate whether the ends of linear DNA are telomeres or broken ends (26). In addition, Cdc13p appears to be a key factor in telomere replication. It interacts with Est1p that is associated with telomerase RNA (27–30) to recruit telomerase to telomeres for replication. This was evidenced in part by the presence of a mutant allele of CDC13, cdc13αα, which causes a gradual loss of the telomere (13). Moreover, Cdc13p could interact with the catalytic subunit of DNA polymerase α, suggesting that it might be involved in a C-strand synthesis of telomeres (28). It might also prevent end-to-end fusion of chromosomes and protect chromosome from degradation by nucleases.

CDC13 is an essential gene that encodes a 924-amino acid protein with a molecular mass of 104,895 Da (25). The Cdc13p fragment ranging from amino acids 451 to 693, Cdc13(451–693)p, contains the telomere-binding region of Cdc13p; it is sufficient to bind single-stranded telomeric DNA in vitro and interacts with telomeres in vivo (24, 31). However, a sequence comparison among Cdc13(451–693)p and known DNA- or RNA-binding proteins did not provide any information on Cdc13(451–693)p responsible for binding to telomeres (24). Thus, Cdc13(451–693)p contains a novel motif for telomere binding. To understand how Cdc13p interacts with telomeres, we used purified Cdc13p and Cdc13(451–693)p to analyze their binding properties. The results of both EMSA and DNase I footprint analysis revealed the specific binding of telomeric DNA by these two proteins.

MATERIALS AND METHODS

Expression and Purification of 6xHis-tagged Cdc13p and Cdc13(451–693)p—A baculovirus system was used to purify Cdc13p. An insect cell line sf21 was used as the host for virus propagation and protein purification. Escherichia coli DH5α was used as a host for plasmid construc-

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1 C.-L. Hsu and J.-J. Lin, unpublished results.

2 The abbreviations used are: EMSA, electrophoretic mobility shift assay; Ni-NTA, nickel-nitrotriacetic acid.
tation and propagation, and BL21(DE3)pLysS was used as the host for Cdc13(451–693)p purification. Plasmid pBac6His-CDC13 was constructed by inserting a 3.0-kilobase pair NcoI-SauI fragment of CDC13 to NcoI- and Suttle-digested pBac6His (modified from pBlueBac4 by J.-J. Lin, Invitrogen). This plasmid enabled the expression of Cdc13p with 6xHis tagged at the N terminus. A recombinant virus that expressed 6xHis-tagged Cdc13p was generated by co-infection of plasmid pBac6His-CDC13 and Bac-N-Blue DNA to sf21 cells (Invitrogen). Plasmid pET6H-CDC13(451–693), which was used to purify the Cdc13p, was constructed by inserting the NcoI-NruI fragment of pTHA-NLS-CDC13(451–693) into NcoI-SmaI-digested pET6H (donated by C.-H. Hu, National Marine University, Taipei, Taiwan). The resulting plasmid was used to express 6xHis-tagged Cdc13p in 40 ml of Buffer A (50 mM Tris-HCl, pH 8.0, 10 mM MgSO₄, 1 mM CaCl₂) and incubated at 25 °C for 10 min. 1 unit of DNase I was added and incubated at 37 °C for another 10 min. The reaction was stopped with 10 μl of 250 mM EGTA. The DNA was then precipitated by adding 1 μl of 10 mg/ml oyster glycogen and 150 μl of ethanol. The precipitant was collected by centrifugation, dried, and analyzed by electrophoresis using a 12% polyacrylamide sequencing gel.

**RESULTS**

**Isolation of Recombinant Cdc13p and Its Specific Binding to Single-stranded TG₁₋₅DNA**—The baculovirus expression system was used to purify Cdc13p. In this study, Cdc13p with six histidines tagged at the N terminus was expressed in insect sf21 cells, and the protein was purified to homogeneity using a Ni-NTA-agarose column (Fig. 1, lane 2). The purified protein has an apparent mass of 105 kDa, which is in agreement with the predicted size of Cdc13p. Western blotting analysis further confirmed that it is Cdc13p (data not shown).

