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A Conserved Motif in the C-terminal Tail of DNA Polymerase α Tethers Primase to the Eukaryotic Replisome*

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Background: Primase initiates DNA replication together with DNA polymerase α and forms part of the eukaryotic replisome.
Results: Primase is tethered by a short motif in pol α that is functionally important.
Conclusion: Tethering primase to the eukaryotic replisome is critical for normal DNA replication.
Significance: Small molecule inhibitors of the primase-pol α interaction might be valuable in antitumor therapies.

The DNA polymerase α-primase complex forms an essential part of the eukaryotic replisome. The catalytic subunits of primase and pol α synthesize composite RNA-DNA primers that initiate the leading and lagging DNA strands at replication forks. The physical basis and physiological significance of tethering primase to the eukaryotic replisome via pol α remain poorly characterized. We have identified a short conserved motif at the extreme C terminus of pol α that is critical for interaction of the yeast ortholog pol1 with primase. We show that truncation of the C-terminal residues 1452–1468 of Pol1 abrogates the interaction with the primase, as does mutation to alanine of the invariant amino acid Phe[1463]. Conversely, a pol1 peptide spanning the last 16 residues binds primase with high affinity, and the equivalent peptide from human Pol α binds primase in an analogous fashion. These *in vitro* data are mirrored by experiments in yeast cells, as primase does not interact in cell extracts with pol1 that either terminates at residue 1452 or has the F1463A mutation. The ability to disrupt the association between primase and pol α allowed us to assess the physiological significance of primase being tethered to the eukaryotic replisome in this way. We find that the F1463A mutation in Pol1 renders yeast cells dependent on the S phase checkpoint, whereas truncation of Pol1 at amino acid 1452 blocks yeast cell proliferation. These findings indicate that tethering of primase to the replisome by pol α is critical for the normal action of DNA replication forks in eukaryotic cells.

The initiation of DNA replication in all living creatures relies on primase, a DNA-dependent RNA polymerase endowed with the unique ability to synthesize an oligonucleotide primer from the ribonucleotide pool (1, 2). The RNA primer is subsequently extended by DNA polymerases on the leading and lagging strand templates (3–5). The antiparallel nature of the DNA double helix and the obligate 5′ to 3′ direction of nucleotide polymerization by DNA polymerase dictate that lagging strand synthesis must be primed repeatedly and frequently by the primase. Thus, the enzymatic activity of the primase is constantly required at DNA replication forks.

Work with *Escherichia coli* showed that the DNA helicase at replication forks is physically coupled to DNA polymerases as part of a multiprotein assembly known as the replisome. Primase is recruited to the bacterial replisome via a highly dynamic interaction with the replicative helicase, and this interaction is important for efficient synthesis of the lagging strand (6). The interaction of primase with helicase is conserved in bacteriophages such as T4, whereas in bacteriophage T7 the primase and helicase are fused into a single polypeptide (7, 8).

The eukaryotic replisome is considerably more complex and less well defined than its prokaryotic counterpart (9), and it appears that primase is recruited to replication forks by a different mechanism that does not involve a direct interaction with the replicative helicase. Instead, primase forms a constitutive complex with DNA pol[4] α, which is uniquely able to extend RNA primers and is needed to begin each new DNA fragment of leading and lagging strands. Previous work indicated that accessory factors such as Go-Ichi-Ni-San (GINS) and Ctf4 link the helicase to the catalytic subunit of pol α at eukaryotic forks (10–12). Thus, primase function is likely to be integrated into the replisome as an essential component of the primosome, a multiprotein complex comprising the catalytic subunit of pol α, the B subunit, and the small and large subunits of the heterodimeric primase (13).

Earlier studies had indicated that primase associates directly with the catalytic subunit of pol α (14) and that a region of about 200 amino acids at the C terminus of the catalytic subunit of pol α is essential for primosome assembly, as it mediates interac-

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[4] The abbreviations used are: pol, DNA polymerase; CTD, C-terminal domain; Ctf4, chromosome transmission fidelity 4; TCEP, *tris*(2-carboxyethyl)-phosphine.

tions with both primase and the B subunit (15–18). Here, we show that primase is linked to the rest of the primosome by a short linear motif at the end of the catalytic subunit of pol α, which has been evolutionarily conserved from yeast to humans. We exploit this structural knowledge to demonstrate that tethering primase to the eukaryotic replisome makes a key contribution to the efficiency of chromosome replication.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The human primase was produced in *E. coli* strain Rosetta2(DE3) using the pRSFDuet-1 vector expressing full-length His-tagged Prim1(1–420) and Prim2(1–462). Amino acids 463–509 of Prim2 were omitted as they are not conserved and are likely to be disordered; amino acids Lys^{72} and Met^{73} of Prim1 were mutated to alanine to prevent proteolytic cleavage during purification. A truncated version of human primase lacking the C-terminal domain of Prim2(1–264; Prim2ΔCTD) was generated using the QuikChange mutagenesis protocol (Stratagene) and expressed in the same way.

The yeast primase was produced in Rosetta2(DE3) *E. coli* strain, using the vector pRSFDuet-1 vector expressing full-length Pri1(1–409) and His-tagged Pri2(49–513). The first 48 and last 15 amino acids of Pri2 were omitted because they are not conserved and are likely to be disordered. In addition, amino acids Arg^{382}, Asn^{383}, and Gly^{384} of Pri2 were excised to prevent proteolytic cleavage of the protein during purification. A truncated version of the primase, lacking the C-terminal domain of Pri2(49–335; Pri2ΔCTD) was generated using the QuikChange mutagenesis protocol and expressed in the same way.

