A Conserved Hsp10-like Domain in Mcm10 Is Required to Stabilize the Catalytic Subunit of DNA Polymerase-α in Budding Yeast*

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Mcm10 is a conserved eukaryotic DNA replication factor that is required for S phase progression. Recently, Mcm10 has been shown to interact physically with the DNA polymerase-α (pol-α)/primase complex. We show now that Mcm10 is in a complex with pol-α throughout the cell cycle. In temperature-sensitive mcm10-1 mutants, depletion of Mcm10 results in degradation of the catalytic subunit of pol-α, Cdc17/Pol1, regardless of whether cells are in G1, S, or G2 phase. Importantly, Cdc17 protein levels can be restored upon overexpression of exogenous Mcm10 in mcm10-1 mutants that are grown at the nonpermissive temperature. Moreover, overexpressed Cdc17 that is normally subject to rapid degradation is stabilized by Mcm10 co-overexpression but not by co-overexpression of the B-subunit of pol-α, Pol12. These results are consistent with Mcm10 having a role as a nuclear chaperone for Cdc17. Mutational analysis indicates that a conserved heat-shock protein 10 (Hsp10)-like domain in Mcm10 is required to prevent the degradation of Cdc17. Substitution of a single residue in the Hsp10-like domain of endogenous Mcm10 results in a dramatic reduction of steady-state Cdc17 levels. The high degree of evolutionary conservation of this domain implies that stabilizing Cdc17 may be a conserved function of Mcm10.

Initiation of DNA replication is a strictly regulated process in eukaryotic cells. In order to prepare replication origins for initiation of DNA replication, a prereplicative complex assembles at origins during the G1 phase of the cell cycle (1). The prereplicative complex is composed of the six-subunit initiator origin recognition complex, Cdc6, Cdt1, and the putative replicative helicase, the minichromosome maintenance complex (2). Since pol-α has known catalytic activity, it is phosphorylated in a cell cycle-specific manner in budding yeast and in human cells (21, 22). This may serve a regulatory role as cells progress through S phase. More recently, Pol12 has been shown to be associated with the origin recognition complex in fission yeast (23). In addition to Pol12, Cdc17/Pol1 also interacts with Pri2, which regulates the primase activity of the catalytic subunit, Pri1 (24).

We and others have recently demonstrated that chromatin association of pol-α/primase requires Mcm10 (7, 25). Mcm10 is a conserved eukaryotic DNA replication factor that is essential for S-phase progression, since it serves a critical role in coordinating replication fork assembly (7). Mcm10 was initially identified in two independent genetic screens in Saccharomyces cerevisiae (26, 27). In addition to pol-α, Mcm10 has been shown to interact with several replication factors, including the Mcm2-7 complex, origin recognition complex, RPA, and Cdc45 (7, 8, 28–34). Furthermore, Mcm10 regulates the stability of the catalytic subunit of pol-α, Cdc17/Pol1, in budding yeast, but not other pol-α subunits such as Pri2 (7). This is of particular interest, given that Cdc17 levels seem to be tightly controlled in the cell. For example, in budding yeast, Cdc17/Pol1 overexpression results in immediate protein degradation, whereas the endogenous protein has a very low protein turnover rate in vivo (35). The same is true for p180, the mammalian counterpart for Cdc17/Pol1 (36). The observation that overexpressed Cdc17/Pol1 is unstable but endogenous Cdc17/Pol1 is stable promotes a model whereby the catalytic subunit must be associated with a limiting stabilizing cofactor in vivo. Our current hypothesis is that this cofactor is Mcm10. However, the domain in Mcm10 required to stabilize Cdc17/Pol1 is currently unknown.

Here, we demonstrate that Mcm10 is indeed required to stabilize Cdc17/Pol1, regardless of whether cells are in the G1, S, or G2 phase of the cell cycle. In an effort to delineate the region in Mcm10 responsible for stabilizing Cdc17/Pol1, we mutated conserved protein domains within Mcm10 to determine which were crucial for regulating Cdc17/Pol1. The high degree of evolutionary conservation of this domain implies that stabilizing Cdc17 may be a conserved function of Mcm10.

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2 The abbreviations used are: RPA, replication protein A; pol, polymerase; HA, hemagglutinin; HU, hydroxyurea; PIP, proliferating cell nuclear antigen-interacting protein; OB-fold, oligonucleotide/oligosaccharide binding fold.
Mcm10 Stabilizes Cdc17 through Its Hsp10-like Domain

