Exposure of Single-stranded Telomeric DNA Causes G₂/M Cell Cycle Arrest in Saccharomyces cerevisiae*

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In Saccharomyces cerevisiae, Cdc13p is a single-stranded TG₁-₃ DNA binding protein that protects telomeres and maintains telomere length. A mutant allele of CDC13, cdc13–1, causes accumulation of single-stranded TG₁-₃ DNA near telomeres along with a G₂/M cell cycle arrest at non-permissive temperatures. We report here that when the single-stranded TG₁-₃ DNA is masked by its binding proteins, such as S. cerevisiae Gbp2p or Schizosaccharomyces pombe Tcg1, the growth arrest phenotype of cdc13–1 is rescued. Mutations on Gbp2p that disrupt its binding to the single-stranded TG₁-₃ DNA render the protein unable to complement the defects of cdc13–1. These results indicate that the presence of a single-stranded TG₁-₃ tail in cdc13–1 cells serves as the signal for the cell cycle checkpoint. Moreover, the binding activity of Gbp2p to single-stranded TG₁-₃ DNA appears to be associated with its ability to restore the telomere-lengthening phenotype in cdc13–1 cells. These results indicate that Gbp2p is involved in modulating telomere length.

Telomeres are the structure at the ends of eukaryotic linear chromosomes (1, 2). In most organisms, the telomeric DNA is composed of short, tandem repeated sequences with a strand rich in guanine residues (G-strand) running 5’ to 3’ toward the end of telomere. For example, the telomeric sequences in the brewers yeast Saccharomyces cerevisiae are ~250–300 base pair-long TG₁₋₃/C₁₋₃:A repeats. Sequences of ciliate telomeres reveal that the G-strand extends beyond the duplex region, creating a short single-stranded 3’-overhang (3–5). In yeast, longer G-strand DNA with varying lengths, presumably an intermediate during telomere replication, is detected during late S phase (6, 7). Telomeres are essential for the maintenance of chromosome integrity. Telomeres protect chromosomes from degradation by nucleases, facilitate complete replication of chromosomes, and differentiate linear chromosome ends from broken ends (1, 2).

Several single-stranded telomeric DNA binding proteins, including Oxytricha α and β subunits (8, 9), Cdc13p (10, 11), Gbp2p (12, 13), hnRNPs (14–16), and Pot1 (17), have been identified in vitro. Among these proteins, Oxytricha α and β subunits have been well characterized because of the abundance of telomeres in this organism. The α subunit binds to the G₄T₄G₄T₄ single-stranded end of the telomere, and the β subunit is required for making the terminus-specific binding (8, 18, 19). Oxytricha α- and β-like-binding proteins represent a novel type of DNA-binding protein because their binding is extremely salt-resistant. The binding of other single-stranded telomeric binding proteins to telomeric DNA is relatively salt-sensitive. A protein Pot1 that shares partial sequence homology with the Oxytricha α subunit has been identified from human and Schizosaccharomyces pombe (17). Mutation in S. pombe POT1 causes loss of telomeric DNA and circulation of chromosomes. In S. cerevisiae, Cdc13p binds specifically to single-stranded TG₁-₃ DNA in vitro and affects telomere function in vivo (10, 11, 20, 21). Interestingly, even though Cdc13p does not share sequence similarity with the ciliate proteins or Pot1, the binding regions of Cdc13p and ciliate proteins that interact with single-stranded telomeric DNA are conserved (22, 23).

A mutation allele of CDC13, cdc13–1, causes yeast cells to arrest at the G₂/M phase of the cell cycle and accumulate single-stranded TG₁-₃ DNA at telomeres (20). Various types of DNA damages, including x-ray, UV, and chemical mutagens, induce DNA damage-dependent cell cycle arrest (24–28). Presumably, these damages on DNA were processed by cellular nucleases to generate single-stranded DNA, which could then serve as the signal for DNA damage-dependent cell cycle arrest (20, 29). Here, we show that S. cerevisiae Gbp2p and S. pombe Tcg1 rescue the growth arrest of cdc13–1 cells and that their single-stranded TG₁-₃ DNA binding activity is required for their complementation. Our results suggest that the appearance of single-stranded telomeric DNA in cdc13–1 cells is the signal that induces the DNA damage-dependent checkpoint. Moreover, we also provide evidence that Gbp2p is involved in modulating telomere length.