The yeast Pol1 CTD-Pol12 complex was produced in *E. coli* strain Rosetta2(DE3) using the vector pRSFDuet-1 vector expressing Pol1 (1263–1468) and His-tagged Pol12 (246–705). A truncated version of the CTD lacking the last 16 amino acids of Pol1 (1453–1468; CTDΔC) was generated using the QuikChange mutagenesis protocol (Stratagene) and the CTDΔC-Pol12 complex was expressed in the same way.

All proteins described above were purified by cobalt-nitrilotriacetic acid-agarose chromatography, heparin-Sepharose chromatography, tobacco etch virus cleavage of the histidine tag, and gel filtration chromatography. Purified protein samples were flash-frozen in liquid nitrogen and stored in small aliquots at −80 °C.

Analytical Gel Filtration—Protein complexes were prepared by mixing equimolar amounts of selected components (Pri1-Pri2, Pri1-Pri2ΔCTD, CTD-B, or CTDΔC-B) and incubating them at 4 °C for 30 min. 100 μl of 14 μM protein complex was injected onto a Superdex 200 10/300 GL gel filtration column (GE Healthcare) and run at 0.5 ml/min in 25 mM HEPES, pH 7.2, 250 mM KCl, 5% glycerol, and 0.5 mM TCEP. Fractions were analyzed by SDS-PAGE and visualized by Coomassie Blue staining.

Pulldown Assays—Amino acids 1453–1468 of Pol1 were expressed in *E. coli* strain BL21(DE3) as a GST-tagged peptide. Five single-point mutations to alanine (V1457A, M1459A, I1462A, F1463A, and F1465A) were generated according the QuikChange mutagenesis protocol and expressed in the same way. Amino acids 1445–1462 of human pol α as well as four single-point mutations to alanine (V1449A, L1451A, L1454A, and F1455A) were also expressed as described above.

To perform the pulldown experiment, an excess of GST fusion protein was bound to 150 μl of glutathione-Sepharose 4B resin (GE Healthcare) and washed three times with buffer A (25 mM HEPES, pH 7.0, 250 mM KCl, 1% BSA, 0.1% Nonidet P-40, 1 mM TCEP). The washed beads were incubated with 400 μl of 20 μM yeast or human primase for 2 h at 4 °C. Beads were washed three times with buffer A, once with 25 mM HEPES, pH 7.0, 250 mM KCl, 0.1% Nonidet P-40, and 1 mM TCEP, and then resuspended in 200 μl of LDS sample loading buffer (Invitrogen). Samples were analyzed by SDS-PAGE and visualized by Coomassie Blue staining.

Fluorescence Polarization—The human (^{1445}GYSEVNLSKLFAGCAVKS^{1462}) and yeast (^{1453}GRRYVDMTSIFDFMLN^{1468}) pol α peptides were synthesized with N-terminal fluorescein labels. Peptide calibration curves were prepared to determine the lowest concentration of peptide at which the binding study could be performed. Fluorescence anisotropy measurements were recorded in a PHERAstar Plus multidetection plate reader (BMG Labtech) equipped with fluorescence polarization optic module (λ_{ex} = 485 nm; λ_{em} = 520 nm) at 25 °C. Each data point is the mean of 200 flashes/well. The voltage gain was set by adjusting the target mP values of fluorescein-labeled peptides relative to that of fluorescein (35 mP). Serial dilutions of proteins were made in 25 mM HEPES, pH 7.2, 250 mM KCl, 1 mM TCEP, and 30 nM fluorescein-labeled peptide. Each data point is the mean of three independent experiments. Monte Carlo curve fitting to the experimental data was performed in ProFit (QuantumSoft).

Chemical Cross-linking of Proteins in Vitro—Proteins and peptides were dialyzed in 25 mM HEPES, pH 7.5, 250 mM KCl, and 2 mM TCEP. Human primase (8 μM) was incubated with equimolar amounts of fluorescein-labeled peptide for 10 min at 4 °C. Complexes were cross-linked by incubation with 0, 0.025, 0.05, 0.1, 0.3, or 1 mM bis(sulfosuccinimidyl) suberate (Sigma-Aldrich) at room temperature for 30 min. Cross-linking was terminated by incubation with 40 mM Tris, pH 7.5, for 15 min, and products were analyzed by SDS-PAGE. The cross-linked peptide were detected by exposing the gel to UV radiation (302 nm), and the proteins were visualized by Coomassie Blue staining.

Yeast Strains and Growth—Table 1 lists the strains that were used in this study. Yeast cultures were grown in rich medium (1% yeast extract, 2% peptone, 40 μg/ml adenine) with 2% glucose (YPD), to provide the carbon source (in none of the experiments did we use raffinose or galactose). Cells were synchronized in G_1 phase by adding 7.5 μg/ml α factor mating pheromone for 70% of a one-generation time, followed by additional aliquots of 3 μg/ml every 40 min up to 1.5 generation times (until 90% of cells were unbudded).