Table 1

| Strain name                        | Relevant genotype                          | Source               |
|------------------------------------|--------------------------------------------|----------------------|
| ABY021                             | W303 bar1Δ                                 | This study           |
| ABY015                             | mcm10-10-td; CDC17-3HA; GAL-UBR1            | This study           |
| BHY100                             | mcm10-1                                     | Homesley et al. (28) |
| ABY013                             | mcm10-1; CDC17-3HA                           | Ricke and Bielinsky (7) |
| ABY005                             | MCM10-9MYC; CDC17-3HA                         | Ricke and Bielinsky (7) |
| ABY070                             | MCM10-9MYC; PRI2-3HA                          | This study           |
| ABY071                             | MCM10-9MYC; POL12-3HA                         | This study           |
| YKL83                              | GAL-UBR1                                    | Labib et al. (42)    |
| ABY074                             | cdc17-td; GAL-UBR1                           | This study           |
| ABY019                             | cdc17-td; MCM10-3HA; GAL-UBR1                | This study           |
| ABY075                             | cdc17-td; PRI2-3HA; GAL-UBR1                 | This study           |
| ABY038                             | POL12-3HA; GAL-UBR1                          | This study           |
| ABY052                             | cdc17-td; POL12-3HA; GAL-UBR1                | This study           |
| OAY644                             | CDC17-3HA                                   | Aparicio et al. (16) |
| ABY035                             | MCM10-3HA                                   | This study           |
| ABY069                             | PRI2-3HA; MCM10-3HA                          | This study           |
| ABY063                             | POL12-3HA; MCM10-3HA                         | This study           |
| ABY056                             | CDC17-3HA; MCM-3HA                           | This study           |
| ABY116                             | ABY013 + pRS426gal-MCM10-3HA                 | This study           |
| ABY128                             | ABY021 + pRS426gal-CDC17-3HA; pRS423cup-MCM10-3HA | This study           |
| ABY081                             | ABY021 + pRS426gal-CDC17-3HA                 | This study           |
| ABY135                             | ABY021 + pRS426gal-CDC17-3HA; pRS423cup-mcm10Δ200-3HA | This study           |
| ABY134                             | ABY021 + pRS426gal-CDC17-3HA; pRS423cup-mcm10Δ250-3HA | This study           |
| ABY137                             | ABY021 + pRS426gal-CDC17-3HA; pRS423cup-mcm10Δ300-3HA | This study           |
| ABY138                             | ABY021 + pRS426gal-CDC17-3HA; pRS423cup-mcm10Δ350-3HA | This study           |
| ABY139                             | ABY021 + pRS426gal-CDC17-3HA; pRS423cup-mcm10Δ260-3HA | This study           |
| ABY143                             | ABY021 + pRS426gal-CDC17-3HA; pRS423cup-mcm10Δ260-3HA | This study           |
| ABY167                             | ABY021 + pRS426gal-CDC17-3HA; pRS423cup-mcm10Δ260-3HA | This study           |
| ABY168                             | ABY021 + pRS426gal-CDC17-3HA; pRS423cup-mcm10Δ260-3HA | This study           |
| ABY169                             | ABY021 + pRS426gal-CDC17-3HA; pRS423cup-mcm10Δ260-3HA | This study           |
| ABY171                             | ABY021 + pRS426gal-CDC17-3HA; pRS423cup-POL12-2HA | This study           |
| ABY219                             | BHY100 + pRS424cup                           | This study           |
| ABY220                             | BHY100 + pRS424cup-MCM10-3HA                 | This study           |
| ABY221                             | BHY100 + pRS424cup-mcm10Δ200-3HA             | This study           |
| ABY222                             | BHY100 + pRS424cup-mcm10Δ250-3HA             | This study           |
| ABY223                             | BHY100 + pRS424cup-mcm10Δ300-3HA             | This study           |
| ABY227                             | BHY100 + pRS423cup                           | This study           |
| ABY228                             | BHY100 + pRS423cup-MCM10-3HA                 | This study           |
| ABY229                             | BHY100 + pRS423cup-mcm10Δ250-3HA             | This study           |
| ABY230                             | BHY100 + pRS423cup-mcm10Δ300-3HA             | This study           |
| ABY240                             | BHY100 + pRS423cup-mcm10Δ250-3HA             | This study           |
| ABY241                             | BHY100 + pRS423cup-mcm10Δ300-3HA             | This study           |
| ABY242                             | BHY100 + pRS423cup-mcm10Δ250-3HA             | This study           |
| ABY243                             | BHY100 + pRS423cup-mcm10Δ250-3HA             | This study           |
| ABY244                             | CDC17-3HA; mcm10-2G261-9MYC                  | This study           |
| ABY245                             | CDC17-3HA; mcm10-2G261A-9MYC                 | This study           |
| ABY246                             | CDC17-3HA; mcm10-2G261B-9MYC                 | This study           |
| ABY247                             | CDC17-3HA; mcm10-2G261C-9MYC                 | This study           |

Pol1 protein levels. Interestingly, we identified an Hsp10-like domain that contributes to Cdc17/Pol1 stability. Mutation of a single residue within the Hsp10-like domain in Mcm10 results in a reduction of steady-state Cdc17/Pol1 protein levels. Importantly, the Hsp10-like domain within Mcm10 is highly conserved across species, implying that this function of Mcm10 may be evolutionarily conserved.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—All strains are isogenic derivatives of W303. The genotypes of the yeast strains utilized in this study are listed in Table 1.

Cell Synchrony and Flow Cytometry—Cells were arrested with α factor (50–100 ng/ml), hydroxyurea (200 mM), or nocodazole (10 μg/ml). Flow cytometry was performed as described (37). Samples were measured using a FACS Calibur (BD Biosciences).

Co-immunoprecipitation—Cells were arrested with α factor, hydroxyurea, or nocodazole. Extracts were prepared using glass beads and chromatin immunoprecipitation lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mm EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, and protease inhibitors) as described (38). For the immunoprecipitations, 4 μg of anti-HA (12CA5; Roche Applied Science) or IgG (Oncogene Research Products) was added to the extract and incubated with shaking at 4 °C for 3 h. Detection of the precipitated proteins was performed using Western blot analysis with anti-Myc (9E11; LabVision Neomarkers) and anti-HA (16B12; Covance) antibodies.

Protein Preparation and Western Blot Analysis—Total protein extracts were prepared from cycling or synchronized yeast cultures. Cell pellets were initially washed with 1 ml of TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) and then washed with 1 ml of 20% trichloroacetic acid. The cell pellet was resuspended in 100 μl of 20% trichloroacetic acid at room temperature. One volume of acid-washed glass beads was added, and the cell wall was disrupted by continuous vortexing for 2 min at 4 °C. Glass beads were washed once with 1 ml of 20% trichloroacetic acid, and the extract was centrifuged for 10 min at 3,000 rpm at room temperature. The protein pellet was resuspended 100 μl of 2X Laemmli buffer (4% SDS, 20% glycerol, 120 mM Tris-Cl, pH 6.8, 200 mM dithiothreitol, 0.1% bromphenol blue). To neutralize the pH, 35–40 μl of 1 M Tris base was added. After boiling for 5 min, the samples were centrifuged at 3,000 rpm for 2 min. The supernatant was retained and subjected to SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and probed with anti-HA (16B12; Covance) for HA-tagged proteins and anti-Myc (9E11; LabVision Neomarkers) for Myc-tagged proteins.

Co-overexpression of Cdc17, Mcm10 and Pol12—Strains carrying plasmids for exogenous expression of Mcm10, Pol12, or Cdc17 were grown...
overnight in minimal medium with 2% raffinose. Cdc17-2HA was overexpressed from a galactose-inducible promoter at 30 °C in the presence of 2% galactose, and Mcm10-3HA or Pol12-2HA were co-overexpressed from a copper-inducible promoter in the presence of 10 μM CuSO₄. Following 2 h of induction, the cells were pelleted and resuspended in YPD with 10 μM CuSO₄. Samples were taken at 0, 15, 30, 60, and 90 min after repression of Cdc17-2HA. Proteins were extracted using the trichloroacetic acid extraction protocol. Cdc17-2HA, Pol12-2HA, and Mcm10-3HA were detected by immunoblotting using anti-HA (16B12) antibodies. Staining of the nitrocellulose membrane with Ponceau S prior to immunoblotting served as a loading control.

