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Replication Protein A-Directed Unloading of PCNA by the Ctf18 Cohesion Establishment Complex

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The replication clamp PCNA is loaded around DNA by replication factor C (RFC) and functions in DNA replication and repair. Regulated unloading of PCNA during the progression and termination of DNA replication may require additional factors. Here we show that a *S. cerevisiae* complex required for the establishment of sister chromatid cohesion functions as an efficient unloader of PCNA. Unloading requires ATP hydrolysis. This seven-subunit Ctf18-RFC complex consists of the four small subunits of RFC, together with Ctf18, Dcc1, and Ctf8. Ctf18-RFC was also a weak loader of PCNA onto naked template-primer DNA. However, when the single-stranded DNA template was coated by the yeast single-stranded DNA binding protein replication protein A (RPA) but not by a mutant form of RPA or a heterologous single-stranded DNA binding protein, both binding of Ctf18-RFC to substrate DNA and loading of PCNA were strongly inhibited, and unloading predominated. Neither yeast RFC itself nor two other related clamp loaders, containing either Rad24 or Elg1, catalyzed significant unloading of PCNA. The Dcc1 and Ctf8 subunits of Ctf18-RFC, while required for establishing sister chromatid cohesion in vivo, did not function specifically in PCNA unloading in vitro, thereby separating the functionality of the Ctf18-RFC complex into two distinct paths.

The process of sister chromatid cohesion ensures that replicated chromosomes are distributed equally to progeny cells during cell division. Cohesion is mediated through a large ring-like structure, cohesin, which is deposited on the chromosomes in the late G1 phase of the cell cycle. Sister chromatid cohesion is established during S phase, presumably by the actual passage of the replication fork. Several models exist by which cohesin is proposed to interact with the duplicated chromosomes in order to mediate cohesion. These models are based either on the idea that one cohesin ring encircles both sister chromosomes or on the idea that cohesion is mediated by the interlocking of two cohesin rings, with each ring more peripherally associated with a daughter chromosome (reviewed in references 33, 34, and 48). If the first type of model is correct, the mere passage of the replication fork through a cohesin ring would invariably ensure that the two sister chromatids stay attached until mitosis. However, the disadvantage of this elegant solution to the chromosome sorting problem is that it may be problematic for the replication fork to pass through the estimated 30- to 40-nm hole of the cohesin ring. Recent studies have shown that cohesin is specifically redistributed along the chromosome to transcription termination sites in a transcription-dependent manner, suggesting that the actual transcription machinery may push the cohesin rings ahead of the transcription bubble (26). If this is caused by steric problems because of the size of the transcription apparatus, similar steric problems may also occur with passage of the replication fork. Failure to establish sister chromatid cohesion would result if fork passage were sterically restricted.

Therefore, it is reasonable to assume that a dedicated apparatus exists to mediate passage of the replication fork through the cohesin ring, and the successful establishment of cohesion depends on the intimate coordination between the establishment and replication machineries.

There are several links between DNA replication and the establishment of sister chromatid cohesion, although a model describing the mechanistic rationale for these links is still lacking. First, the Ctf7 acetyltransferase is required for the establishment of chromatid cohesion (20, 44, 46). Temperature-sensitive mutations in the essential *CTF7* gene are synthetic lethal with conditional mutations in *POL30* (PCNA), while *POL30* overexpression suppresses the temperature sensitivity of *CTF7* mutants (44). Second, mutations in the *POL2* gene for the leading-strand DNA polymerase ε show cohesion establishment defects (9). Third, the cohesion establishment protein Ctf4 has been identified as a DNA polymerase α-interacting protein and *CTF4* mutants show replication-related defects (12, 19). Fourth, cohesion establishment factors Ctf18, Dcc1, and Ctf8 form a complex with the four small subunits (Rfc2-5) of replication factor C (RFC) (30). A temperature-sensitive mutation in *RFC4* showed defects in the establishment of chromatid cohesion and resulted in premature sister chromatid separation (30).

RFC loads PCNA onto DNA in an ATP-dependent manner. This RFC-dependent loading is essential for all PCNA-related processes during DNA replication and repair (reviewed in reference 27). It is not entirely clear whether RFC can also efficiently unload PCNA from DNA, either during DNA replication or after completion of DNA replication or repair. During lagging-strand DNA replication, unloading of PCNA by RFC from one completed Okazaki fragment may provide the required PCNA for the next Okazaki fragment to be synthesized. However, proof for this model is still lacking. Re-

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modeling of the replication fork during cellular stress, DNA damage, or as a more regular aspect of replication progression may also depend on the temporary unloading of PCNA. Unloading of PCNA by RFC has been demonstrated in the human system. RFC mediates unloading of PCNA from fully replicated simian virus 40 chromatin (40). In model loading/unloading systems, human RFC has been shown to unload PCNA from template-primer DNA in an ATP-dependent reaction (6, 50). However, unloading efficiencies and rates, and therefore, physiological relevance of this type of unloading, have not been addressed. Curiously, in several unpublished studies, we have never been able to detect any significant PCNA unloading catalyzed by *Saccharomyces cerevisiae* RFC.