Immunoprecipitation of Proteins from Yeast Extracts and Mass Spectrometry—Yeast cell extracts were made from 250 ml of mid-log culture (2.5×10^9 cells) in the presence of 100 mM potassium acetate as described previously (19). Chromosomal DNA was then digested for 30 min at 4 °C with 800 units of Benzonase (71206-3; Merck) before immunoprecipitation of

TABLE 1
Yeast strains used in this study
All strains are isogenic to W303-1a.

Strain	Genotype
W303–1a	*MAT***a** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100*
YGDP1102	*MAT***a** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 / MAT***α** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pol1 (1–1452) (HIS3MX)*
YGDP1127	*MAT***a** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 / MAT***α** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pol1-F1463A (hphNT)*
YGDP1135	*MAT***a** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pol1-F1463A (hphNT)*
YGDP1146	*MAT***a** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::URA3 PRI1–9MYC (K.l.TRP1) / MAT***α** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::URA3 PRI1–9MYC (K.l.TRP1)*
YGDP1167	*MAT***a** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::URA3 PRI1–9MYC (K.l.TRP1) / MAT***α** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::URA3 PRI1–9MYC (K.l.TRP1) POL1–6HA (His3MX)*
YGDP1169	*MAT***a** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::URA3 PRI1–9MYC (K.l.TRP1) / MAT***α** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::URA3 PRI1–9MYC (K.l.TRP1) pol1 (1–1452)-6HA (His3MX)*
YGDP1191	*MAT***a** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::URA3 PRI1–9MYC (K.l.TRP1) / MAT***α** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::URA3 PRI1–9MYC (K.l.TRP1) pol1-F1463A-6HA (hphNT)*
YGDP1290	*MAT***a** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::ADE2 / MAT***α** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::ADE2*
YGDP1291	*MAT***a** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::ADE2 / MAT***α** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::ADE2 POL1–9MYC (HIS3MX)*
YGDP1292	*MAT***a** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::ADE2 / MAT***α** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::ADE2 pol1 (1–1452)-9MYC (HIS3MX)*
YGDP1293	*MAT***a** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::ADE2 / MAT***α** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 pep4Δ::ADE2 pol1-F1463A -9MYC (hphNT)*
YGDP1453	*MAT***a** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 sml1Δ::HIS3 mec1Δ::ADE2 / MAT***α** *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can1–100 sml1Δ::HIS3 pol1-F1463A (hphNT)*

tagged proteins using magnetic beads coupled to the monoclonal antibodies 9E10 (anti-MYC) or 12CA5 (anti-HA).

For the experiment in Fig. 4*A*, each sample was run for 1 cm in one lane of an SDS-polyacrylamide gel that was then cut into 10 bands before digestion with trypsin and analysis of the protein content by mass spectrometry as described previously (20). The mass spectrometry analysis was performed by the company MS Bioworks. For the experiment in Fig. 4*Bii*, cells were treated with 1% formaldehyde for 25 min at 24 °C, and extracts were then prepared as described previously (14).

Detection of Proteins by Immunoblotting—The indicated proteins in Fig. 4 were detected by the corresponding sheep polyclonal antibodies (20), or mouse monoclonal antibodies specific for Pol12 (21), the HA epitope from influenza virus hemagglutinin (12CA5), or the c-Myc epitope (9E10).

Flow Cytometry—Samples were fixed with 70% ethanol and then processed as described previously (22) before analysis with a Becton Dickinson FACScan machine and CellQuest software.

RESULTS

Identification of a Conserved Motif in the C-terminal Tail of pol α—Electron microscopy of the yeast primosome revealed a dumbbell-shaped particle (18), with one lobe representing the catalytic domain of pol1 and the other lobe comprising the CTD of Pol1 bound to the B subunit Pol12 and the heterodimeric primase Pri1-Pri2. The latter feature agrees with previous evidence that the CTD of mouse and human pol α is important for its interaction with the B subunit and primase (15, 16). Interestingly, crystallographic analysis of Pol1 CTD in complex with Pol12 (17) revealed that the ordered part of the CTD terminates at residue Cys^{1452}, 16 amino acids before the end of the protein (Fig. 1*A*). The conservation of several hydrophobic and aromatic positions in the unstructured C-terminal tail of Pol1 suggested that residues 1453–1468 might harbor a novel protein-protein interaction motif (Fig. 1*B*).

The C-terminal Tail of pol α Is Required for Primosome Assembly—To determine whether the short motif at the end of the CTD is important for the association of pol α with primase in budding yeast, we used recombinant proteins to reconstitute a heterotetrameric assembly of the heterodimeric primase and the B subunit bound to the CTD of pol α. Successful reconstitution of the heterotetrameric complex was verified by analytical gel filtration chromatography (Fig. 2). As expected, an equimolar mixture of primase and CTD-B subunit complex eluted as a single peak. Compared with the elution profiles of the isolated primase and CTD-B subunit complex, the elution volume of the mixture was clearly indicative of complex formation (Fig. 2; compare Pri1-Pri2 and CTD-Pol12 with Pri1-Pri2 + CTD-Pol12).