Construction of the mcm10 Mutants—Mutations within the Hsp10-like domain of MCM10 were constructed using QuikChange mutagenesis (Stratagene). The Hsp10-like domain was altered to encode the following mutations: G261D, G261A, N268D, and N268I. The zinc finger mutant (mcm10-45) was subcloned from pRS315-mcm10-45, a kind gift from M. Lei (39). This allele carries mutations within the zinc finger domain, such that 309CX₁₀CX₁₁CX₂H₃₃₅ is altered to 309CX₁₀YX₁₁GX₈L₃₃₅ (39).

Complementation of mcm10-1 Temperature Sensitivity—Copper-inducible Mcm10 expression plasmids were transformed into the BY100 yeast strain (mcm10-1). 10-fold serial dilutions of cells were spotted onto minimal medium supplemented with 0.2 mM copper sulfate. Plates were incubated for 2–3 days at 30 or 37 °C. To analyze protein expression, log phase cultures were induced with 10 μM CuSO₄ for 2 h at 30 °C. Protein extracts were prepared using the trichloroacetic acid extraction protocol. Cdc17-3HA, Pol12-3HA, and Mcm10-td-HA were detected by immunoblotting using anti-HA (16B12) antibodies. Staining of the nitrocellulose membrane with Ponceau S prior to immunoblotting served as a loading control.

FIGURE 1. Mcm10 serves as a nuclear chaperone for Cdc17 throughout the cell cycle. A, ABy015 cells (Cdc17-3HA, mcm10-td, GAL-UBR1) were blocked in the cell cycle under permissive conditions (room temperature, 2% raffinose) with α factor (α), HU, or nocodazole (Noc). Following cell cycle arrest, the cultures were shifted to 37 °C in the presence of 2% galactose to induce Mcm10-td protein degradation. DNA content was analyzed throughout the course of the experiment using flow cytometry. B, the stability of Cdc17-3HA and Mcm10-td-HA proteins were analyzed by Western blot with anti-HA antibodies (16B12). Ponceau S staining of the membrane prior to Western blotting served as a loading control. C, BTY100 (CDC17⁺, POL12⁺, mcm10-1), ABy013 (CDC17⁺, POL12⁺, mcm10-1), and ABy079 (CDC17⁺, POL12⁺, mcm10-1) cells were blocked in the cell cycle at room temperature (RT) with α factor, HU, or nocodazole. Once arrested, cultures were shifted to 37 °C. Cell cycle synchrony was monitored using flow cytometry. D, the protein stability of Cdc17-3HA and Pol12-3HA was assessed by Western blot with an anti-HA antibody (16B12). Ponceau S staining served as a loading control.
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RESULTS

Mcm10 Serves as a Nuclear Chaperone for Cdc17 throughout the Cell Cycle—We recently observed that the degradation of Mcm10 from cycling yeast cells results in the co-depletion of the catalytic subunit of pol-α, Cdc17/Pol1 (hereafter referred to as Cdc17) (7). All previous experiments analyzing the stability of Cdc17 were performed either in asynchronously growing or in hydroxurea (HU)-treated cells. Importantly, since cells accumulate in S phase in the absence of Mcm10 (7, 27), we were unsure if this function of Mcm10 was restricted to S phase or if Mcm10 is required to stabilize Cdc17 throughout the cell cycle. To experimentally address this question, Mcm10 protein was depleted from temperature-sensitive mcm10 mutants arrested in G1, S, or G2 phase of the cell cycle. First, cultures were treated with the respective cell cycle inhibitor (α-factor, HU, or nocodazole) under permissive conditions and then shifted to 37 °C in the continued presence of the inhibitor. Cell cycle arrests were confirmed by monitoring DNA content using flow cytometry (Fig. 1, A and C). In both the mcm10-1 and mcm10-td strains, degradation of Mcm10 resulted in the destabilization of Cdc17 regardless of whether cells were blocked in G1, S, or G2 phase (Fig. 1, B and D). It has been shown previously that the mcm10-1 and mcm10-td temperature-sensitive strains deplete cellular pools of Mcm10 under nonpermissive conditions, albeit through different mechanisms (7, 34). This indicates that Mcm10 serves to stabilize Cdc17 throughout the cell cycle.

To ascertain that the observed effect was specific for Cdc17, we analyzed the stability of the B-subunit of pol-α, Pol12, in the mcm10-1 strain. Whereas shifting cells to the restrictive temperature did not significantly affect Pol12 protein levels, depletion of Mcm10 did affect the phosphorylation status of Pol12 (Fig. 1D). It has been previously reported that Pol12 is phosphorylated as cells progress through the cell cycle (40). Similarly, we observed two forms of Pol12 in cells blocked in S phase with HU, consistent with Pol12 becoming phosphorylated (Fig. 1D). In addition, the entire Pol12 pool was phosphorylated in cells arrested in G1/M phase with nocodazole (Fig. 1D). However, in the absence of Mcm10, the amount of phosphorylated Pol12 decreased in cells blocked in S and G2/M phases. In cells treated with HU, the entire Pol12 pool was dephosphorylated in the absence of Mcm10, whereas in cells treated with nocodazole, about half of the Pol12 pool was dephosphorylated (Fig. 1D). Interestingly, Ferrari et al. (21) reported that Pol12 phosphorylation was dependent on pol-α complex formation. Given that Cdc17 stability was severely compromised in the absence of Mcm10, Pol12 dephosphorylation probably occurred in response to Cdc17 degradation. Moreover, we observed that Pol12 was dephosphorylated in cycling cells depleted solely of Cdc17 (see Fig. 3B). Taken together, these data further support the hypothesis that Mcm10 regulates the stability of Cdc17 throughout the cell cycle.