Several RFC-like complexes have recently been identified, all of which retain the four small subunits of RFC: the Rfc2-5 core. A complex of Rfc2-5 with *S. cerevisiae* Rad24 (Rad17 in human and *Schizosaccharomyces pombe*) functions in the DNA damage checkpoint, whereas the chromosome maintenance function of a third complex, consisting of Elg1 and Rfc2-5, has been less clearly defined (reviewed in reference 27). Finally, a complex consisting of the Rfc2-5 core, Ctf18, Dec1, and Ctf8 (Ctf18-RFC) is required for the establishment of sister chromatid cohesion in yeast (19, 30). This seven-subunit complex is conserved from yeast to mammals. In an effort to understand the function of this cohesion-related clamp loader, we have purified this complex and studied its biochemical properties. Surprisingly, we find that Ctf18-RFC efficiently unloads PCNA from DNA. Unloading requires the energy of ATP hydrolysis. We discuss our results in light of two recent reports that conclude that the human Ctf18-RFC is primarily a loader of PCNA (2, 41). Furthermore, we propose that regulated unloading of PCNA may serve an important function during the establishment of sister chromatid cohesion.

**MATERIALS AND METHODS**

**Strains, plasmids, DNA, and proteins.** *S. cerevisiae* strain BI2168 (MATa ura3-52 trpl-289 leu2-3,112 his3-112 prc1-407 pep4-3) was used for overexpression purposes. Complementation studies were carried out in strain Y06212 (muta his3-A11 leu2-3,112 met15-10 ura3-200 ctf18-A11)kanMX4) from Eurocarls. All genes to be expressed and purified were cloned into multicopy yeast shuttle vectors. The *C82* contained a frameshift mutation resulting in an 82-amino-acid polypeptide. A 2-kb plasmid containing a BbvC1 restriction endonuclease site (pUC19b1) was treated with either the BbvC1 or BbvC1 nickase (New England Biolabs) and reisolated by phenol extraction and ethanol precipitation to give nicked DNA.

**Protein expression and purification of clamp loaders.** Strain BI2168 was transformed with plasmid pBL422 (2 µM ori TRIP1 RFC2 RFC3 RFC4 RFC5) with the four small-subunit genes under control of the GAL1-10 promoter (14). This strain was transformed with plasmid pGL438 to produce the five-subunit Ctf18-RFC, with pBL441 and pBL440 to produce the seven-subunit Ctf18-RFC, and with pBL448 to produce Elg1-RFC. Cell growth, induction with galactose, and crude extract preparation by dry ice breakage of 150 g of yeast cell paste were carried out as described previously (4). The crude lysate was adjusted to 150 mM ammonium sulfate and stirred with 45 µg/ml of 10% Polymyx P for 15 min at 0°C to precipitate nucleic acids. After centrifugation at 39,000 × g for 45 min, protein pellets were resuspended and diluted in buffer A (50 mM HEPES-NaOH (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM EGTA, 0.05% Tween 20, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 3 mM dithiothreitol, 10 mM NaHSO4, 5 µM pepstatin A, 10 µM leupeptin, 2.5 mM benzamidine, 5 µg/ml chymostatin, 0.5 µM phenylmethylsulfonyl fluoride) to a conductivity equal to that of 150 mM NaCl.

Glutathione-Sepharose (5 ml; Amersham Pharmacia) equilibrated in A150 was washed with 20 volumes of 150 mM NaCl, and protein was eluted with buffer B (buffer A at pH 8.0 with 150 mM NaCl, 20 mM glutathione, and 0.05% amphoteries [pH = 3 to 10]) [Amersham Pharmacia] and no benzamidine or phenylmethylsulfonyl fluoride). Clamp loader containing fractions were identified by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and treated with PreScission protease on ice overnight (Amersham Pharmacia).

Protein fractions from the affinity column were diluted with C80 (buffer B, pH 7.5, without glutathione) to a conductivity equivalent to that of C100 before further purification. Ctf18-RFC and Elg1-RFC were further purified on a 1-m1 monosQ column, while Ctf18-RFC(5) was further purified on a 5-m1 heparin agarose column.

**PreScission cleavage.** Ctf8-DCcl heterodimer was overproduced from plasmid pBL446 in E. coli strain BL21(DE3)-RIL using a standard induction protocol at 20°C to improve complex solubility and purified using Ni-agarose chromatography. The protein was eluted with a 100 mM imidazole buffer and further purified by MonoS fast-performance liquid chromatography.

**Reconstitution of the seven-subunit Ctf18-RFC complex.** His6-Ctf8-DCcl (50 pmol) was incubated with 20 pmol of either Ctf18-RFC(5), Ctf18-AC18(5), or Rfc2-5 in 100 µl of buffer C500 containing 7 mM MgAc2 and 1 mM ATP where indicated for 1 h at 0°C. The reaction mixture was further incubated for 1 h at 4°C on a shaker with 100 µl of Ni2+-nitrilotriacetic acid (NTA) agarose (QIA-GEN), previously equilibrated in buffer C500 containing 20 mM imidazole. The Ni2+-NTA agarose, with bound protein, was washed three times with 200 µl of buffer C500 containing 20 mM imidazole before protein was eluted with 200 µl of buffer C500 containing 100 mM imidazole. The protein composition of the eluates was precipitated with 10% trichloroacetic acid and analyzed by 15% SDS-PAGE and Coomassie staining. No binding of clamp loader complexes to the Ni2+-NTA agarose was observed when the procedure was repeated in the absence of His6-Ctf8-DCcl.