When reconstitution was attempted with a version of the CTD that lacked the last 16 amino acids (CTDΔC), no single peak corresponding to the heterotetrameric assembly was detected; instead, the primase and the CTDΔC-B subunit complex eluted as separate, overlapping peaks (Fig. 2; compare Pri1-Pri2 and CTDΔC-Pol12 with Pri1-Pri2 + CTDΔC-Pol12). Importantly, CTDΔC retained its ability to bind to Pol12 (Fig. 2; CTDΔC-Pol12). Furthermore, the interaction between primase and the CTD-B subunit complex was maintained when the iron-sulfur domain of Pri2 (23) was deleted (Fig. 2; Pri1-Pri2ΔCTD + CTD-Pol12). These results indicate that the last

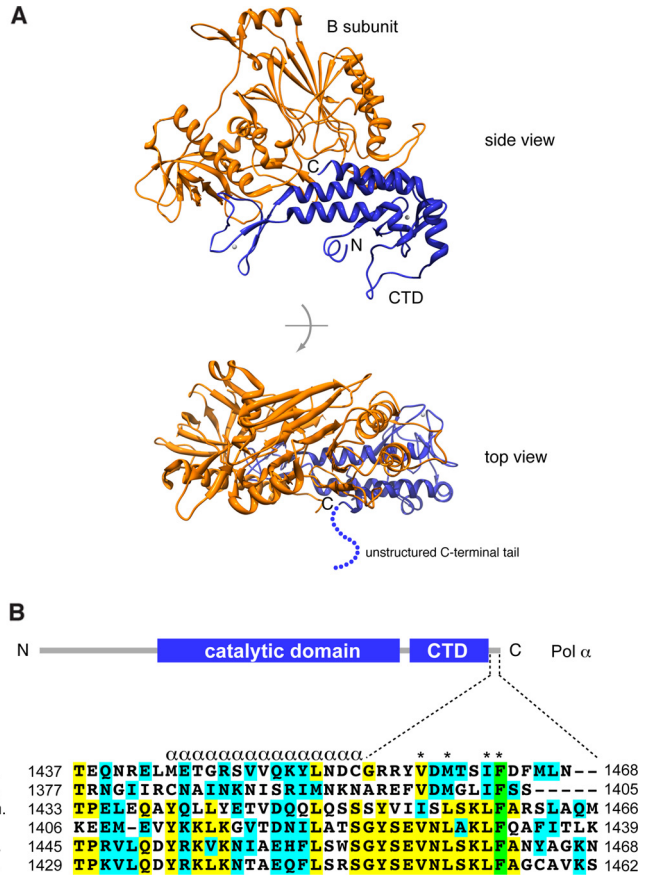

FIGURE 1. **Evolutionary conservation of a short, conserved motif at the extreme C terminus of pol α.** *A*, two views of the crystal structure of the yeast pol α CTD-B subunit complex (17). The polypeptide chains are drawn as orange (*B*) and blue (*CTD*) ribbons. The position of the natively unstructured tail at the C terminus of the CTD is indicated with *blue dots* in the *top view panel*. *B*, multiple sequence alignment of the C terminus of pol α (*S.c.*: *Saccharomyces cerevisiae*; *S.p.*: *Schizosaccharomyces pombe*; *D.m.*: *Drosophila melanogaster*; *D.r.*: *Danio rerio*; *R.n.*: *Rattus norvegicus*; *H.s.*: *Homo sapiens*). The position of the α-helix that represents the last structured segment of the CTD is indicated above the alignment. *Asterisks* mark conserved hydrophobic amino acids in a natively unstructured motif at the extreme C terminus of pol α.