The binding specificity of Cdc13p to telomeric DNA was determined using EMSA analysis. Purified protein was mixed with several concentrations of different competitors before incubating with 100 nM purified Cdc13p. Competitors were yeast TG15, vertebrate (T₅AG₃)₅, Oxytricha (T₄G₅)₂, Tetrahymena (T₄G₅)₂, and total yeast RNA (Ysc RNA). The gel shift assay was then carried out as shown in autoradiogram, B, quantification of the Cdc13p binding activity. The amount of 32P-labeled TG15 bound to the protein was quantified by a PhosphorImager, and binding without any competitor was taken as 100% (A, lane 2). Data are the average of three experiments.

**B**

- **A**
- **B**
- **C**
- **D**

**FIG. 1. Purification of Cdc13p and Cdc13(451–693)p.** Cdc13p and Cdc13(451–693)p with 6xHis tag were purified from sf21 and E. coli using a Ni-NTA-agarose column, respectively (see under “Materials and Methods”). A Coomassie Blue-stained 10% SDS-polyacrylamide gel is given. Lane 1 shows the molecular mass markers. Lanes 2 and 3 were 2 μg each of purified Cdc13p and Cdc13(451–693)p, respectively.

**FIG. 2. Specific binding of Cdc13p to single-stranded TG₁₋₅DNA.** Competition analysis with various telomeric DNA was used to determine binding specificity. A, 20 nm 32P-labeled TG15 were mixed with several concentrations of different competitors before incubating with 100 nM purified Cdc13p. Competitors were yeast TG15, vertebrate (T₅AG₃)₅, Oxytricha (T₄G₅)₂, Tetrahymena (T₄G₅)₂, and total yeast RNA (Ysc RNA). The gel shift assay was then carried out as shown in autoradiogram, B, quantification of the Cdc13p binding activity. The amount of 32P-labeled TG15 bound to the protein was quantified by a PhosphorImager, and binding without any competitor was taken as 100% (A, lane 2). Data are the average of three experiments.
**Cdc13p Binds Telomeres**

**TABLE I**

| Name | Sequences |
|------|------------|
| TG10 | 5’-GGGTGGTGGTGG-3’ |
| TG13 | 5’-GGGTGGTGGTGGTG-3’ |
| TG15 | 5’-GGGTGGTGGTGGTGGTG-3’ |
| TG20 | 5’-GGGTGGTGGTGGTGGTGGTG-3’ |
| TG25 | 5’-GGGTGGTGGTGGTGGTGGTGGTG-3’ |
| TG30 | 5’-GGGTGGTGGTGGTGGTGGTGGTGGTG-3’ |
| TG35 | 5’-GGGTGGTGGTGGTGGTGGTGGTGGTGGTG-3’ |
| (TG)_{12} | 5’-GGGTGGTGGTGGTGGTGGTGGTGGTGGTG-3’ |
| (TGG)_{6} | 5’-GGGTGGTGGTGGTGGTGGTGGTGGTGGTG-3’ |
| (TGGG)_{6} | 5’-GGGTGGTGGTGGTGGTGGTGGTGGTGGTG-3’ |
| TG15 | 5’-GGGTGGTGGTGGTGGTGGTGGTGGTGGTG-3’ |
| TG13 | 5’-GGGTGGTGGTGGTGGTGGTGGTGGTGGTG-3’ |
| TG10 | 5’-GGGTGGTGGTGGTGGTGGTGGTGGTGGTG-3’ |

**TABLE II**

**Binding affinity of Cdc13p and Cdc13(451–693)p to DNA substrates**

The apparent binding constant (Kd_{app}) for each substrate was determined from an average of 2–3 experiments.