**ATPase assays.** Assays (25 µl) in buffer H50 (30 mM HEPES-NaOH [pH 7.5], 0.5 mM EDTA, 10% glycerol, 10 mM magnesium acetate, 5 mM dithiothreitol, 0.1% amphoteries pH 10, 0.05% Nonidet P-40, 10 µg/ml bovine serum albumin (50 mM NaCl)) contained 100 µM [γ-32P]ATP, 10 nM [3H]2′-deoxyribonucleoside 5′-triphosphate, 200 nmol deoxyribonucleoside triphosphate, 100 µmol MgAc2, and 500 pmol of Ctf18-RFC. Reactions were incubated at 30°C, and after 2, 4, and 6 min, 5-µl aliquots were removed, quenched with 2.5 µl of 50 mM EDTA, 1% SDS, and 25 mM cold ATP and...
ADP, and processed for polyethyleneimine cellulose thin-layer chromatography and phosphorimager analysis. Linear initial rates were calculated from each time course.

Isolation of DNA-PCNA complexes. The standard 100-μl reaction mixture contained in buffer H$_2$ was as follows: 500 fmol of blueprints SKI$^+$ ssDNA primed by 10 evenly spaced 30-mer primers (5 pmol primer termini), 60 pmol of RPA if present, 6 pmol of PCNA (containing when present 0.5 pmol of $^{32}$P-PCNA as a radioactive tracer), 3 pmol of RFC or Ctf18-RFC, and 1 mM ATP, 5 min at room temperature. The mixture was filtered through a 2-ml Biogel A5m column equilibrated in buffer H$_2$, at room temperature. Four-drop fractions were collected; the void volume containing DNA-protein complexes eluted in fractions 9 to 12, and the unbound protein in fractions 16 to 22. After counting of the fractions, complexes were precipitated with an equal volume of 20% trichloroacetic acid and analyzed by SDS-PAGE and Coomasie staining and, after drying of the gel, by phosphorimaging analysis (see Fig. 3).

PCNA-nicked DNA complexes and PCNA-gapped DNA complexes used for our unloading studies were isolated similarly after loading $^{32}$P-PCNA (2.5 pmol) onto 500 fmol of nicked pUC19B1 DNA by 200 fmol of RFC and 1 mM ATP at 30°C for 5 min. Complex-containing fractions were identified with a Geiger counter and immediately used further in unloading assays.

PCNA unloading assays using magnetic beads. Streptavidin-coated magnetic beads were charged with a 286-mer ssDNA via a 5'-terminal biotin linkage and $^{32}$P-PCNA was loaded by RFC onto either RPA-coated deca-primed blueprints ssDNA, gapped blueprints DNA (90-nucleotide [nt] ssDNA gap), or nicked DNA, and the PCNA-DNA complexes were purified by BioGel A5m gel filtration as described above. Complexes in buffer UB were incubated with RFC or Ctf18-RFC and the indicated nucleotide, and after the indicated time, the reaction was stopped by adding 1% final glutaraldehyde. After 30 to 60 min on ice, loading buffer containing 0.2% SDS and 10 mM EDTA (final concentrations) was added, and the samples were electrophoresed at 1 V/cm for 16 h on a 1 to 1.5% agarose gel in 50 mM Tris-borate-EDTA buffer. The gel was dried and radioactivity quantitated on a phosphorimager (see Fig. 5 and 6). Migration positions of glutaraldehyde-cross-linked PCNA and Ctf18-RFC-PCNA were determined independently.

Rates of disappearance of the DNA-PCNA complex were calculated using standard first-order kinetics, and these rates were taken to represent rates of PCNA unloading.

Quantitation and visualization techniques. All SDS-PAGE gels were stained with colloidal Coomassie and photographed with a charge-coupled-device camera under linear response conditions. Bands were quantitated using NIH-image software. Gels stained with SYPRO orange were quantitated using a STORM phosphorimager, using the blue fluorescence mode, and ImageQuant software. Gels stained with SYPRO orange were quantitated using NIH-image and Canvas software.

RESULTS

Yeast clamp loaders have the Rfc2-5 core assembly in common. We overproduced the five-subunit Ctf18-RFC(5) complex in yeast from multicopy plasmids with each gene placed downstream of the galactoside-inducible GAL1-10 promoter. A cleavable GST tag was added to the CTF18 gene to aid in purification (see Materials and Methods). Following the glutathione affinity column, the GST tag was proteolytically cleaved and the remaining complex further purified by HPLC ion exchange chromatography. The seven-subunit Ctf18-RFC(5) complex was similarly purified from a system in which in addition the CTF8 and DCC1 genes were overexpressed. We also overexpressed and purified the five-subunit Elg1-RFC complex using the same methodology as was used to obtain Ctf18-RFC(5). A comparative electrophoretic analysis of these purified clamp loaders with those previously isolated, i.e., RFC and Rad24-RFC, showed that all clamp loaders have the Rfc2-5 core in common. All subunits unique to the individual clamp loaders are present at equal stoichiometry, within an error of 20%, with the Rfc2-5 core (Fig. 1). The Ctf8 subunit of Ctf18-RFC was the only exception. Based on staining of the SDS-polyacrylamide gel, this subunit appeared to be overrepresented in the complex at a ratio of 1.6:1 (Coomassie staining) or 1:4:1 (SYPRO orange staining; data not shown) compared to Rfc2-5.