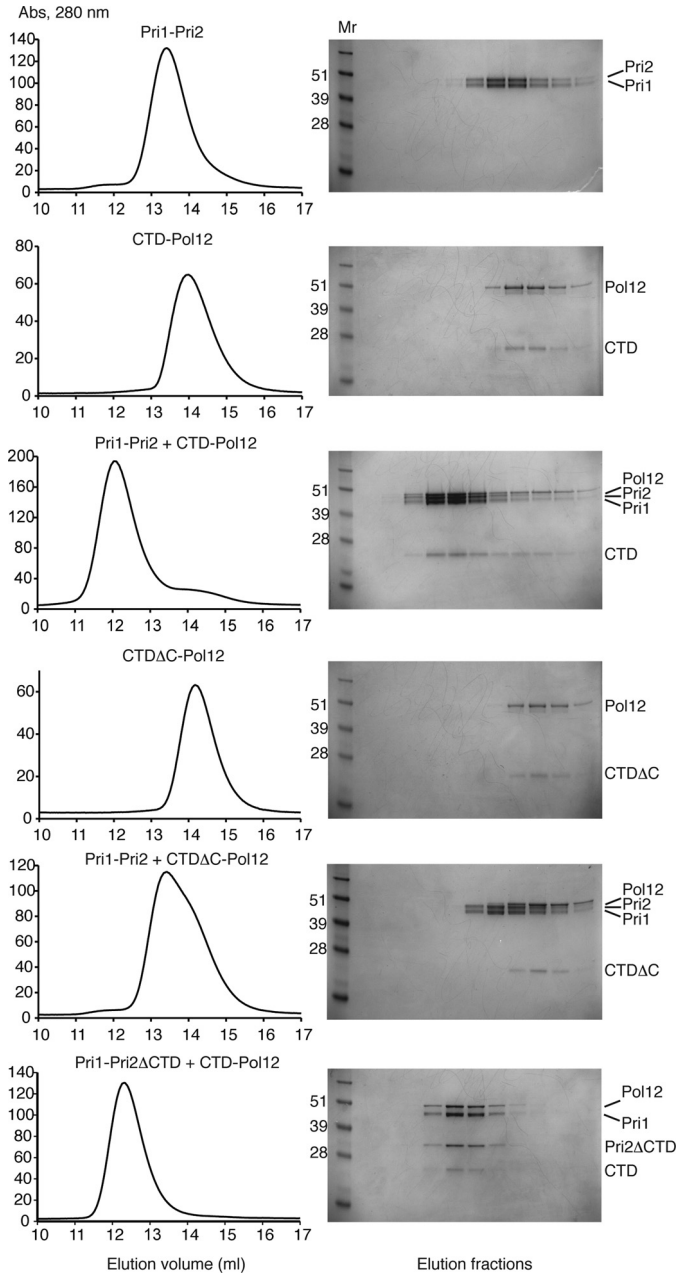

FIGURE 2. **Gel filtration analysis of the heterotetrameric assembly reconstituted from heterodimeric Pri1-Pri2 primase and the Pol1 CTD-Pol12 complex.** For each experiment, the chromatogram and the SDS-PAGE analysis (gels stained with Coomassie Blue) of the relevant fractions are shown.

16 amino acids of Pol1 are essential for its specific association with the primase.

The C-terminal Tail of pol α Binds Primase Directly—To determine whether the C-terminal tail of Pol1 is able to support interaction with primase, the Pol1 sequence ^{1453}GRRYVDMTSIFDFMLN^{1468} was fused to the C terminus of GST and used as bait for *in vitro* binding assays with recombinant primase. A strong interaction was observed in a pulldown experiment on glutathione-Sepharose between primase and the C-terminal motif of Pol1, which was not seen with free GST (Fig. 3*A*; compare WT and GST only), indicating that amino acids 1453–1468 of Pol1 are able to bind directly to the primase.

A common hallmark of protein regions involved in protein-protein interactions is the presence of solvent-exposed hydrophobic and aromatic residues that become buried at the interface upon complex formation. The occurrence of several conserved hydrophobic and aromatic amino acids in the primase binding motif of pol α prompted us to dissect their individual role in the interaction. We created a set of alanine point mutants that targeted Pol1 residues Val^{1457}, Met^{1459}, Ile^{1462}, Phe^{1463}, and Phe^{1465} and tested their effect on primase binding in the pulldown assay. All of the alanine mutants showed a reduced ability to interact with the primase, albeit to a different extent (Fig. 3*A*). The F1463A mutation was particularly effective in destabilizing the association with the primase, pointing to a critical role of this highly conserved aromatic residue in the pol α-primase interaction.

Consistent with these data, the orthologous sequence ^{1445}GYSEVNLSKLFAGCAVKS^{1462} of human pol α also bound to human primase in pulldown experiments (Fig. 3*B*). Mirroring the results obtained with the yeast proteins, alanine mutations of conserved hydrophobic amino acids Val^{1449}, Leu^{1451}, Leu^{1454}, and Phe^{1455} (equivalent to Phe^{1463} in yeast pol α) greatly diminished the interaction (Fig. 2*B*). Affinity measure-

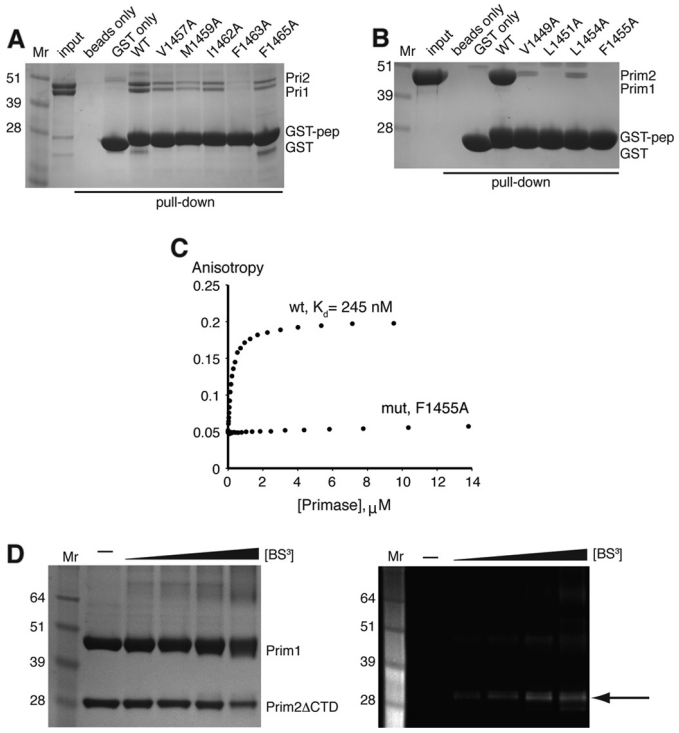

FIGURE 3. **The C-terminal motif of pol α supports primase binding.** *A*, peptides corresponding to wild-type and mutated versions of Pol1 residues 1453–1468 were expressed as GST fusions and tested in binding assays with yeast primase (SDS-PAGE stained with Coomassie Blue). *B*, same as *A*, but measuring the interaction of wild-type and mutated versions of amino acids 1445–1462 of human pol α with human primase. *C*, fluorescence anisotropy binding curves of human primase to fluorescein-labeled wild-type and F1455A mutant peptides spanning amino acids 1445–1462 of human pol α. *D*, cross-linking analysis of the interaction between human primase and a fluorescein-labeled peptide spanning amino acids 1445–1462 of human pol α. The result of the experiment is shown by SDS-PAGE (*left panel*, stained with Coomassie Blue) and UV fluorescence of the fluorescein-labeled peptide (*right panel*). A truncated version of human primase lacking the iron-sulfur domain (Prim2ΔCTD) was used to allow the unambiguous identification of the cross-linked primase subunit. The *arrow* indicates the position of the cross-linked peptide.