Based upon these results, we predicted that Mcm10 should be associated with pol-α primase in a cell cycle-independent manner. Thus, we monitored the ability of Mcm10 to physically associate with Cdc17 as well as Cdc17-interacting subunits, Pol12 and Pri2, in cells blocked in G1, S, and G2/M phases. Regardless of whether Pri2, Pol12, or Cdc17 was immunoprecipitated, Mcm10 co-precipitated with pol-α, independently of the stage at which the cell cycle was arrested (Fig. 2). These data therefore support a model whereby Mcm10 physically interacts with the complex throughout the cell cycle. Furthermore, we believe that Mcm10 and pol-α primase associate with each other independently of DNA, since we showed previously that DNase treatment did not interfere with complex formation in S phase extracts (7).

Cdc17 Does Not Control Mcm10 Protein Levels—The finding that Mcm10 associates with pol-α throughout the cell cycle raised the question of whether Cdc17 was required to maintain Mcm10 protein stability. Therefore, we examined Mcm10 stability in the absence of Cdc17 by constructing a Cdc17 degron strain (cdc17-td). At the restrictive temperature, Cdc17 was efficiently depleted, particularly when Ubr1, the E3 ligase required for N-end rule degradation, was overexpressed on a galactose-inducible promoter (Fig. 3A) (41, 42). Depletion of Cdc17 in cycling cells did not affect the protein levels of either Mcm10 or the primase regulatory subunit, Pri2 (Fig. 3A). Therefore, it appears that Cdc17 does not control Mcm10 protein levels.

Exogenous Overexpression of Mcm10 Restores Cdc17 Levels in the mcm10-1 Mutant—Because Mcm10 was required to stabilize Cdc17, we wished to explore whether we could rescue Cdc17 protein degradation in the temperature-sensitive mcm10-1 mutant by overexpressing exogenous Mcm10 before the cells were shifted to the nonpermissive temperature. To this end, we expressed Mcm10 with three C-terminal hemagglutinin (HA) epitope tags from a galactose-inducible promoter (Fig. 3C). However, in the absence of Cdc17, the entire Pol12 pool was dephosphorylated in the absence of Mcm10, whereas in cells treated with HU, the entire Pol12 pool was dephosphorylated in the absence of Mcm10, whereas in cells treated with nocodazole, about half of the Pol12 pool was dephosphorylated (Fig. 1D). Interestingly, Ferrari et al. (21) reported that Pol12 phosphorylation was dependent on pol-α complex formation. Given that Cdc17 stability was severely compromised in the absence of Mcm10, Pol12 dephosphorylation probably occurred in response to Cdc17 degradation. Moreover, we observed that Pol12 was dephosphorylated in cycling cells depleted solely of Cdc17 (see Fig. 3B). Taken together, these data further support the hypothesis that Mcm10 regulates the stability of Cdc17 throughout the cell cycle.

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Stabilize Overexpressed Cdc17

In order to ensure that this activity was specific for Mcm10, we also monitored whether overexpression of a different pol-α-interacting protein would result in stabilization of Cdc17. Therefore, we overexpressed the regulatory subunit for pol-α, Pol12, and monitored whether co-overexpression of Pol12 could stabilize Cdc17. Again, we overexpressed Cdc17 fused with two C-terminal HA epitope tags from a galactose-inducible promoter, whereas Pol12 fused with two C-terminal HA epitope tags was expressed exogenously from a copper-inducible promoter in an otherwise wild-type yeast strain. After 2 h of co-overexpression, Cdc17 expression was repressed with 2% glucose, whereas Pol12 expression was continuously induced with copper. At 15 min, 81% of Cdc17 remained after the addition of glucose, and by 90 min, only 14% of Cdc17 remained (Fig. 5B). Although the kinetics of Cdc17 degradation were slightly altered when Pol12 was co-overexpressed compared with Cdc17 expressed alone (Fig. 5D, 15 min), Pol12 co-overexpression does not result in stabilizing Cdc17 (Fig. 5D). This was despite the fact that Pol12 overexpression yielded higher protein expression than Mcm10 overexpression from a copper-inducible promoter (Fig. 5C). We conclude that Mcm10, but not Pol12, is necessary to stabilize Cdc17.

A Conserved Hsp10-like Domain in Mcm10 Is Responsible for Stabilizing Cdc17—In budding yeast, Mcm10 is a 571-amino acid peptide that contains one predicted oligonucleotide/oligosaccharide binding fold (OB-fold) from amino acids 201 to 297 (that was identified using the protein motif search in a hidden Markov model), a zinc finger motif (amino acids 309–335) (39), and two C-terminal nuclear localization signals (amino acids 435–451 and 512–527) (44) (NLS in Fig. 6A). Although OB-fold domains share little sequence homology from pro-
Mcm10 Stabilizes Cdc17 through Its Hsp10-like Domain

FIGURE 4. Exogenous expression of Mcm10 rescues Cdc17 stability in the mcm10-1 mutant. A, experimental design to determine whether exogenous expression of Mcm10 from a galactose-inducible promoter will restore Cdc17 protein levels in the mcm10-1 mutant. ABY116 (mcm10-1, GAL::MCM10-3HA, CDC17-3HA) cells were induced for Mcm10-3HA expression in 2% galactose (Gal) or were repressed in 2% glucose (Gluc) at room temperature (RT) for 3 or 6 h in minimal medium. Cultures were then shifted to 37 °C in YP-Gal (Gal) or YPD (Gluc.) for 90 min to deplete endogenous Mcm10-1 protein pools. B, protein levels of Cdc17-3HA and Mcm10-3HA were monitored by Western blot with an anti-HA antibody (16B12) when Mcm10 expression was induced 3 h prior to the temperature shift. C, protein levels of Cdc17-3HA and Mcm10-3HA were detected by Western blot when Mcm10 expression was induced 6 h prior to the temperature shift.