MATERIALS AND METHODS

Yeast Strains—Yeast strain W13a (MATα cdc13-1 his7 leu2-3, 112 ura3-52 trp1-299) and the wild-type CDC13 version of W13a, strain 4055-5-2a, were used as the hosts in complementation tests (provided by L. Hartwell, Fred Hutchinson Cancer Research Center). Strain YEM1a (MATα his3Δ1 trp1-1 leu2::LexAop6-LEU2 ura3-1::URA3- pLexAop8-LacZ) was used in the two-hybrid method (30).

Plasmids—Plasmids pTHA, pTHA-CDC13 (expressing Cdc13p under the control of GAL1 promoter; Ref. 11) and pGAL-GBP2 (expresses GBP2 under the control of GAL1 promoter; Ref. 12) were described previously. To express S. cerevisiae HRB1 in S. cerevisiae, HRB1 was first PCR amplified with primers HRB15 (5’-CCCATGGTTGATCGTCGCTTGGT-3’) and HRB13 (5’-AGGCTATAGCGTTACGCGA-3’) using Vent DNA polymerase (New England Biolabs). The ~1.1-kbp HRB1 PCR fragment was cloned directly into pGEM-T (Promega) to generate pGEM-HRB1. To construct GAL1 promoter-driven HRB1, the Neo-StuI HRB1 DNA-containing fragment from pGEM-HRB1 was ligated into the Neo- and Smal-digested pSH380.¹ DNA fragments used in two-hybrid analysis were subcloned into

¹ S. Hahn, unpublished data.
Cloning and Sequencing of S. pombe TCG1—To clone S. pombe genes that complement the cdc13-1 temperature-sensitive phenotype, an S. pombe cDNA library (32) was transformed into W13a. Transformants were then plated, incubated at 25 °C for 16 h, and then at 30 °C for 4 days. A total of 72 colonies grew at 30 °C from ~120,000 transformants.

DNAs from these 72 colonies were prepared and transformed into Escherichia coli XL1-Blue. Plasmid DNAs were prepared and transformed back into W13a to retest their temperature sensitivity at 30 °C. Only one clone grew at 30 °C after the retransformation of purified plasmid. A 1.4-kbp HindIII-HindIII DNA insert within this complementing plasmid, pDB-TCG1, was subcloned into HindIII-digested pVZ1 to generate pVZ-TCG1. The sequence of this DNA was determined by the exonuclease III deletion method and sequenced using Sequenase (United Biotech). To construct pGST-TCG1, the TCG1 fragment was first PCR amplified with primers TG5 (5'-GCGAATTCTAGATTCCGCGGCGAC-3') and TG3 (5'-GCAATTGCGAGAAGGAATTA-3'). A PCR-amplified ~1-kbp DNA fragment was digested with EcoRI and SalI and ligated with the EcoRI- and XhoI-digested pGEX-4T-1 to generate pGST-TCG1.

Site-specific Mutagenesis—PCR-based site-specific mutagenesis was applied to generate mutations on the RNA recognition motifs (RRMs) of GBP2. Mutations on residues Arg-181 and Arg-248 of GBP2 were changed to Ala on plasmid pMAL-GBP2 to generate pMAL-GBP2, and pMAL-GBP2 and pMAL-GBP2, respectively. The mutation pMAL-GBP2 and pMAL-GBP2 by changing Arg-161 to Ala. A similar approach was used to generate mutations on pGAL-GBP2, pGAL-GBP2, pGAL-GBP2, and pGAL-GBP2. All the mutations were confirmed by DNA sequencing.

Protein Purification—To purify glutathione S-transferase (GST)-fused Teg1, a 1000-ml culture of E. coli harboring pGST-TCG1 was grown at 30 °C to an 0.8 of 0.5 and induced with the addition of 1 mM IPTG. The cells were grown at 30 °C for another 3 h before being harvested by centrifugation. Cells were resuspended in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 50 mM NaOAc, 20% glycerol, 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 get rid of soluble cell fractions. The pellet was then extracted with sonication buffer with 0.5% Tween 20 and 0.1 mM EDTA to get GST-TCG1 fusion protein. Purified protein was dialyzed against storage buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 50 mM NaOAc, 50% glycerol), aliquoted, and frozen by a dry ice-ethanol bath.