| Telomeric DNA | Cdc13p | Cdc13(451–693)p |
|---------------|--------|----------------|
| TG10          | 310 ± 99 | 364 ± 23       |
| TG13          | 89 ± 25  | 240 ± 140      |
| TG15          | 46 ± 7   | 72 ± 6         |
| TG20          | 106 ± 5  | 161 ± 36       |
| TG25          | 55 ± 10  | 70 ± 2         |
| TG30          | 43 ± 7   | 41 ± 15        |
| TG35          | 61 ± 2   | 65 ± 9         |
| (TG)_{12}     | 30 ± 0   | 288 ± 0        |
| (TGG)_{6}     | 35 ± 1   | 313 ± 71       |
| (TGGG)_{6}    | 53 ± 8   | 409 ± 40       |

(T_{2}G_{4}) telomeric DNA did not compete for the binding of TG15 to Cdc13p (Fig. 2A, lanes 7–12). Total yeast RNA, single-stranded C_{1–3}A DNA, or duplex TG_{1–3}C_{1–3}A DNA did not compete for Cdc13p binding (Fig. 3A, lanes 16–18) (data not shown). Interestingly, Cdc13p formed two complexes with TG15, although the nature of these multiple complexes is unclear. Nevertheless, these results indicated that Cdc13p bound specifically to single-stranded TG_{1–3} telomeric DNA.

DNA encoding Cdc13(451–693)p with 6xHis tag was expressed in E. coli (BL21(DE3)pLysS). Although Cdc13(451–693)p formed insoluble aggregates at 37 °C (24), a sufficient amount of soluble Cdc13(451–693)p, however, could be obtained at 25 °C (Fig. 1, lane 3). We then investigated the binding properties of the purified Cdc13(451–693)p. Similar to Cdc13p, purified Cdc13(451–693)p bound specifically to single-stranded TG_{1–3} telomeric DNA (Fig. 3). However, only one distinct complex was observed. This result further confirmed our conclusion that the telomeric DNA-binding domain of Cdc13p is located within amino acids 451–693. Identical results were previously obtained with Cdc13p fusion protein and chemically renatured Cdc13(451–693)p (14, 24). Previously, E. coli extracts containing Cdc13p fused to glutathione S-transferase was used to show that Cdc13p bound specifically to single-stranded TG_{1–3} DNA in vitro (14), and the renatured Cdc13(451–693)p was used to demonstrate that this region contained the telomere-binding domain of Cdc13p, and its binding to telomeres was specific (24).

**Binding of Cdc13p and Cdc13(451–693)p to Long Telomeric DNA**—To determine whether Cdc13p and Cdc13(451–693)p could bind to long single-stranded TG_{1–3} telomeric DNA, Cdc13p and Cdc13(451–693)p were mixed with telomeric DNA substrates with different lengths, and the complexes were analyzed. The results shown in Fig. 4 demonstrated that both Cdc13p and Cdc13(451–693)p were capable of forming complexes with long telomeric DNA. Multiple protein-DNA complexes were apparent in long DNA substrates, and the patterns of the complexes suggested that more than one protein could bind to a single DNA molecule. We also determined the binding affinity of Cdc13p and Cdc13(451–693)p to these DNA substrates. An apparent binding constant was determined from the Hill plot. As shown in Table II, Cdc13(451–693)p appears to bind to telomeric DNA with an affinity similar to that of Cdc13p. Cdc13p required telomeric sequences longer than 13 bases for proper binding, whereas Cdc13(451–693)p required 15 bases for proper binding.

**Telomeric DNA Binding Properties of Cdc13p and Cdc13(451–693)p**—The telomeric DNA sequences of S. cerevisiae are combinations of TG, TGG, and TGGG repeats. To test if Cdc13p and Cdc13(451–693)p have a sequence preference, the binding affinities of these two proteins for (TG)_{12}, (TGG)_{6}, or (TGGG)_{6} were determined. As shown in Table II, the apparent binding constants of Cdc13p to these three substrates were similar to other telomeric DNA substrates. This result sug-
gested that Cdc13p did not favor either repeat for binding. However, Cdc13(451–693)p bound to these three substrates with affinities significantly lower than binding to other telomeric DNA substrates, suggesting that the telomeric DNA-binding domain alone preferred TG1–3 sequences for binding.