ment by fluorescence polarization resulted in a dissociation constant of 245 nM for binding of peptide ^{1445}GYSEVNLSKLFAGCAVKS^{1462} to human primase, whereas the peptide bearing the F1455A mutation did not display a measurable level of binding (Fig. 3*C*). Considered together, these data indicate that the C-terminal motif of pol α tethers the protein to primase and that conserved hydrophobic and aromatic residues play a prominent role at the pol α-primase interface.

pol α Binds to the Large Subunit of the Primase—Having determined the existence of a C-terminal motif in pol α that serves to tether the catalytic subunit to primase, we sought to identify which of the two primase subunits is responsible for binding pol α by chemical cross-linking experiments.

Primase was incubated with the C-terminal pol α peptide in the presence of bis(sulfosuccinimidyl) suberate cross-linker, and the products of the cross-linking reaction were resolved by SDS-PAGE and visualized under UV light, exploiting the presence of a fluorescein tag on the peptide. Given the absence of lysine residues in the C-terminal motif of yeast pol α, cross-linking was performed with human primase and the human pol α peptide. To be able to differentiate between subunits of the human primase, cross-linking was performed using primase with truncated Prim2 lacking the Fe-S domain (Prim2ΔCTD). As shown by the gel filtration analysis in Fig. 2, the Fe-S domain is not required for the interaction with the CTD-B subunit complex, and therefore its absence was not expected to interfere with binding. A predominant species was observed, clearly indicating that the pol α peptide had cross-linked to Prim2ΔCTD (Fig. 3*D*). The result of the cross-linking experiment indicates that pol α interacts prevalently with the large subunit of the primase.

The C-terminal Tail of pol α Tethers Primase to the Replisome in Vivo—To examine the importance of tethering primase to pol α and thus to the rest of the replisome, we generated diploid yeast strains in which one copy of the *POL1* gene was modified either to remove the sequence encoding the last 16 amino acids (*pol1 1–1452/POL1*) or else to introduce the F1463A mutation (*pol1-F1463A/POL1*). Immunoprecipitation and mass spectrometry analysis of epitope-tagged wild-type or mutant Pol1 from yeast cell extracts showed that Pol1 1–1452 and Pol1-F1463A associated with the B subunit Pol12 as efficiently as wild-type Pol1 (Fig. 4*A*). Critically, however, neither of the mutated alleles of Pol1 co-purified with primase (Fig. 4*A*), consistent with our *in vitro* data.

We also used immunoblotting to confirm that Pol1, Pol1 1–1452, and Pol1-F1463A all associated equally well with Pol12 (Fig. 4*Bi*). Moreover, both of the mutant proteins were still able to co-purify with other replisome components, although the interaction appeared slightly weaker than for wild-type Pol1 (Fig. 4*Bi*). In contrast, neither of the mutated forms interacted with primase in native cell extracts (Fig. 4*Bi*) or in extracts of cells that had been treated with formaldehyde to trap weaker interactions between proteins (Fig. 4*Bii*). These data indicated that the C-terminal tail of Pol1 is required *in vivo* to tether primase to the rest of the primosome and thus to the replisome.

Functional Significance of Tethering Primase to the Eukaryotic Replisome—Sporulation of diploid strains with one copy of wild-type *POL1* and one copy of *pol1 1–1452* (lacking any epitope tag) showed that *pol1 1–1452* cells were not able to form colonies (Fig. 5*A*, *upper panels*). Cells lacking the C-terminal tail of Pol1 germinated but then died in the first cell cycle (Fig. 5*A*, *lower panel*), indicating that the primase binding motif of pol α is essential for cell proliferation.

In a similar fashion, we sporulated diploid cells with one copy of *POL1* and one copy of *pol1-F1463A*. Interestingly, *pol1-F1463A* cells were viable and grew as rapidly as control cells (Fig. 5*B*). Moreover, we synchronized control and *pol1-F1463A* in G_1 phase and found that both strains completed S phase and cell division with similar kinetics (Fig. 5*C*). These data indicated that chromosome replication still progresses in a timely and efficient fashion, even under conditions where primase is displaced from the replisome.

To determine whether displacement of primase from the replisome caused subtle defects in the action of DNA replication forks, despite the efficient progression of bulk DNA synthesis, we determined whether viability of the *pol1-F1463A* allele required the S phase checkpoint pathway, which becomes essential in response to DNA replication stress. As shown in Fig. 4*D*, the viability of *pol1-F1463A* was completely dependent upon the Mec1 checkpoint kinase that is the yeast ortholog of