Amino acid 247 in budding yeast Mcm10 is predicted to contain at least six major β strands and two minor β strands (Fig. 6B). We also compared the OB-fold in Mcm10 with the DNA binding domains found in the largest subunit of RPA, RPA70 (Fig. 6C). Like RPA70, Mcm10 contains a conserved phenylalanine (Phe247) following β strand 3 (Fig. 6B). It is, however, worthwhile to note that this is not a shared feature of all OB folds, since the OB folds in Cdc13 and Cdc9 do not contain an aromatic residue immediately following β strand 3 (data not shown). Analysis of eukaryotic RPA and prokaryotic single-stranded binding protein indicate that aromatic residues within the fold are critically important for DNA binding activity (47, 48). All four DNA binding domains found in the large subunit of RPA contain an aromatic residue following β strand 3 (49). In budding yeast, mutation of the phenylalanine following β strand 3 (Phe247) in DNA binding domain A of RPA70 results in lethality (49). In order to assess whether this phenylalanine in Mcm10 contributes to binding DNA, we introduced an alanine substitution and assayed whether exogenous expression of the mcm10Δ247A allele could complement the temperature sensitivity of mcm10Δ1 cells. We observed that overexpression of Mcm10Δ247A resulted in full complementation (data not shown), indicating that the mutant retains DNA binding activity, which is thought to be essential for S phase progression (7). Our results suggest that the OB-fold domain in Mcm10 functions differently from the DNA binding domain A domain in RPA70. This is perhaps not surprising, because, unlike other DNA-binding proteins, such as RPA, Mcm10 has additional interaction domains within the OB-fold (Fig. 7A).

In addition to the OB-fold, Mcm10 contains three other protein motifs that appear to be conserved in all eukaryotes (Fig. 7A) (30). The zinc finger motif has been shown to be required for homocomplex formation (39). Our laboratory has also observed that a modified form of Mcm10 interacts with proliferating cell nuclear antigen through its proliferating cell nuclear antigen-interacting protein (PIN) box (3). Using the NCBI conserved domain search, we also identified a motif in Mcm10 that shares homology with heat shock protein 10 (Hsp10) from position 261 to 268 (Fig. 7C). Whether this putative Hsp10-like domain in Mcm10 was of any functional relevance remained unclear, although it was intriguing, because Hsp10 and its binding partner, Hsp60, are well characterized proteins that function in protein folding in the mitochondria (for a review, see Ref. 50). The respective domain in Hsp10 is part of an 18-amino acid, highly hydrophobic, mobile loop that mediates the interaction with Hsp60 (51–53). Mutational analysis of Hsp10 in S. cerevisiae and the Escherichia coli homolog (GroES) identified a conserved glycine as a critical residue within this domain, since it was observed in both systems that mutation of the glycine to an aspartic acid resulted in temperature sensitivity (51, 54). Importantly, the Hsp10-like domain in Mcm10 contains only the second portion of this loop, including the conserved glycine residue (Fig. 7C). In addition to Gly261, budding yeast Mcm10 contains two other exact matches to those found in Hsp10 from S. cerevisiae and the E. coli homolog GroES, namely Val263 and Asn268 (Fig. 7C). All three amino acids are highly conserved in Mcm10 from various species, including humans (Fig. 7C).

To determine whether the Hsp10-like domain or any of the other characterized domains in Mcm10 had a role in stabilizing Cdc17, we asked if stoichiometric amounts of respective mutant Mcm10 proteins could stabilize Cdc17 by co-overexpression. To monitor whether the zinc finger makes any significant contribution to stabilizing Cdc17, we expressed a mutant (Mcm10-45) that contained several amino acid substitutions within the domain such that the consensus changed from 309CX10CX11CX12H335 to 309CX10XY11CX12 (39). This mutant has been shown to be defective in the formation of Mcm10 homocomplexes (39). In order to assay whether the Hsp10-like domain was required for stabilizing Cdc17, we created a single amino acid change within the domain at the conserved glycine residue (G261D). In addition, to further identify regions important for stabilizing Cdc17 that did not fall within the already documented domains, we truncated Mcm10

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from its N terminus. All of the mcm10 mutants that we constructed in this study retained an intact C terminus that allowed the proteins to localize to the nucleus. The mcm10 mutants were fused with three C-terminal HA epitope tags and expressed exogenously from a copper-inducible promoter, whereas the full-length CDC17 was fused with two C-terminal HA epitope tags and expressed exogenously from a galactose-inducible promoter. The endogenous MCM10 and CDC17 genes in the strain remained unchanged. We reasoned that mutants in which the domain of interest was altered would lose the ability to stabilize Cdc17. Given that Mcm10 stabilizes overexpressed Cdc17 when protein levels are stoichiometric (Fig. 5), we were careful to ensure that mutant proteins were expressed at levels similar to the wild-type protein. Unfortunately, two of the N-terminal truncation mutants were not expressed at sufficiently high levels (mcm10/H9004300 and mcm10/H9004350) (Fig. 7B), and thus, we omitted mutants mcm10/H9004300 and mcm10/H9004350 from further analyses. However, the other two N-terminal truncation mutants (mcm10/H9004200 and mcm10/H9004250) exhibited expression levels comparable with wild-type Mcm10 (Fig. 7B). Importantly, substitution mutations within the Hsp10-like (G261D) and the zinc finger domain (Mcm10-45) did not aberrantly affect protein expression, since all were expressed at levels similar to wild-type Mcm10 (Fig. 7B). Each mutant was significantly compromised in its ability to stabilize overexpressed Cdc17, such that 90 min after the addition of glucose, only 18% of Cdc17 remained with co-overexpression of mcm10/H9004250 (Fig. 7, D (vii) and E), 21% remained with co-overexpress-

