The protein Cdc13p binds telomeres in vivo and is essential for the maintenance of the telomeres of Saccharomyces cerevisiae. In addition, Cdc13p is known to bind single-stranded TG$_{1-3}$ DNA in vitro. Here we have shown that Cdc13p also binds DNA quadruplex, G-quartet, formed by TG$_{1-3}$ DNA. Moreover, the binding of Cdc13p causes a partial denaturing of the G-quartet DNA. Formation of DNA quadruplexes may involve the intermolecular association of TG$_{1-3}$ DNA and inhibit the extension of telomeres by telomerase. Thus, our finding suggests that Cdc13p may disrupt telomere association and facilitate telomere replication.

Telomeres, the ends of eukaryotic chromosomes, are essential for the stability of linear chromosomes (1, 2). Telomeric DNA sequences consist of simple sequences repeated with one strand rich in guanine. For example, the telomeres of the budding yeast Saccharomyces cerevisiae contain ~300 base pairs of double-stranded (TG$_{1-3}$/C$_{1-3}$A) sequences. In most of the eukaryotic telomeres, the G-rich strand protrudes as a 3’ overhang. Single-stranded telomeric DNA is capable of forming multimeric structures. It could form G-quartet structures within a single DNA molecule or involving two or four DNA molecules (3). Formation of G-quartet structures in vitro requires high DNA concentrations and long incubation time. A G-quartet consists of four guanines arranged in a square-planar array, and each guanine serves as both hydrogen bond acceptor and donor in a Hoogsteen base pair. Successive layers of G-quartets stack on each other to form folded structures (4–8).

The biological role of G-quartet remains unknown. Nevertheless, evidence has been presented to indicate that G-quartet indeed may exist in vivo. For instance, both the β-subunit of the Oxytricha telomere-binding protein and the Rap1p of S. cerevisiae promote the formation of G-quartet at a very low G-rich DNA concentration in vitro (9–11). Moreover, proteins that bind to G-quartets were identified in several organisms, although their biological role in telomeres needs to be determined (12–19). Thus, formation of G-quartet in vivo is likely to occur and is supposed to be involved in telomere-telomere interaction.

Cdc13p is a single-stranded TG$_{1-3}$-binding protein that interacts with telomeres in S. cerevisiae (20–22). It has 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 G$_2$/M phase at nonpermissive temperatures (23). Binding of Cdc13p to telomeres may cause yeast cells to differentiate whether the ends of linear DNA are telomeres or broken ends (24). In addition, Cdc13p appears to be a key factor in telomere replication (25–27). It might also prevent end-to-end fusion of chromosomes and thus protect chromosomes from degradation by nucleases.

CDC13 is an essential gene that encodes a 924-amino acid protein with a molecular mass of 104,895 Da (23). The Cdc13p fragment ranging from amino acids 451 to 693, Cdc13-(451–693)p, contains the telomere binding region of Cdc13p. This fragment is sufficient to bind single-stranded telomeric DNA in vitro, and it interacts with telomeres in vivo (28–30). Herein we have demonstrated that Cdc13p also binds intermolecular G-quartet and partially denatures the structure. This observation implies that Cdc13p may have a role in vivo in regulating the association of yeast telomeres and the accessibility of telomerase to telomeres.

MATERIALS AND METHODS

Expression and Purification of 6xHis-tagged Cdc13p and Cdc13-(451–693)p—Insect cell line sf21 was used as the host for baculovirus propagation, expression, and purification of Cdc13p. Plasmid pBac6His-CDC13 was constructed by inserting a 3.0-kilobase pair NcoI-Sall fragment of CDC13 into NcoI- and Sall-digested pBac6His (constructed by J.-J. Lin, modified from pBlueBac4, Invitrogen). This plasmid enabled the expression of Cdc13p with a 6xHis tag at the N terminus. Recombinant virus that express 6xHis-tagged Cdc13p was generated by co-infection of plasmid pBacHis-CDC13 and Bac-N-Blue DNA to sf21 cells (Invitrogen). To purify 6xHis-tagged Cdc13p, ~5 × 10$^7$ sf21 cells were infected with recombinant virus for 4 days. Cells were washed with phosphate-buffered saline and then lysed by adding Nonident P-40 lysis buffer (50 mM Tris, pH 8.0, 250 mM imidazole, and 20% glycerol. Purified protein was generated by co-infection of plasmid pBacHis-CDC13 and Bac-N-Blue and propagation, and BL21(DE3)pLysS was used as the host for Cdc13-(451–693)p purification. Plasmid pET6H-CDC13-(451–693), which was used to purify the Cdc13-(451–693)p, was constructed by inserting the NcoI-Sall fragment of pTHA-NLS-CDC13-(451–693) into NcoI-MsiI-digested pET6H (a gift from C.-H. Hu, National Marine University, Taiwan). The resulting plasmid was used to express 6xHis-tagged Cdc13-(451–693)p under the control of the T7 promoter (28, 29). To
purify 6xHis-tagged Cdc13p-(451–693)p, a 1-liter culture of *E. coli* harboring pET6H-CDC13-(451–693) was grown at 25 °C to an *A*_{600} of 0.5 and induced with the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were grown at 25 °C for another 4 h before harvesting by centrifugation. Cells were resuspended in 10 ml of sonication buffer (50 mM NaOH, pH 7.8, 300 mM NaCl, 5 mM β-mercaptoethanol, 1× protease inhibitors (Calbiochem)) and sonicated to release the cell contents. The sonicated cells were centrifuged at 13,000 × g for 15 min at 4 °C to obtain total cell-free extracts. 0.5 ml of Ni-NTA-agarose (Qiagen) was added to the total cell-free extracts and incubated at 4 °C for 1 h. The resin was washed and eluted with 2 ml of buffer containing 50 mM NaOH, pH 8.0, 250 mM imidazole, and 20% glycerol. Purified protein was aliquoted and frozen by dry ice-ethanol bath. The yield of Cdc13p-(451–693)p was ~4.2 mg from 1 liter of *E. coli* culture.