The seven-subunit complex could also be reconstituted by mixing the Ctf18(5) complex, purified from yeast, with Ctf8-Dcc1 overproduced in E. coli. Because only Ctf8 contained a heptahistidine tag, complex formation was simply assessed by determining retention of Ctf18-RFC(5) onto a Ni-agarose column. Retention of Ctf18-RFC(5) on this column did not require ATP (Fig. 2, lane 5 versus lane 6). The four-subunit Rfc2-5 core complex failed to interact with Dcc1-Ctf8 (lane 8). Successful reconstitution required the C-terminal 82-amino-acid tail of Ctf18. A Ctf18 truncation mutant lacking this tail was not retained on the column (lane 7). In addition, we were unable to purify a seven-subunit complex from yeast when CTF18-ΔC82 replaced wild-type CTF18 in our overexpression system (data not shown). This lack of interaction was unlikely caused by misfolding of Ctf18-ΔC82, because the truncated complex was fully active in PCNA loading and unloading (data not shown).
Since the two Ctf18-containing complexes, Ctf18-RFC(5) and Ctf18-RFC(7), showed very similar biochemical properties with regard to PCNA loading and unloading, primarily those studies carried out with Ctf18-RFC(7) will be reported. This seven-subunit complex will be referred to as Ctf18-RFC.

RPA inhibits PCNA loading by Ctf18-RFC. Recently a human Ctf18-RFC complex was identified, and two studies showed that this complex can function, albeit inefficiently, as a loader of PCNA (2, 32, 41). Moreover, this loaded PCNA acts as a processivity factor for DNA Pol β, indicating that it is loaded in the proper orientation at the 3′-primer-template junction. This was surprising to us because we had consistently failed to observe evidence for a processive PCNA-Pol δ complex in a coupled replication assay with yeast Ctf18-RFC as the putative loader (data not shown). In this coupled assay, PCNA was loaded by the clamp loader of interest onto RPA-coated single-stranded DNA, and loading was assessed indirectly by measuring processive DNA synthesis by Pol β. However, such a coupled assay, because of its indirectness, did not allow us to determine whether PCNA had actually been loaded or not, since it could be present on the DNA in a form unable to interact with Pol δ.

In order to determine PCNA loading directly, we separated the products of the loading reaction on a gel filtration column in order to separate DNA-bound PCNA from other forms of PCNA, either free or in a complex with the respective clamp loader (Fig. 3A). A radiolabeled form of PCNA was used to facilitate detection (10). We observed virtually no loading of PCNA by Ctf18-RFC onto multiply primed ssDNA, which was coated with RPA, the eukaryotic ssDNA binding protein. Surprisingly, a reasonable loading efficiency was observed when we carried out the reaction on naked DNA substrate. This loading of PCNA was ATP dependent. The observation that Ctf18-RFC loaded PCNA only onto naked template-primer DNA was very surprising, because in contrast, PCNA loading by its normal partner, RFC, proceeded with much higher efficiency when the ssDNA was coated with RPA, as shown before in many studies (Fig. 3A) (11, 25, 47, 51). The isolated DNA-PCNA complex formed by Ctf18-RFC action was active for processive DNA replication upon addition of RPA and Pol δ, indicating that PCNA had been loaded properly for interaction with Pol δ (data not shown).

One possible explanation for these results may be that the PCNA-Ctf18-RFC complex is incapable of binding RPA-coated primed DNA. The deca-primed ssDNA was coated with RPA where indicated. Loading of 32P-PCNA by RFC (open symbols) or Ctf18-RFC (filled symbols) and ATP where indicated and BioGel-A5m gel filtration were as described in Materials and Methods (see inset for a scheme of the assay). The amount of PCNA (fmol) in each fraction is indicated. The dashed line shows background loading of PCNA by RFC or by Ctf18-RFC without ATP present (results were identical). (B) DNA was incubated with increasing levels of RPA at 30°C for 10 min prior to loading, and gel filtration was done as for (A). The void volume fractions (DNA-PCNA) were acid precipitated and analyzed by SDS-PAGE as described in Materials and Methods. The gel was stained with colloidal Coomassie (upper panel), dried, and subjected to phosphorimager analysis (lower panel). Saturation binding of the ssDNA was achieved with ~45 pmol of RPA (22). The open arrow to the right indicates a proteolytic fragment of RPA. It migrates close to, but is distinct from, PCNA.
TABLE 1. Specificity parameters for PCNA loading

<table>
<thead>
<tr>
<th>Condition</th>
<th>SSB</th>
<th>Loader</th>
<th>Amt of PCNA loaded (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deca-primed SKII+ ssDNA</td>
<td>None</td>
<td>RFC</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>RPA</td>
<td>RFC</td>
<td>1,240</td>
</tr>
<tr>
<td></td>
<td>RPA(1-ΔN)</td>
<td>RFC</td>
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</tr>
<tr>
<td></td>
<td>E. coli SSB</td>
<td>RFC</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>T4 gp32</td>
<td>RFC</td>
<td>980</td>
</tr>
<tr>
<td>No ATP</td>
<td>None</td>
<td>Ctf18-RFC</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>Ctf18-RFC</td>
<td>460</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>Ctf18-RFC</td>
<td>410⁺</td>
</tr>
<tr>
<td></td>
<td>RPA</td>
<td>Ctf18-RFC</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>RPA</td>
<td>Ctf18-RFC</td>
<td>30⁺</td>
</tr>
<tr>
<td></td>
<td>RPA(1-ΔN)</td>
<td>Ctf18-RFC</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>E. coli SSB</td>
<td>Ctf18-RFC</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>T4 gp32</td>
<td>Ctf18-RFC</td>
<td>320</td>
</tr>
<tr>
<td>Nicked pUC19B1 DNA</td>
<td>None</td>
<td>RFC</td>
<td>360</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>Ctf18-RFC</td>
<td>7</td>
</tr>
</tbody>
</table>

* All loading reactions were as described in Materials and Methods with 5 pmol template-termini, 6 pmol of PCNA, and 3 pmol of RFC or Ctf18-RFC, except where indicated.