*Oxytricha* telomere-binding proteins were shown to bind to T4G4 telomeric DNA at a high concentration of salt (22, 32, 33). We then investigated whether the affinity of Cdc13p or Cdc13(451–693)p for TG15 may depend on NaCl concentrations. As shown in Fig. 5, both proteins dissociated from TG15 at a NaCl concentration higher than 0.3 M. However, Cdc13(451–693)p appeared to tolerate NaCl better than Cdc13p.

To evaluate the stability of the protein-DNA complex, the dissociation rate of protein-DNA complex was measured. Cdc13p or Cdc13(451–693)p was first bound to 32P-labeled TG15, and then an excess amount of unlabeled TG15 was added to prevent a re-association of protein to labeled DNA. The dissociation rate was estimated as the time required for half of the protein-DNA complex to dissociate. As shown in Fig. 6, both Cdc13p and Cdc13(451–693)p had dissociation rates ~ 30 min, suggesting that these two proteins bound to telomeric DNA with similar stability.

*Cdc13p Does Not Require a 3' end for Binding—To determine if single-stranded TG1–3 has to locate at the 3' end for Cdc13p binding, we synthesized oligonucleotides, 5'-Tel, Int-Tel, and 3'-Tel (Table I), of identical size harboring telomeric sequences at various locations. They were tested for Cdc13p binding by EMSA. Because all three substrates bound to Cdc13p or Cdc13(451–693)p to the same extent (Fig. 7), the location of TG1–3 in a single-stranded DNA does not appear significant for Cdc13p binding.

In Fig. 7, the three oligonucleotides migrated differently on a polyacrylamide gel even though they have the same size and were heat-denatured and quick-cooled on ice before the binding assay. This result suggested an intrinsic position-dependent structure of these oligonucleotides. Also, although Int-Tel DNA gave the slowest mobility on the polyacrylamide gel, it had a slightly faster mobility upon binding to Cdc13(451–693)p. This migration behavior suggested that Cdc13(451–693)p bound to

![Fig. 4. Binding of Cdc13p and Cdc13(451–693)p to single-stranded TG1–3 DNA with a different length. 5 nM each of 32P-labeled TG10, TG15, TG20, TG25, TG30, and TG35 were mixed with several concentrations of the purified Cdc13p (A) or Cdc13(451–693)p (B) before subjecting to gel shift assay. The concentrations of Cdc13p or Cdc13(451–693)p used in each set of experiments were 0, 31, 63, 125, 250, and 500 nM. Autoradiograms are shown here.](image)

![Fig. 5. Both Cdc13p and Cdc13(451–693)p prefer low salt for binding. 5 nM 32P-labeled TG15 were incubated with 50 nM of Cdc13p or Cdc13(451–693)p at room temperature for 10 min. 1 μM unlabeled TG15 was added to the reaction mixtures, and gel shift assay then was performed. The binding was quantified by a PhosphorImager, and the activity without NaCl was taken as 100%.](image)

![Fig. 6. Slow dissociation of Cdc13p and Cdc13(451–693)p from single-stranded TG1–3DNA. 15 nM 32P-labeled TG15 were incubated with 50 nM Cdc13p or Cdc13(451–693)p at room temperature for 10 min. 1 μM unlabeled TG15 was then added (t = 0). Aliquots of the mixtures were withdrawn at indicated time points and were loaded onto a running gel. After electrophoresis, the amount of 32P-labeled TG15 that remained bound was quantified by a PhosphorImager, and the binding at t = 0 was taken as 100%.](image)

![Fig. 7. Cdc13p does not require a 3' end for binding. The binding of 5'-Tel, Int-Tel, or 3'-Tel by Cdc13p (A) and Cdc13(451–693)p (B) is shown. 5 nM each of 32P-labeled DNA were mixed with several concentrations of the purified Cdc13p or Cdc13(451–693)p, and gel shift assay was then carried out. The concentrations of Cdc13p and Cdc13(451–693)p used in each set of experiments were 0, 10, 40, and 160 nM. An autoradiogram is presented.](image)
telomeric DNA and minimized the position-dependent structure of the DNA substrates. Moreover, faster mobility caused by protein binding to the middle of the DNA fragment would imply a protein-induced structural alteration of the DNA.