**DNA Preparation**—Oligonucleotide Sacc1 (5’-ACTGTCTGACTTTGATATGGGTGTGTGTGGG-3’ (31)) was labeled with [γ-32P]ATP (3000 mCi/μM, PerkinElmer Life Sciences) using T4 polynucleotide kinase (New England Biolabs) and subsequently purified from a 10% sequencing gel after electrophoresis. The labeled single-stranded Sacc1 DNA was mixed with an equal volume of 2 mM NaCl to give a final concentration of 4 μg/μl Sacc1 DNA in 10 mM Tris, pH 8.0, 0.1 mM EDTA, and 1 mM NaCl. Samples were then sealed in a 0.5 ml microcentrifuge tube using Parafilm and incubated at 37 °C for 72 h. The G4 DNA was then purified from a 5% nondenaturing polyacrylamide gel after electrophoresis.

**Electrophoretic Mobility Shift Assay**—To perform the assays (29, 32), Cdc13p or Cdc13-(451–693)p in Buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, and 1 μg of heat-denatured poly(dI-dC)) was mixed with 0.5 μg labeled DNA with a total volume of 15 μl. The reaction mixture was incubated at room temperature for 10 min. Then the mixtures were loaded directly on an 8% nondenaturing polyacrylamide gel, which was prerun at 125 V for 10 min. Electrophoresis was carried out in TBE (89 mM Tris borate, 2 mM EDTA, and 1× protease inhibitors (Calbiochem)) and sonicated to release the cell contents. The sonicated cells were centrifuged at 13,000 × g for 15 min at 4 °C to obtain total cell-free extracts. 0.5 ml of Ni-NTA-agarose (Qiagen) was added to the total cell-free extracts and incubated at 4 °C for 1 h. The resin was washed and eluted with 2 ml of buffer containing 50 mM NaOH, pH 8.0, 250 mM imidazole, and 20% glycerol. Purified protein was aliquoted and frozen by dry ice-ethanol bath. The yield of Cdc13p-(451–693)p was ~4.2 mg from 1 liter of *E. coli* culture.

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The apparent binding constant of Cdc13p and Cdc13-(451–693)p to DNA substrate was determined using electrophoretic mobility shift assay and quantified by a PhosphorImager. DNA substrates used were at 0.5 μm in all the experiments. Values presented in Table I were determined from interpolation on a Hill plot. Each value was the average of two to four experiments.

**Dimethyl Sulfate Footprint**—Labeled single-stranded or G4 Sacc1 DNA was mixed with Cdc13p or Cdc13-(451–693)p in 50 mM cacodylate buffer, pH 7.0, and incubated at 37 °C for 10 min. Dimethyl sulfate (DMS) was added to a final concentration of 0.05% and incubated at 22 °C for another 15 min. The mixtures were ethanol-precipitated, resuspended in 50 μl of 10% piperidine, incubated at 95 °C for 15 min, and separated in 12% denaturing gels. The gels were dried and analyzed with a PhosphorImager (Molecular Dynamics).