* Reaction mixtures contained 2.5 pmol of PCNA and 5 pmol of Ctf18-RFC.

3B). However, further coating of the ssDNA led to a dramatic decrease in Ctf18-RFC binding and an even more dramatic decrease in PCNA loading. At saturated RPA levels, PCNA loading was inhibited 82 to 96% compared to naked DNA (six determinations). In contrast, RFC-mediated loading of PCNA was strongly stimulated at all levels of RPA (Fig. 3A, data not shown).

We wondered whether the observed inhibition was specific for yeast RPA or whether any ssDNA binding protein would inhibit loading by Ctf18-RFC. The latter scenario would suggest that Ctf18-RFC preferred binding to a template-primer junction with adjacent naked ssDNA. Therefore, the loading reaction was carried out with several different ssDNA binding proteins, and the PCNA loaded was quantitated by gel filtration analysis, similarly to loading shown in Fig. 3A (Table 1). For each ssDNA binding protein, a plateau value of PCNA loading efficiency was reached upon saturation binding of the ssDNA, and addition of up to a twofold excess was without further effect. Surprisingly, while wild-type RPA strongly inhibited PCNA loading, a mutant RPA (RPA-1ΔN) lacking the N-terminal regulatory domain of Rpa1 (Δ1-180) allowed Ctf18-RFC binding to the DNA and completely supported PCNA loading even though this mutant coated the ssDNA as efficiently as wild-type RPA (Table 1, data not shown). The N-terminal 180-amino-acid domain of Rpa1 mediates many of the known interactions with other replication proteins including a direct interaction with Rfc4, a component of Ctf18-RFC (23). The implication of these experiments is that the interactions between the N terminus of Rpa1 and one or more subunits of Ctf18-RFC may serve to inhibit the PCNA loading function of this complex. Consistent with this hypothesis, we found that compared to RPA, two heterologous ssDNA binding proteins, *E. coli* SSB and bacteriophage T4 gp32, did not cause a strong inhibition of PCNA loading by Ctf18-RFC (Table 1). Control experiments showed that compared to naked DNA, all four ssDNA binding proteins stimulated PCNA loading by RFC, with RPA-1ΔN being the most stimulatory (Table 1).

We also determined whether Ctf18-RFC was able to load PCNA onto a nicked DNA substrate. Loading at a nick may be important for the proper function of several DNA metabolic pathways including base excision repair and nucleotide excision repair, and RFC was shown to be a proficient PCNA loader onto a nicked substrate (Table 1) (6, 37). In contrast, Ctf18-RFC completely failed to load PCNA onto a nick.

**ATPase activities of clamp loaders.** Table 2 summarizes the ATPase activities of all known clamp loaders, including the four-subunit Rfc2-5 core. The ATPase activity of RFC was stimulated about 20-fold when both PCNA and effector DNA were present in the assay, indicating efficient loading of PCNA. Similarly, the ATPase of Ctf18-RFC was also strongly stimulated by PCNA and DNA, but only when the ssDNA was naked or coated with RPA-1ΔN. In contrast, when the ssDNA was coated with RPA, the ATPase in the presence of cofactors was not significantly different from that without (Table 2). This lack of ATPase stimulation strongly suggests that Ctf18-RFC does not bind RPA-coated DNA and therefore does not load PCNA onto such a substrate.

Although different in quantitative detail, the ATPase activities of the other two clamp loaders, Rad24-RFC and Elg1-RFC, showed similar responses to the presence of PCNA and DNA as observed for the Rfc2-5 core. The ATPase activity of each of these three complexes was stimulated severalfold by addition of PCNA to the assay, indicating that these complexes do interact with PCNA (17). However, this ATPase was not further stimulated in the additional presence of template-primer DNA, suggesting that the bound PCNA was not loaded onto the DNA. Indeed, in PCNA-loading assays as described in the legend to Fig. 3A, neither Rad24-RFC nor Elg1-RFC showed evidence for stable loading of PCNA (data not shown) (28).

**TABLE 2. ATPase activities of clamp loaders**

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>RFC</th>
<th>Ctf18-RFC</th>
<th>Elg1-RFC</th>
<th>Rad24-RFC</th>
<th>Rfc2-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>−RPA</td>
<td>6</td>
<td>32</td>
<td>33</td>
<td>−RPA</td>
<td>−RPA</td>
</tr>
<tr>
<td>+RPA</td>
<td>4</td>
<td>35</td>
<td>4</td>
<td>+RPA</td>
<td>+RPA</td>
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<tr>
<td>PCNA</td>
<td>12</td>
<td>30</td>
<td>29</td>
<td>21</td>
<td>23</td>
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<tr>
<td>DNA</td>
<td>90</td>
<td>89</td>
<td>92</td>
<td>3</td>
<td>9</td>
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<tr>
<td>DNA + PCNA</td>
<td>95</td>
<td>138</td>
<td>142</td>
<td>19</td>
<td>25</td>
</tr>
</tbody>
</table>

* Turnover numbers (ADP formed/min/RFC) are given. Standard errors are 5%. See Materials and Methods for details.

* Data for Rad24-RFC from reference 28.
Unloading of PCNA by Ctf18-RFC. Rather than being a loader, we reasoned that Ctf18-RFC might actually be a PCNA unloader. To test this hypothesis, we developed a rapid and convenient assay for PCNA unloading. Radiolabeled PCNA was loaded by RFC onto RPA-coated template-primer DNA attached to magnetic beads, and RFC was removed by washing of the beads (17). PCNA unloading was determined in a subsequent assay as loss of bead-associated radioactivity (Fig. 4A).