![FIGURE 5. Stoichiometric amounts of Mcm10, but not Pol12, are required to stabilize overexpressed Cdc17. A, ABy128 (Cu-MCM10-3HA, GAL-CDC17-2HA), and ABy081 (GAL-CDC17-2HA) cells were induced to co-overexpress Cdc17-2HA and Mcm10-3HA as described. Samples were taken at 0, 15, 30, 60, and 90 min after the addition of glucose. Cdc17-2HA and Mcm10-3HA were detected by Western blot using anti-HA (16B12) antibodies. Ponceau S staining served as a loading control. B, ABy171 (Cu-POL12-2HA, GAL-CDC17-2HA) cells were induced to co-overexpress Pol12-2HA and Cdc17-2HA as described. Samples were taken at 0, 15, 30, 60, and 90 min after the addition of glucose. Cdc17-2HA and Pol12-2HA were detected by Western blot using anti-HA (16B12) antibodies. Ponceau S staining served as a loading control. C, ABy128 and ABy171 cells were induced to overexpress Mcm10-3HA and Pol12-2HA, respectively, by the addition of 10 μM CuSO4 for 2 h in the presence of 2% glucose. Mcm10-3HA and Pol12-2HA were detected by Western blot with an anti-HA (16B12) antibody. D, the average percentage of Cdc17-2HA remaining was plotted versus time. The error bars indicate one S.D.](image-url)
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FIGURE 6. Structural features of Mcm10. A, schematic of the protein motifs identified in Mcm10. Mcm10 is a 571-amino acid protein that contains one predicted OB-fold from amino acid 201 to 297 and two C-terminal nuclear localization signals (NLS) (amino acids 435–451 and 512–527) (44). B, secondary structure prediction for the OB-fold in Mcm10. The secondary structure of the OB-fold found in Mcm10 from S. cerevisiae was predicted using the PROF algorithm (46). The black arrows represent β strands. The larger arrows represent stretches of 6 residues or more. Unlike canonical β barrel structures associated with OB-folds, the PROF algorithm did not identify any α helices in this region of Mcm10. The positions of residues known to be important for function in budding yeast RPA (49) are highlighted with a light gray box. C, secondary structure prediction for one of the four OB-folds (DNA binding domain A; DBD-A) found in the large subunit of RPA from budding yeast. The black arrows represent β strands, and the large gray box represents an α helix.

As previously mentioned, the Hsp10-like domain is located within the Mcm10 OB-fold, and secondary structure predictions indicate that part of the Hsp10-like domain in Mcm10 probably forms a β strand and may contribute to the β barrel structure of the OB-fold. One possible consequence of mutating the Hsp10-like domain is that the structure of the OB-fold will be aberrantly altered. At least two pieces of evidence discount this possibility. First, the predicted secondary structure of the OB-fold in Mcm10 is not affected by mutations in Gly261 or Asn268 (data not shown). Second, the truncation analysis of Mcm10 indicates that whereas the Mcm10Δ250 protein contains only half of the OB-fold, it is functional at stabilizing Cdc17 (Fig. 7D, ii). In order to determine whether an intact OB-fold is required for cell proliferation, we assessed whether exogenous expression of truncated Mcm10 could complement the temperature sensitivity of the mcMin10-1 allele. Although all of the truncated proteins were expressed at similar levels (Fig. 8B), only two mutants (mcm10Δ50 and mcm10Δ150) rescued the temperature-sensitive phenotype of mcm10-1 cells at 37 °C (Fig. 8A). The observation that the N-terminal 150 amino acids in Mcm10 are not required for cell proliferation is consistent with another report (55). The protein lacking the N-terminal 250 amino acids cannot support cell growth (Fig. 8A), presumably because it lacks a functional OB-fold and/or PIP box. However, this truncated protein is capable of stabilizing Cdc17 (Fig. 7, ii), arguing that the Hsp10-like domain, and not the OB-fold, is required for Mcm10-mediated stabilization of Cdc17.

To further investigate whether mutations in the Hsp10-like domain in Mcm10 affected protein function, we performed a similar complementation test with the mcm10-1 strain as described above. We transformed mcm10-1 cells with plasmids allowing for the copper-inducible expression of Mcm10. Importantly, mutations in the zinc finger (Mcm10-45) and the Hsp10-like domain (G261D, G261A, N268D, and N268I) did not affect expression levels in the presence of 10 μM CuSO4 as detected by Western blot analysis (Fig. 8C). As expected (39), exogenous expression of Mcm10–G45 could not support cell growth at the nonpermissive temperature (Fig. 8A). Overexpression of Mcm10G261A, Mcm10G261D, Mcm10N268D, and Mcm10N268I only partially restored viability of mcm10-1 cells at 37 °C (Fig. 8A). Moreover, some mutants (mcm10G261D) complemented better than others (mcm10N268D) (Fig. 8A), indicating functional differences between the alleles. Together, these observations support the notion that the Hsp10-like domain contributes to Mcm10 function in the cell.

Despite all the evidence indicating that the Hsp10-like domain plays a role in stabilizing overexpressed Cdc17, it was still unclear whether mutations in this domain have any effect on endogenous Cdc17 levels. To address this question, we introduced mutations in the Hsp10-like domain into the endogenous MCM10 locus in a strain in which CDC17 was fused in-frame with three HA epitope tags. In addition, wild-type MCM10 and mcm10 mutants were fused to nine C-terminal Myc epitope tags for detection. All resulting strains were viable, did not display any temperature sensitivity, and grew with kinetics similar to wild-type cells (data not shown). However, when we analyzed Mcm10 and Cdc17 expression in these cells, we found that steady-state levels of Cdc17 were reduced by 75% in the mcm10G261D mutant, whereas the mcm10G261A mutants showed a 21% reduction, mcm10N268D cells showed a 27% reduction, and mcm10N268I cells showed a 34% reduction compared with wild type (Fig. 8, D and E). Importantly, mutation of the Hsp10-like domain did not alter steady-state levels of Mcm10 (Fig. 8D). Again, this suggests functional differences among these mcm10 alleles. These results clearly demonstrate that the Hsp10-like domain in Mcm10 plays a role in stabilizing Cdc17 in vivo. Moreover, our findings are consistent with a previous report demonstrating that budding yeast cells are able to proliferate with greatly reduced amounts of Cdc17 (59).