**RESULTS**

The proteins used in this study, Cdc13p and Cdc13-(451–693)p, were expressed in insect sf21 cells and *E. coli*, respectively. They contain a six-histidine tag at the N terminus and were purified to homogeneity using a Ni-NTA-agarose column (Fig. 1A). These two proteins were capable of binding to single-stranded TG₃₋₃ DNA (29). The DNA probe used in this study, Sacc1, has 16 bases of TG₃₋₃ at the 3’ end, preceded by 16 bases of nontelomeric DNA. It was shown to be capable of forming intermolecular parallel G-quartet structure in the presence of Na⁺ (31). Indeed, a slow migration band was apparent when Sacc1 was incubated in 1 mM NaCl at 37 °C for 72 h (Fig. 1B). In agreement with the previous report (31), the slow mobility, the heat sensitivity (Fig. 1B), and the DMS footprint (see Fig. 3) indicated that the band was an intermolecular parallel G-quartet DNA, G4 Sacc1. A model for the structure of G4 Sacc1 is illustrated in Fig. 1C.

To determine whether Cdc13p and Cdc13-(451–693)p could bind to G-quartet DNA, the proteins were mixed with single-stranded DNA substrate, and the G4 Sacc1 and the complexes were analyzed. The result, as shown in Fig. 2, demonstrated that both Cdc13p and Cdc13-(451–693)p were capable of forming complexes with single-stranded (Fig. 2A) and G-quartet DNA (Fig. 2B). We also determined the binding affinity of Cdc13p and Cdc13-(451–693)p to these DNA substrates. The apparent binding constant was determined from a Hill plot. Compared with Cdc13p, Cdc13-(451–693)p appears to bind this single-stranded telomeric DNA with lower affinity. Both proteins bind to G-quartet DNA with their affinity similar to that of single-stranded DNA (Table I).

To investigate whether the binding of Cdc13p and Cdc13-(451–693)p might affect the structure of G-quartet DNA, the protein-G4-DNA complex was deproteinized using phenol extraction, and the resulting DNA products were subjected to electrophoretic mobility shift assay analysis. As shown in Fig. 3, the level of single-stranded Sacc1 was increased upon incubation with Cdc13-(451–693)p. Quantification of the gel indicated that the proportion of the single-stranded Sacc1 within the DNA sample was increased by ~10% after being incubated with Cdc13-(451–693)p. The result suggested that the G-quartet was partially denatured into single-stranded DNA upon binding of Cdc13p.

One of the assays for the presence of G-quartets was to establish whether the N-7 of guanines is utilized for Hoogsteen-type base pairing. The accessibility of the N-7 of guanines therefore was probed using DMS (33). The methylation protection study was carried out using purified single-stranded DNA and the G4 Sacc1, and the result of the footprint analysis is shown in Fig. 4. In the single-stranded Sacc1 substrate, all guanines are methylated to the same extent indicating that the oligonucleotide is unfolded (Fig. 4A, lane 2). In contrast, the guanines within the telomeric region of G4 Sacc1 DNA became
inaccessible to methylation, although the guanines in the non-telomeric region remained accessible to methylation (Fig. 4, A, lane 3, and B). This result is in agreement with the conclusion that the Sacc1 oligonucleotide folded into a G-quartet structure.

To test whether the binding of Cdc13p and Cdc13-(451–693)p would actually affect the structure of G-quartet DNA, the protein-DNA complexes were subjected to DMS footprint analysis. Autoradiograms of the footprints for Cdc13p and Cdc13-(451–693)p complexing with G-quartet DNA are shown in Fig. 4 A, and the tracing of these results is shown in Fig. 4 B. In the presence of Cdc13p or Cdc13-(451–693)p, the methylation pattern of G-quartet DNA was changed. A few telomeric guanines now become accessible to DMS, suggesting that both Cdc13p and Cdc13-(451–693)p partially denature the G-quartet.

**DISCUSSION**

In vivo studies show that CDC13 is essential for the maintenance of S. cerevisiae telomeres (21, 23, 25–27). In vitro binding and footprint studies have shown that Cdc13p binds to single-stranded telomeric DNA, suggesting that Cdc13p is a telomere-binding protein of yeast (20, 21, 28–30). Herein we demonstrate that Cdc13p could also bind to the G-quartet DNA and change its structure.

Denaturing of G-quartet structure by Cdc13p could have several biological implications. In S. cerevisiae, telomeres acquire long G-rich tails at late S phase (34). Such a single-stranded tail could contribute to the formation of ring-like DNA structure in linear plasmids bearing a single-stranded TG1–3 tail (34, 35). Since G-quartet structure is not the substrate of telomerase (36, 37) and it is extremely stable once it is formed (6, 7, 36, 38), the G-quartet structure therefore has to be disassembled to allow for the extension by telomerase. The participation of Cdc13p in the binding and denaturing of G-quartets may provide a possible means for regulating the accessibility of telomeres by telomerase. Moreover, since G-quartet structure was implicated in the association among telomeres, including pairing between telomeres of sister chromatids (39), Cdc13p may also play a role in the regulation of
the organization of telomeres. In any case, binding of G-quartet DNA by a telomere-binding protein implies that such structure is biologically relevant in cells and that Cdc13p may be involved in the maintenance of such structure.

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