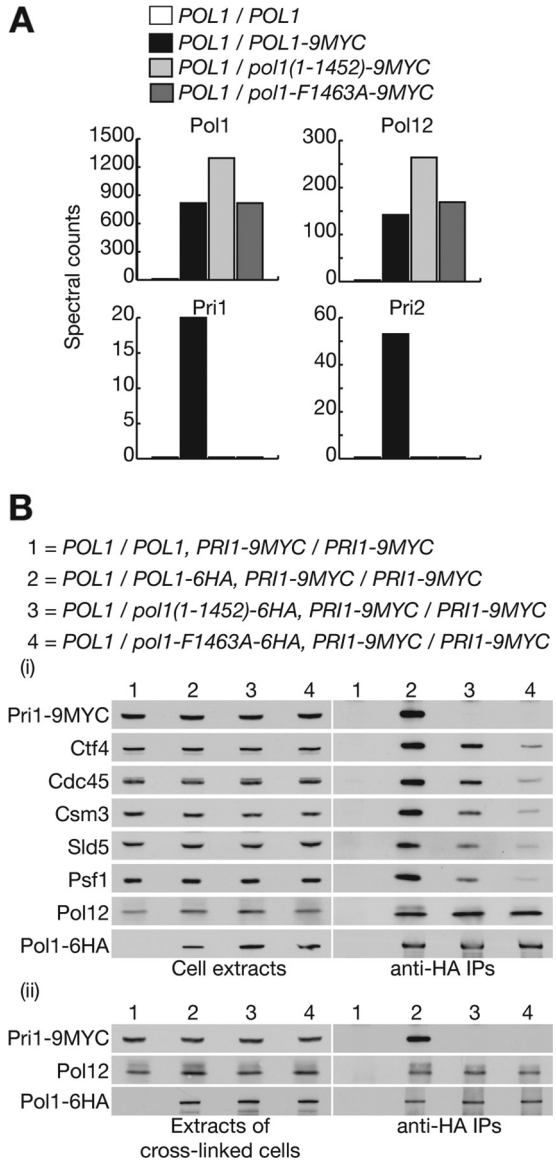

FIGURE 4. **The C-terminal motif of yeast pol α is essential for recruitment of primase to the replisome *in vivo*.** *A*, extracts were made from asynchronous cultures of the indicated diploid yeast strains, before immunoprecipitation on anti-Myc beads. Proteins were separated by SDS-PAGE and digested with trypsin before detection of peptides by mass spectrometry (displayed as "spectral counts"). *B*, *i*, extracts from asynchronous cultures of the indicated diploid strains were subjected to immunoprecipitation on anti-HA beads. The indicated proteins were detected by immunoblotting using the corresponding antibodies as described under "Experimental Procedures." *ii*, an equivalent experiment was performed using extracts of cells that had been treated with the cross-linking agent formaldehyde.

ATR (we also deleted the *SML1* gene that encodes an inhibitor of ribonucleotide reductase, because Mec1 is normally needed to degrade Sml1 in each S phase). The combination of *pol1-F1463A* and *mec1*Δ caused cells to die after a couple of rounds of cell division (Fig. 5*D*, *lower panels*), indicating that the untethered primase in *pol1-F1463A* causes DNA replication stress.

DISCUSSION

In this study we have investigated the molecular basis and physiological relevance of the constitutive association between pol α and primase, two essential components of the eukaryotic replisome. We have identified a short sequence motif in the C-terminal tail of pol α that is conserved from yeast to humans and is critical for the interaction with primase. In agreement with the *in vitro* data, a version of Pol1 bearing the F1463A mutation is unable to associate with the primase in yeast cells, and the *pol1-F1463A* strain becomes dependent on the S phase checkpoint for survival. The functional importance of the primase binding motif of pol α is further highlighted by the observation that removal of the last 16 amino acids of Pol1 blocks yeast cell proliferation. Taken together, our data indicate that primase recruitment to the eukaryotic replisome is critically dependent on a short conserved motif in the C-terminal tail of the catalytic subunit of pol α (Fig. 5*E*).

Our findings agree with and extend earlier observations that the C-terminal 200 amino acids of pol α mediate interaction with primase and B subunit (15–18). We note that a previous study showed that removal of the last 67 amino acids from a recombinant form of human pol α abolished interaction with the B subunit (16). Association of the truncated protein with primase was greatly reduced but not completely abolished (19), and we cannot exclude the possibility that the critical interaction of primase with the C-terminal tail of pol α might be fortified in the case of the human complex by additional contacts within the C-terminal region of the catalytic subunit.

The important role of short linear motifs in mediating protein-protein interactions is increasingly being recognized (24, 25). The primase binding sequence of pol α displays the salient features observed in this class of protein-protein interactions. It is natively unstructured, as it was disordered in the crystal structure of the yeast CTD-B subunit complex. Its conserved positions are enriched for hydrophobic and especially aromatic residues. Finally, primase binding relies critically on "hot spot" residues that make a dominant contribution to the binding energy (24). In fact, primase binding is effectively abrogated by the point mutation of an invariant phenylalanine, F1463A in Pol1 or Phe^{1455} in human pol α, as determined by GST pull-down, fluorescence anisotropy, and immunoprecipitation from yeast cells.