**DISCUSSION**

Cdc13p is an essential sequence-specific DNA-binding protein involved in a wide range of telomere functions including telomere length maintenance (13, 27, 28), telomere position effect (14), and cell cycle regulation (25). Therefore, it is important to elucidate the interaction of this protein with telomeric DNA. In this study, we purified Cdc13p in native form and its telomere-binding domain, Cdc13(451–693)p. Using these purified proteins, the telomeric DNA binding properties were characterized. Both Cdc13p and Cdc13(451–693)p bound to telomeric DNA with similar specificity and stability. Furthermore, DNase I footprints showed virtually identical binding of telomeric DNA by both Cdc13p and Cdc13(451–693)p, indicating that amino acid 451–693 is the region within Cdc13p that contacts telomere DNA.

A dissociation constant of $\sim 10^{-7}$ M is corresponding to $\sim 10$ kcal/mol of binding energy. Binding energies of this magnitude are certainly common among macromolecule-macromolecule interactions. We proposed that the binding of Cdc13p to telomeric DNA was the result of at least two factors, specific bond formation to nucleic acid bases and nonspecific electrostatic interaction. The binding to yeast telomeric DNA was not reduced by competition with telomeric DNA from other species, suggesting that the binding is sequence-specific, and specific interaction between Cdc13p and the bases of nucleic acids should be critical for telomere binding. Moreover, because the binding is sensitive to ionic strength, electrostatic interaction must contribute significantly to the binding. This property is significantly different from that of Oxytricha telomere-binding protein, which used a series of aromatic amino acid residues to interact with the extended bases of single-stranded T,G,T DNA (34). In Oxytricha, the phosphodiester-sugar backbone that contributes to electrostatic interaction was largely solvent-exposed. It could be worthwhile in future work to gain more precise information on this protein-DNA interaction.

In Fig. 7, EMSA analysis implicated a structure alteration of telomeric DNA induced by Cdc13(451–693)p. Similarly, DNase I footprint analysis also supports the presence of protein-induced alteration on DNA (Figs. 8 and 9). The footprint assay was done using 5 nM each of 5'-Tel, Int-Tel, or 3'-Tel. The concentrations of Cdc13(451–693)p were 40 nM (lane 3) and 160 nM (lane 4), respectively. The protected regions are bracketed, and the asterisks indicate the positions of the hypersensitive sites.
structure. For example, DNA bends on double-stranded DNA usually cause hypersensitive sites by DNase I digestion. Although we do not know the exact nature of the structural change in the single-stranded telomeric DNA induced by Cdc13p, it appeared that Cdc13p indeed caused structural alteration in telomeric DNA. Such alteration caused by Cdc13p may be an important feature of telomeres. Protein-induced DNA distortion at specific sites is considered to be an important mechanism for promoting the multiprotein interactions involved in regulation of gene activity (35, 36), initiation of replication (37–39), site-specific recombination (40, 41), or recognition of DNA damage (42). Cdc13p was shown to interact with Stn1p (43), the catalytic subunit of polymerase a (44). Cdc13p was shown to interact with Stn1p (43), the catalytic subunit of polymerase α, and Est1p (28). Moreover, Cdc13p was proposed to mediate telomerase access to telomeres (27). Thus, the alteration of telomeric DNA by Cdc13p might facilitate the formation of a multiprotein complex on telomeres. Alternatively, the single-stranded telomeric tail was shown to loop back to the double-stranded region of telomeres to form a “t-loop” structure in mammalian telomeres (44). In yeast, telomeres are folded back to form a looped structure (45, 46). By cooperating with the double-stranded telomeric DNA bends induced by Rap1p (47, 48), the alteration of single-stranded telomeric DNA by Cdc13p might facilitate the formation of such a structure in yeast.

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