To purify maltose-binding protein (MBP)-fused Gbp2p, 100 ml culture of E. coli harboring pMBP-GBP2 was grown at 30 °C to an 0.5 of 0.6 and induced with the addition of 1 mM IPTG. The cells were grown at 30 °C for another 16 h before being harvested by centrifugation. Cells were resuspended in 10 ml of sonication buffer (50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 50 mM NaOAc, 50% glycerol), aliquoted, and frozen by dry ice-ethanol bath.

At 125 V for 105 min. The gels were dried and autoradiographed.

RESULTS AND DISCUSSION

In S. cerevisiae, a cdc13-1 mutation causes yeast cells to arrest at the G2/M phase and accumulate single-stranded TG1 DNA at telomeres at non-permissive temperatures (20). Analogous to what is caused by various types of DNA damage, the accumu-
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Arg-161 and Arg-259 are required for binding to single-stranded TG₃₃ DNA. a, domain structure of Gbp2p and sequence alignment of RRM motifs from hnRNP A1 (Ref. 1) and Gbp2p (Gbp2p Ref. 1) and Gbp2p (Ref. 2). Arrows indicate positions of residues Arg-161 and Arg-259. B, purification of wild-type and mutant Gbp2p proteins. The wild-type (lane 3) and mutant proteins (lanes 4–6) were expressed as MBP fusion proteins in E. coli and purified using amylose-agarose resins. 2 μg each of the purified protein was analyzed on a 10% SDS-polyacrylamide gel, and the Coomassie blue-stained gel is shown. Positions of the alterations on Gbp2p are indicated above the lanes. c, the single-stranded TG₃₃ DNA binding activity of Gbp2p and its mutants. −5 nt each of 32P-labeled TG₃₃ was mixed with several concentrations of the purified MBP, wild-type Gbp2p, Gbp2pR161A, Gbp2pR259A, Gbp2pR161A, R259A, and the gel shift assay was then carried out. Proteins used in each set of experiments were 300, 100, and 33 ng. An autoradiogram is shown here.

The single-stranded TG₃₃ binding activity of Gbp2p is required to complement the cdc13-1 mutant. As in Fig. 1, yeast W13a (cdc13-1) carrying plasmids pSH380, pGAL-GBP2, pGAL-GBP2R161A, pGAL-GBP2R259A, or pGAL-GBP2R161A, R259A was spotted on YC-Leu plates with the addition of 2% glucose (left) or 3% galactose (right) and grown at 25°C (top) or 30°C (bottom) until colonies formed (a). Yeast strains 4053–5-2a (CDC13) carrying pSH380 (vector) or W13a (cdc13-1) carrying plasmids pSH380, pGAL-GBP2, pGAL-GBP2R161A, pGAL-GBP2R259A, or pGAL-GBP2R161A, R259A were grown in the presence of 2% glucose at 25°C for 3 days. Cells were then washed, resuspended in YC-Leu medium with 3% galactose and continued to grow at 30°C for another 6 h. Total yeast DNA was then isolated from these cells, digested with XhoI, run in a 1% agarose gel, and analyzed by Southern blotting under denatured (b) or native (c) conditions. The blots were hybridized with a 32P-labeled C₁₃₋₄ probe. Arrow indicates the Y'-bearing telomeres.

At 30°C but could not complement cdc13-1 at 33°C (Fig. 1d). It should be noted that the above complementation is specific to single-stranded telomeric DNA-binding proteins because other RRM-containing proteins that do not bind single-stranded telomeric DNA, including Ssb1p, Pub1p, or Hrb1p, did not complement the growth defect of cdc13-1 at 30°C (Fig. 1a, and data not shown).

Gbp2p contains two RRM motifs. The RRM motif, an ~90-amino acid module, is used for RNA binding in many RNA-binding proteins (33). The signature of the RRM motif is the two consensus sequences, RNP1 and RNP2, located about 30 amino acids apart in the RRM domain (Fig. 2a). These two conserved regions are involved in making contacts with RNA. In U1 small nuclear RNP, the Arg residue in RNP1 forms a salt bridge with the phosphate of U1 RNA, whereas residues in RNP2 form hydrogen bonds with the RNA (34). We changed both Arg-161 and Arg-259, the conserved Arg residues within the two RRM motifs of Gbp2p, to Ala (Fig. 2a), and tested the effect on the single-stranded TG₃₃ binding activity in a gel mobility shift assay using purified mutant proteins (Fig. 2, b and c). The results indicate that the R259A mutation greatly reduced the binding of Gbp2p to single-stranded TG₃₃ DNA, whereas the R161A mutation only partially inactivated Gbp2p. Mutation on a non-
conserved residue, Arg-127, did not affect the binding activity (data not shown). These results indicate that Arg-161 and Arg-259 are important for binding to a single-stranded TG$_{1-3}$ DNA.