DISCUSSION

In this study, we have further substantiated the finding that Mcm10 is required to stabilize Cdc17 in vivo. Importantly, Mcm10 specifically affected Cdc17 stability as none of the other replication factors analyzed so far have been affected by depletion of Mcm10, including Cdc45, Pri2,
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**FIGURE 7.** The region in Mcm10 required for Cdc17 stabilization maps to a conserved Hsp10-like domain. A, schematic of the protein motifs identified in Mcm10. Mcm10 is a 571-amino acid protein that contains one predicted OB-fold from amino acid 201 to 297, a PIP domain (amino acids 239–245), a Hsp10-like domain from amino acids 309–335, and two C-terminal nuclear localization signals (NLS) (amino acids 435–451 and 512–527) (44). B, asynchronous cultures of ABY128 (Cu-MCM10-2HA, GAL-CDC17-2HA), ABY133 (Cu-mcm10-2HA, GAL-CDC17-2HA), ABY134 (Cu-mcm10-2HA, GAL-CDC17-2HA), ABY137 (Cu-mcm10-3HA, GAL-CDC17-2HA), ABY138 (Cu-mcm10-3HA, GAL-CDC17-2HA), ABY163 (Cu-mcm10-10A-3HA, GAL-CDC17-2HA), ABY167 (Cu-mcm10-10A-3HA, GAL-CDC17-2HA), and ABY169 (Cu-mcm10-10A-3HA, GAL-CDC17-2HA) were grown overnight in minimal medium with 2% raffinose. Cdc17-2HA remains at 90 min after repression serving as a loading control. Using anti-HA (16B12) antibodies. Ponceau S stain-90 min after repression of Cdc17-2HA. Cdc17-2HA des-cribed samples were taken at 0, 15, 30, 60, and 90 min after repression of Cdc17-2HA. Cdc17-2HA, and ABY169 (Cu-mcm10-10A-3HA, GAL-CDC17-2HA) were grown overnight in minimal medium with 2% glucose. Expression of full-length (FL) Mcm10-3HA and mcm10 mutants was induced with 10 μM CuSO4 for 2 h at 30°C. Protein extracts were separated on a 12% SDS-polyacrylamide gel. Full-length wild-type Mcm10-3HA and mcm10 mutants were detected by Western blot with an anti-HA (16B12) antibody. The asterisks indicate two nonspecific bands, and the black diamond marks a degrada-tion product of Mcm10. C, the Hsp10-like domain in Mcm10 is aligned with Hsp10 from budding yeast and the Hsp10 homolog in E. coli (GroES). Also shown is the alignment of the Hsp10-like domain in Mcm10 across species. Amino acids conserved from Hsp10/GroES to Mcm10 are marked in large boldface type. D, the strains described in B were grown overnight in minimal medium with 2% raffinose. Cdc17-2HA and mcm10 mutants were co-overexpressed as described. Samples were taken at 0, 15, 30, 60, and 90 min after repression of Cdc17-2HA. Cdc17-2HA and MCM10-3HA were detected by Western blot using an anti-HA (16B12) antibody. Ponceau S staining served as a loading control. E, the percentage of Cdc17-2HA remaining at 90 min after repression of Cdc17 expression is plotted for each of the full-length mcm10 mutants. The average of two inde-pendent experiments is shown, and the error bars indicate one S.D.

Pol12, Orc2, and Mcm7 (7). Moreover, although Pol12 stability was unaffected by Mcm10 depletion, Pol12 was dephosphorylated in the absence of Mcm10. Given that Pol12 phosphorylation requires pol-α complex formation (21), it seems likely that Pol12 dephosphorylation is due to co-depletion of Cdc17. This was further supported by our finding that depletion of Cdc17 results in Pol12 dephosphorylation, without affecting Mcm10 protein levels (Fig. 3B). Although it remains unclear what dephosphorylates Pol12 in the absence of Cdc17, our results also demonstrated that the kinetics of Cdc17 degradation and Pol12 dephosphorylation differed, since we observed an almost complete degradation of Cdc17 but only a 50% reduction in Pol12 phosphorylation. This indicates that the two pathways are coupled but regulated independently, arguing that binding to Cdc17 is a prerequisite but not sufficient to determine the phosphorylation status of Pol12. This is consistent with previous reports showing that Pol12 is part of the pol-α-prime complex throughout the cell cycle but is not phosphorylated until cells have entered S phase.

The ability of overexpressed Mcm10 to restore Cdc17 protein levels in the mcm10-1 mutant promotes the idea that Cdc17 stabilization is a unique feature of Mcm10. One interesting finding is that increasing the induction time of Mcm10-3HA overexpression resulted in greater stabilization of Cdc17 (Fig. 4). Since MCM10-3HA was expressed from a galactose-inducible promoter, Mcm10-3HA protein levels presumably far exceeded the protein levels of endogenous Mcm10-1 protein in the cell. Had there been free and efficient exchange of the overexpressed MCM10-3HA protein with the MCM10-1 protein already in a complex with pol-α-prime, then a short burst of MCM10-3HA overexpression should have been sufficient to restore Cdc17 protein levels. However, what we observed was that increasing the time of MCM10-3HA overexpression prior to degrading MCM10-1 resulted in greater Cdc17 stabi-lization.
FIGURE 8. The Hsp10-like domain in Mcm10 plays a role in stabilizing endogenous Cdc17.

A, ABy227 (pRS423cup), ABy240 (pRS423cup-mcm10Δ50-3HA), ABy241 (pRS423cup-mcm10Δ150-3HA), ABy242 (pRS423cup-mcm10Δ200-3HA), ABy243 (pRS423cup-mcm10Δ250-3HA), ABy244 (pRS423cup-mcm10Δ300-3HA), ABy245 (pRS423cup-mcm10ΔG261D-3HA), ABy219 (pRS424cup), ABy221 (pRS424cup-mcm10ΔG261A-3HA), ABy222 (pRS424cup-mcm10ΔG261I-3HA), and ABy220 (pRS424cup-MCM10-3HA) cells were serially spotted in 10-fold dilutions onto minimal medium plates containing 0.2 mM CuSO4. The plates were incubated at 30 or 37 °C for 2–3 days.

B, cultures of ABy227 (pRS423cup), ABy240 (pRS423cup-mcm10Δ50-3HA), ABy241 (pRS423cup-mcm10Δ150-3HA), ABy242 (pRS423cup-mcm10Δ200-3HA), ABy243 (pRS423cup-mcm10Δ250-3HA), ABy244 (pRS423cup-mcm10Δ300-3HA), ABy219 (pRS424cup), ABy221 (pRS424cup-mcm10ΔG261A-3HA), ABy222 (pRS424cup-mcm10ΔG261I-3HA), and ABy220 (pRS424cup-MCM10-3HA) were induced with 10 μM CuSO4 for 2 h. Mcm10-3HA was detected by Western blot with anti-HA antibodies (16B12). The asterisk denotes a degradation product. Ponceau S staining of the membrane prior to Western blot served as a loading control.