PCNA unloading by Ctf18-RFC was observed in a reaction that required hydrolysis of ATP, since ATPyS was inactive (Fig. 4B). Unloading did not require the Dcc1 and Ctf8 subunits of the seven-subunit complex, because a five-subunit Ctf18-RFC complex was also proficient in unloading PCNA (Fig. 4D). Unloading did not require the Dcc1 and Ctf8 subunits, as well as in PCNA loading (data not shown).

However, an intact ATP-binding domain in Ctf18 was required for activity. We mutated the invariant lysine in the Walker A motif of this ATP-binding domain to glutamic acid. Ctf18-K189E, which was inactive in subsequent unloading reaction. Ctf18-RFC unloaded PCNA (Fig. 2). Furthermore, a C-terminal truncation mutant of Ctf18 abolished all interaction with Dcc1 (Fig. 2). The five-subunit Ctf18-ΔC82-RFC complex was also proficient in PCNA unloading, as well as in PCNA loading (data not shown).

Defects in the establishment of sister chromatid cohesion are associated with sensitivity to the topoisomerase I drug camptothecin (35). When assayed in a yeast ctf18 deletion strain, ctf18-K189E failed to rescue the camptothecin sensitivity of the deletion mutant (Fig. 4D). The C-terminal truncation ctf18-ΔC82 mutant also failed to rescue camptothecin sensitivity of the deletion strain, suggesting that the chromatin establishment function of the Dcc1 and Ctf8 subunits requires their participation in the seven-subunit Ctf18-RFC complex.

None of the other clamp loaders was able to unload PCNA. Addition of RFC and ATP or ATPyS in the assay merely resulted in a reloading of the 10 to 20% PCNA that freely slid off the DNA during the assay period (Fig. 4E). Neither the four-subunit Rfc2-5 core complex nor the checkpoint clamp loader Rad24-RFC was able to unload PCNA. A fourth putative clamp loader, Elg1-RFC, was also inactive in unloading PCNA. In addition, in an experiment identical to that shown in Fig. 3A, loading of PCNA by Elg1-RFC was also not observed (data not shown). This complex also failed to load the DNA damage checkpoint clamp Rad17/Mec3/Ddc1 onto primed DNA, under conditions that gave efficient loading by Rad24-RFC, leaving it currently as the only clamp loader with no specific biochemical function (data not shown) (29).

In order to test the DNA substrate preference for PCNA unloading by Ctf18-RFC, we used an assay in which radiolabeled PCNA encircling DNA was quantitated by glutaraldehyde cross-linking, followed by agarose gel electrophoresis and phosphorimager analysis (Fig. 5A). This assay was carried out at 20°C, because initial assays showed that unloading proceeded too rapidly at 30°C to be measured reliably by this assay. Control experiments showed that addition of glutaraldehyde promptly inactivated the loader, with the reaction being complete within 10 s. First, we assessed unloading from circular, double-stranded DNA containing a 90-nt ssDNA gap. PCNA was loaded onto the gapped DNA by RFC, and the DNA-bound PCNA was purified by gel filtration and used in a subsequent unloading reaction. Ctf18-RFC unloaded PCNA from the gapped DNA, and this unloading required a hydrolyzable form of ATP, since ATPyS-mediated unloading was inefficient (Fig. 5B, lane 5 versus lane 6).

The unloaded PCNA was released in a complex with Ctf18-RFC; this complex was efficiently cross-linked by glutaraldehyde and migrated at a unique position in the agarose gel.
Unloading by Ctf18-RFC was rapid, being complete after 1 min (Fig. 5C). Unloading also appeared to be stoichiometric rather than catalytic; an excess of loader over PCNA was required to completely unload the clamp (Fig. 5C). Although one explanation of these results could be that the act of PCNA unloading inactivates Ctf18-RFC, we think that based upon inhibition data presented below, lack of turnover is caused by the extremely slow PCNA product dissociation.

Surprisingly, under these same reaction conditions, yeast RFC failed to significantly unload PCNA even though several groups have reported that human RFC can unload PCNA in an ATP-dependent reaction (6, 40, 50). Since most of the unloading assays reported by these groups were carried out with nicked plasmid DNA, we repeated our unloading studies with nicked DNA plasmids. During prolonged incubation with substoichiometric concentrations of RFC, multiple PCNA trimers are loaded onto nicked plasmids because rapid turnover of RFC occurs. Consequently, the PCNA-DNA complexes isolated by gel filtration are virtually free of RFC (data not shown). When this isolated complex was incubated with ATP and an excess of Ctf18-RFC, rapid unloading was detected (Fig. 6A). However, when the complex was incubated with ATP and RFC, we merely observed a reloading of the small fraction of PCNA that had spontaneously dissociated from the DNA during the complex isolation procedure. Mere incubation of the isolated complex with or without ATP resulted in a very slow spontaneous dissociation of PCNA with an estimated half-life of 40 to 60 min at 20°C (Fig. 6A and data not shown, respectively). Similar unloading assays with RFC were also carried out at very low DNA-PCNA concentrations, since unloading might be favored under these conditions (see Discussion). Even at the lowest concentration practical (0.3 nM DNA-PCNA), RFC still was more proficient in reloading spontaneously dissociated PCNA than in active unloading (Fig. 6B, experiment 2). Unloading by Ctf18-RFC was still proficient at these low concentrations (experiment 1).