Each Gbp2p binding mutant was tested for efficiency in complementing the growth defect of cdc13–1 cells. The experiments were conducted in the presence of glucose or galactose, because GBP2 expression was driven by the GAL1 promoter. As shown in Fig 3a, whereas wild-type GBP2 complemented cdc13–1 even under the non-inducing condition (– Leu + Gal, 30 °C) and gbp2R161A complemented only under the inducing condition (– Leu + Gal, 30 °C), neither gbp2R259A nor gbp2R161A/R259A was able to complement cdc13–1 at 30 °C. These results suggested that complementation depends on the single-stranded telomeric DNA binding activity.

We investigated the effect of mutant or wild-type Gbp2p on the cdc13–1-associated telomere-lengthening phenotype (35). In S. cerevisiae, middle repetitive sequences known as Y′ elements are found in the subtelomeric regions of most chromosomes. As shown in Fig. 3b, in CDC13/pSH380 cells a XhoI digest produces a ~1.3 kbp fragment from the ends of Y′-bearing chromosomes that contains ~950 bp of Y′ and the terminal tract of ~350 bp of TG$_{1-3}$/C$_{1-3}$.A DNA. However, the length of Y′-bearing telomeres in cdc13–1 cells appeared to be longer and more heterogenous. Expression of the wild-type GBP2 or gbp2R161A, but not gbp2R259A or gbp2R161A/R259A suppressed telomere lengthening in cdc13–1 cells. Expressing Gbp2p in wild-type cells or cells with gbp2A or gbp2Δ cdc13–1 mutations did not affect the telomere length (data not shown). The effect of expressing Gbp2p on the accumulation of single-stranded telomeric DNA in cdc13–1 cells at 30 °C was also evaluated. The cdc13–1 cells, but not the wild-type cells, accumulate abnormally high levels of single-stranded telomeric DNA at 30 °C (Fig. 3c). By taking the single-stranded TG$_{1-3}$ DNA level in cdc13–1 cells at 30 °C as 100%, the single-stranded TG$_{1-3}$ DNA level in wild-type CDC13 cells, cdc13–1 cells overexpressing wild-type GBP2, R161A, R259A, or R161A/R259A mutants was 26%, 45%, 50%, 102%, and 91%, respectively. Thus, expressing the wild-type Gbp2p or R161A mutant moderately decreased the levels of single-stranded telomeric DNA, whereas R259A or R161A/R259A mutants of Gbp2p did not have this effect. These results demonstrate that the single-stranded TG$_{1-3}$ DNA binding activity of Gbp2p is required for suppressing the telomere lengthening of cdc13–1 cells and affecting the accumulation of single-stranded telomeric tails. The presence of single-stranded DNA has been speculated as being the signal for the DNA-damage checkpoint (29) because it is present at the replication fork when replication is incomplete. Moreover, single-stranded DNA is a common intermediate in many repair pathways, including double-strand break repair, excision repair, and recombination repair (36–38). Here we have shown that expressing two single-stranded TG$_{1-3}$ binding proteins, S. cerevisiae Gbp2p and S. pombe Tgc1, complemented the defects of cdc13–1 cells. In addition, the single-stranded TG$_{1-3}$ DNA binding activity of Gbp2p is required for this complementation. Because the cell cycle defect in cdc13–1 is dependent on the DNA-damage checkpoint, our results strongly suggest that the presence of single-stranded TG$_{1-3}$ DNA in cdc13–1 at a non-permissive temperature sends the signal for the DNA damage checkpoint. In wild-type cells, the binding of Cdc13p to the telomeric tail may convey a message to sensor proteins of the DNA-damage checkpoint, thus marking telomeres different from broken DNA ends. In cdc13–1 cells, masking the arrest signal with other single-stranded TG$_{1-3}$ DNA binding activity is necessary at non-permissive temperatures to circumvent the chromosome-surveillance mechanism and prevent cell cycle arrest.
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