Although the *pol1-F1463A* strain is viable, its dependence for survival on the checkpoint kinases Mec1 and Rad53 (data not shown) implies the existence of a subtle replication defect. The difference in the phenotype of the *pol1-F1463A* and *pol1 1–1452* alleles is in intriguing contrast with their similar biochemical effects. The inviability of the *pol1 1–1452* allele might reflect additional interactions that are mediated by the C-terminal tail of pol1. Alternatively, pol1-F1463A might retain a minimal level of primase binding that is still able to support a viable level of primase incorporation in the replisome, even though this is not detectable experimentally. We note that the mutant pol1 proteins showed reduced levels of association with other core components of the replisome. Previous work had indicated that Pol1 incorporation into the replisome is critically dependent on Ctf4, via an interaction mediated by the N-terminal region of Pol1 (10). It is plausible that, in addition to the known Pol1-Ctf4 interaction, stable integration of the pol α-primase complex into the replisome might require additional

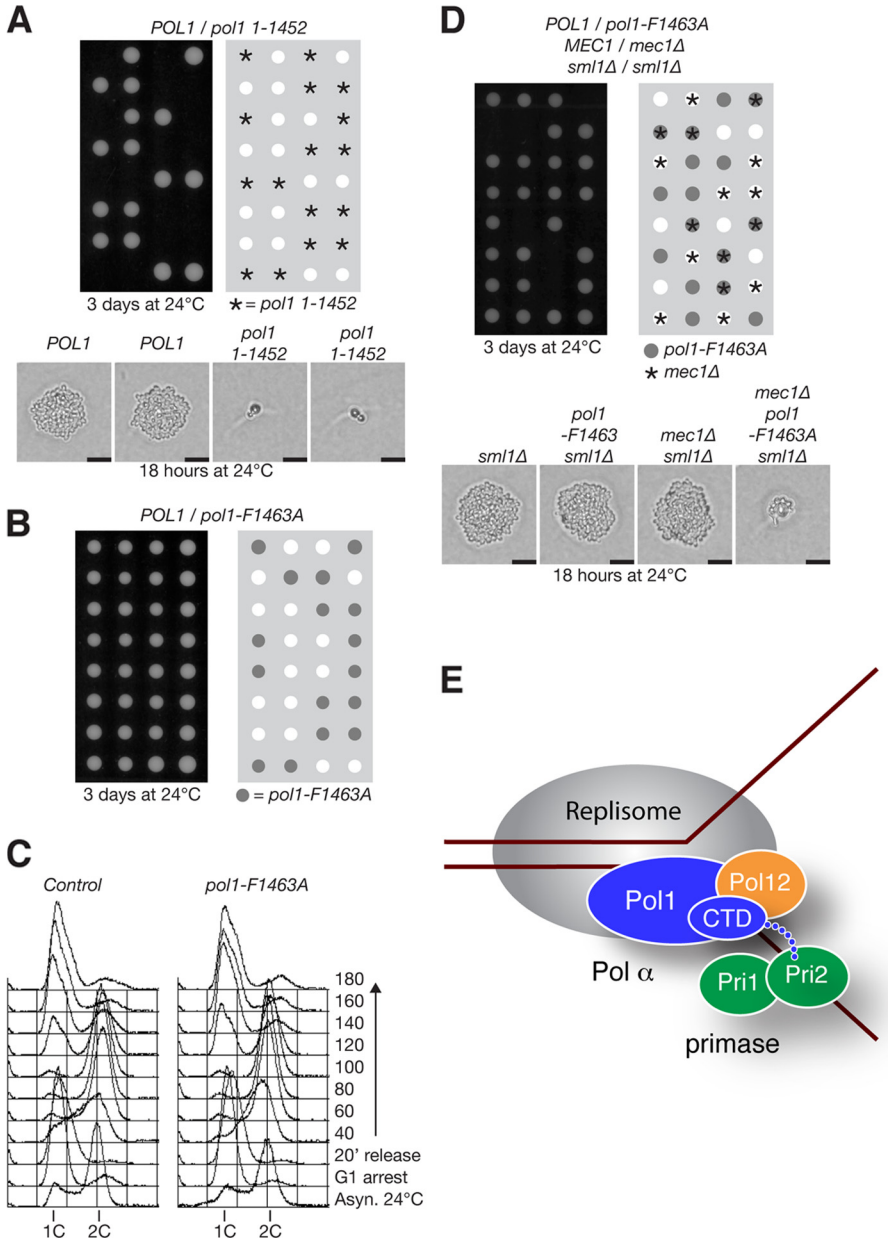

FIGURE 5. **Functional significance of tethering primase to the replisome in yeast cells.** *A*, tetrad analysis of the meiotic progeny of a diploid strain with one copy of wild-type *POL1* and one copy of *pol1 1–1452*. *Scale bars*, 20 μm. *B*, tetrad analysis of the meiotic progeny of a diploid strain with one copy of wild-type *POL1* and one copy of *pol1-F1463A*. *C*, control and *pol1-F1463A* synchronized in G_1 phase with mating pheromone and then allowed to progress through a complete round of the cell cycle. Mating pheromone was added again at the 60-min time point so that cells completing cell division would not enter a second cell cycle. DNA content was monitored throughout the experiment by flow cytometry. *D*, tetrad analysis of the meiotic progeny of a diploid strain with the indicated genotype. *Scale bars*, 20 μm. *E*, a model for primase recruitment to the eukaryotic replisome. A conserved motif in the C-terminal tail of the catalytic subunit of pol α tethers primase to the replication fork. The names are those of the *S. cerevisiae* proteins.

contacts with replisome components that are mediated by primase.

Our findings highlight the importance of tethering primase to the eukaryotic replisome during chromosome replication. The limited size of the primase-binding epitope of pol α suggests that it might be possible to design small molecule inhibitors that disrupt the pol α-primase interface. As loss of the interaction of primase with pol α makes cells dependent upon the S phase checkpoint response, such inhibitors might have therapeutic potential in the treatment of human cancers with inherent defects in chromosome replication.

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