The rates of PCNA unloading from different DNA substrates by Ctf18-RFC were determined. At 3 nM DNA-PCNA, unloading from a DNA nick was rapid, with a half-time of unloading of ~20 s at 20°C (Fig. 6A). In contrast, unloading from RPA-coated primed ssDNA was much slower, with a half-time of ~3 min (Fig. 6C, experiment 5). The apparent stoichiometric nature of PCNA unloading by Ctf18-RFC, as suggested by the data in Fig. 5C, indicates that Ctf18-RFC forms an unusually stable complex with PCNA. Possibly, the
nature of the unloading reaction itself proceeded to yield PCNA–Ctf18-RFC as a very stable complex, thereby effectively inactivating Ctf18-RFC. This complex was detected as an uniquely migrating band by agarose gel electrophoresis (Fig. 5B and 6A). Alternatively, this type of stable complex could also be formed simply by mixing Ctf18-RFC with PCNA without nucleotide. Indeed, when Ctf18-RFC was preincubated also be formed simply by mixing Ctf18-RFC with PCNA without nucleotide. Indeed, when Ctf18-RFC was preincubated with a twofold molar excess of PCNA prior to its addition to an unloading assay, the observed rates of unloading were substantially decreased. Half-times of unloading increased from 20 s to \( \sim 10 \) min for nicked DNA and from 3 min to \( > 30 \) min for RPA-coated primed DNA (Fig. 6B, experiment 4, and Fig. 6C, experiment 6, respectively). Inhibition was not alleviated by increasing the temperature of the assay, from 20°C to 30°C, or by increasing salt to 100 mM NaCl (data not shown).

**DISCUSSION**

**Comparison between yeast and human clamp loaders.** During our investigation of the yeast Ctf18-RFC complex, two biochemical studies of the human complex were published that require a more in-depth comparative discussion (2, 41). In addition, earlier work had indicated that human RFC itself may function as an unloader of PCNA (6, 40, 50). To put those data in the context of the present study, we will first address the clamp unloading activities of RFC and then the activities of Ctf18-RFC.

The kinetic mechanism of PCNA loading by yeast RFC has been studied in detail and shown to follow a multistep ordered pathway with each step fueled by binding of ATP (17, 18). Initially, RFC forms a complex with PCNA; this PCNA-RFC complex then binds template-primer DNA. Hydrolysis of the bound ATP mediates release of RFC from the DNA-PCNA complex. All of these steps are proposed to take place on a msec time scale. However, the observation that RFC exhibits a high level of steady-state ATPase activity in the presence of PCNA and template-primer DNA is an indication that RFC can interact with the DNA-PCNA complex in one or more reactions that turn over ATP, with unloading of PCNA likely being one of them. Assuming that three ATPs are consumed during loading and three during unloading, yeast RFC would cycle through a loading/unloading cycle once every 3 to 4 s (Table 2) (39). If this is the case, why then do we consistently fail to observe unloading of PCNA by yeast RFC while unloading by human RFC can be readily demonstrated?

Both loading and unloading of PCNA catalyzed by RFC and ATP can be represented by the following two irreversible reactions:

\[
\text{PCNA} + \text{DNA} + \text{ATP} \rightarrow \text{DNA-PCNA} + \text{ADP} \tag{1}
\]

\[
\text{DNA-PCNA} + \text{ATP} \rightarrow \text{PCNA} + \text{DNA} + \text{ADP} \tag{2}
\]

If ATP is present at saturation, the rate of loading is a function of the concentrations of three variables, PCNA, DNA, and RFC, while unloading rates depend on the concentrations of only two variables: the DNA-PCNA complex and RFC. An equilibrium is reached when loading and unloading rates are equal. However, this is not an equilibrium in the sense of a classical enzymatic reaction, because each independent irreversible direction is driven by ATP hydrolysis. Therefore, the equilibrium may be different for each species of RFC, depending on how competent this RFC is in loading and unloading, respectively. At equilibrium, for any given RFC species, the fraction of DNA-PCNA complex over total PCNA should largely be a function of the PCNA, the DNA, and the DNA-PCNA concentrations (equations 1 and 2).

This equilibrium should shift towards unloading at very low DNA-PCNA concentrations, because the reverse reaction, i.e., reloading, should slow down substantially when the concentrations of the substrates, PCNA and DNA, are below their \( K_d \) values for binding. These \( K_d \) values are 1.3 nM and 5 nM, respectively, for RFC binding to ATP and PCNA-RFC binding to DNA (17). Yet even at a starting concentration of 0.3 nM DNA-PCNA in the unloading assay, reloading by RFC of free PCNA that was either generated by spontaneous dissociation or by unloading was the predominant reaction (Fig. 6B, experiment 2). We also did not obtain evidence for PCNA unloading by RFC when the DNA-PCNA substrate was gapped DNA or primed ssDNA (Fig. 4E and data not shown). We have carried out these studies with wild-type RFC and with a truncated form of RFC, RFC-1ΔN, which removes an N-terminal 272-amino-acid domain of Rfc1 that is somewhat inhibitory to PCNA loading (Fig. 1) (16, 38, 49). Both forms of RFC exhibited the same behavior. Therefore, while the large stimulation of the steady-state ATPase activity of RFC by PCNA and effector DNA suggests that RFC catalyzes cycles of PCNA loading and unloading, our failure to measure significant unloading per se indicates that the efficiency of loading far outweighs the efficiency of unloading.