C, ABy227 (pRS423cup), ABy229 (pRS423cup-mcm10Δ45-3HA), ABy230 (pRS423cup-mcm10Δ90-3HA), ABy222 (pRS424cup-mcm10ΔG261D-3HA), ABy223 (pRS424cup-mcm10ΔG261I-3HA), and ABy220 (pRS424cup-MCM10-3HA) cells were induced with 10 μM CuSO4 for 2 h. Mcm10-3HA was detected by Western blot with anti-HA antibodies (16B12). Ponceau S staining of the membrane prior to immunoblot served as a loading control.

D, asynchronous cultures of ABy003 (CDC17-3HA, MCM10-9MYC), ABy244 (CDC17-3HA, MCM10ΔG261D-9MYC), ABy245 (CDC17-3HA, MCM10ΔG261I-9MYC), ABy246 (CDC17-3HA, MCM10ΔG261D-9MYC), and ABy247 (CDC17-3HA, MCM10ΔG261I-9MYC) were grown in complete medium overnight to an A600 of 0.6. The protein levels of Cdc17-3HA and Mcm10-9Myc were assayed by Western blot using anti-HA (16B12) and anti-Myc (9E11) antibodies, respectively. Ponceau S staining of the membrane prior to immunoblot served as a loading control. E, the percentage of Cdc17-3HA is plotted for each of the mcm10 mutants. The amount of Cdc17 in wild-type cells was set to 100%. The averages of two exposures of a representative experiment are shown, and the error bars indicate one S.D.
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zation, suggesting that overexpressed Mcm10-3HA did not substitute with Mcm10-1 that was already in a complex with pol-α. The rescue kinetics that we observed are consistent with a model in which overexpressed Mcm10-3HA competes with the endogenous Mcm10-1 protein pool to associate with newly synthesized Cdc17. Then stabilizing Cdc17 would require a relatively longer induction of Mcm10-3HA expression, because basal levels of Cdc17 are low (35). This model agrees with what we observed in this study (Fig. 4).

It has been reported for yeast and mammalian cells that overexpression of the catalytic subunit of pol-α results in its rapid degradation (35, 36). That overexpressed Cdc17 is highly unstable, whereas endogenous Cdc17 has a long half-life, supports the hypothesis that Cdc17 is associated with a partner protein conferring stability in vivo. We estimated the relative abundance of Mcm10 and Cdc17 and found that Mcm10 protein levels exceeded those of Cdc17 by 1.7–2.4-fold (data not shown). Recent work by the O’Shea and Weissman laboratories in which protein levels were measured genome-wide in cycling budding yeast cells estimates Cdc17 as the limiting factor in the pol-α complex (56). In addition to serving as a stability factor for Cdc17, Mcm10 does have other pol-α-independent functions, which explains why eukaryotic cells may require more Mcm10 than Cdc17. For example, Mcm10 is required for the sequential association of other factors, such as Cdc45, with chromatin (5, 6) prior to pol-α recruitment. This pool of Mcm10 most likely associates with chromatin independently of pol-α. We hypothesize that approximately half of the Mcm10 protein pool is associated with Cdc17, and the fraction of the Mcm10 pool that is not bound to pol-α participates in preparing replication origins for DNA replication initiation (7).

The actual domain in Mcm10 required for stabilizing Cdc17 was unknown until now. To the best of our knowledge, this is the first example of a protein without chaperone function containing a protein domain found in Hsp10. Importantly, the corresponding domain in E. coli GroES and budding yeast Hsp10 share a high degree of conservation. E. coli GroES cannot functionally substitute for Hsp10 in yeast unless the mobile loop in GroES is altered to exactly correspond to the domain found in Hsp10 (53). We also found that the domain found in budding yeast Hsp10 (53) is crucial for Mcm10 binding to Cdc17. We hypothesize that approximately half of the Mcm10 protein pool is required for the sequential association of other factors, such as Cdc45, with chromatin (5, 6) prior to pol-α recruitment. This pool of Mcm10 most likely associates with chromatin independently of pol-α. We hypothesize that approximately half of the Mcm10 protein pool is associated with Cdc17, and the fraction of the Mcm10 pool that is not bound to pol-α participates in preparing replication origins for DNA replication initiation (7).

Although we do not yet understand exactly how this domain contributes to Cdc17 stability, one possible explanation is that this domain is crucial for Mcm10 binding to Cdc17. We hypothesize that the hydrophobic nature of the Hsp10-like domain interacts with a hydrophobic patch in Cdc17. It is therefore conceivable that loss of Mcm10 exposes a hydrophobic region in Cdc17, which may trigger a conformational change in the protein that is then recognized as an aberrantly folded protein. Alternatively, it is also possible that binding of Mcm10 protects Cdc17 from proteolytic degradation by masking a degradation signal in Cdc17. We are currently in the process of testing these models. In addition, we would also like to note the high degree of conservation of the Hsp10-like domain in Mcm10 (Fig. 7C), which suggests to us that this function of Mcm10 may be important in higher eukaryotes as well.

One point that this study raises is the question of why the cell regulates pol-α protein levels. A recent report has linked genomic instability, including chromosomal translocations and breaks, with low levels of pol-α (59). Two regions of the genome were observed to be particularly sensitive to such perturbations and were suggested to be fragile sites (59). Chromosome fragility at these sites probably resulted from incomplete DNA replication (59). Whereas there is significant evidence to suggest that the S phase checkpoint contributes to the expression of fragile sites (60), fragile sites were initially identified as regions in the human genome that were sensitive to aphidicolin, an inhibitor of pol-α (61, 62). In addition to fragile site expression, mutations in CDC17 have also been correlated with microsatellite instability in budding yeast (63). One particularly intriguing finding was that protein levels of Cdc17 were diminished in one of the cdc17 mutants analyzed, providing another link between pol-α protein levels and genomic instability (63). Finally, by electron microscopy, it was observed that a primase mutant, pri1-M4, shows an increased amount of single-stranded regions at the replication fork, similar to rad53 mutants (64). Given that DNA primase subunits are required for pol-α chromatin association (65), we conclude that regulation of the pol-α-prime complex is important to complete genome duplication in the face of replication stress. Taken together, this suggests that Mcm10 may regulate pol-α levels to maintain genome stability.

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