**PCNA loading and unloading by Ctf18-RFC.** While our results strongly indicate that under physiological conditions Ctf18-RFC is an unloader rather than a loader of PCNA, the studies of the human Ctf18-RFC complex are in part at odds with our conclusions. Both Hurwitz and colleagues and Tsurimoto and colleagues have studied the PCNA loading properties of the human five-subunit enzyme, and the Hurwitz group also studied the seven-subunit enzyme (2, 41). Similar to our studies, no fundamental biochemical differences were detected between the five-subunit and the seven-subunit human enzymes. However, both groups detected significant loading of PCNA onto primed DNA in a reaction which was not appreciably inhibited by addition of human RPA. In our system, subsaturating RPA actually stimulated PCNA loading, and it was only upon complete coating of the ssDNA with RPA that the strong and specific inhibition by RPA could be demonstrated (Fig. 3B).

However, in actuality our data on the yeast cohesion clamp loader may not be as much in disagreement with those on the human enzyme as they initially appear to be. It is evident from our studies that yeast Ctf18-RFC cycles in an equilibrium between PCNA loading and PCNA unloading and that this equilibrium is shifted strongly towards unloading in the presence of RPA. A similar equilibrium may also exist for the human complex; however, this equilibrium may be less severely disturbed by the presence of human RPA. It will be interesting to see whether the human Ctf18-RFC complex also unloads PCNA and how RPA controls these two opposing processes.

**Role of RPA in regulating Ctf18-RFC function.** Yeast Ctf18-RFC is both a clamp loader and a clamp unloader, and each direction of this PCNA cycle is driven by the energy of ATP.
PCNA + DNA₁ + ATP → DNA₁-PCNA + ADP (3)
DNA₂-PCNA + ATP → PCNA + DNA₂ + ADP (4)

where DNA₁ is naked template-primer DNA and DNA₂ is nicked DNA or RPA-coated template-primer DNA.

Only template-primer DNA with an adjacent naked ssDNA region or coated with a mutant RPA or heterologous ssDNA binding protein is a proficient substrate for PCNA loading by Ctf18-RFC in vitro. Generally, it is assumed that any ssDNA in the cell is bound up with RPA, which should inhibit the loading activity of Ctf18-RFC. Since Ctf18-RFC is also unable to load PCNA at a nick (Table 1), it appears that so far there is no demonstrated substrate for PCNA loading. On the other hand, Ctf18-RFC efficiently unloads PCNA from nicked and gapped substrates and somewhat less efficiently from RPA-coated primed ssDNA (Fig. 5 and 6).

Surprisingly, however, we found that the inhibition by RPA is not a mere consequence of coating the ssDNA. Whereas coating by E. coli SSB and phage T4 gp32 protein caused only a minor inhibition of PCNA loading, coating by yeast RPA-1ΔN actually stimulated PCNA loading by Ctf18-RFC (108 to 150% in three determinations) (Table 1). The N-terminal domain of Rpa1, lacking in RPA-1ΔN, while not required for ssDNA binding, has been implicated in protein-protein interactions with the Rfc4 subunit of Ctf18-RFC (23). Mutations in the rfc4-2 mutation. Singly, these mutants show both replication defects and DNA damage checkpoint defects; the possible defects in chromatic cohesion were not investigated (23). Currently, it is not clear how the Rpa1-Rfc4 interaction could function to inhibit Ctf18-RFC while stimulating RFC, and additional specific interactions may exist. Another relevant question is whether yeast has a pathway that relieves this inhibition by a modification of Rpa1 and/or Ctf18-RFC? So far no in vivo modification of the Ctf18 subunit has been reported. Although Rpa1 does not appear to be phosphorylated during normal DNA replication in the absence of stress or DNA damage, phosphorylation of the Rpa2 subunit persists throughout the S phase of the cell cycle (8).

A proposed role for PCNA unloading in the establishment of sister chromatic cohesion. Previous proposals for the function of Ctf18-RFC have focused on its PCNA-loading function and the potential of this loader to target establishment-specific protein(s) to the newly loaded PCNA onto replicating DNA (2, 9, 30). Specific localization to replicating DNA at cohesin binding sites may be imparted by interactions between Ctf18-RFC and cohesin (2). Since PCNA is loaded by Ctf18-RFC in the same orientation and at the same 3’-DNA structure as it is by RFC, localization of these putative proteins cannot just depend on PCNA binding, or else loading by RFC would suffice; further specificity would have to be imparted by interactions with Ctf18-RFC. Currently, it is not known which factor is supposed to be targeted to this newly loaded PCNA. One function of this factor could be to mediate bypass of the cohesin ring past the replication fork (Fig. 7). While it is still controversial whether the association of cohesin with chromosomes is peripheral in nature or whether the cohesin ring actually encircles the chromosome, as shown in Fig. 7, in both models restructuring of the fork and/or the action of specific factors may be required to allow the replication fork to bypass the obstacle formed by the cohesin ring (reviewed in reference 33). However, if cohesin does encircle the replicating chromosome, an alternative model requiring partial fork disassembly is suggested by the necessity for the DNA replication apparatus to thread through the cohesin ring.

Cohesin is loaded around the chromatin as a large 30- to 40-nm-diameter ring during late G1. Subsequently, sister chromatid cohesion would be established simply by passage of the replication fork through the cohesin ring (reviewed in references 34 and 48) (Fig. 7). Support for this model is derived from the ability of the cohesin ring to slide along chromatin both before and after DNA replication (26). Moreover, the act of transcription relocates cohesin rings to intergenic regions of convergent transcription, where the transcription machineries collide. A persuasive reasoning for this transcription-dependent cohesin sliding is that the transcription machinery is
simply too large to pass through the cohesin ring without disturbing its position on the chromatin. However, the replication machinery may still be larger in size than that. Polypeptides known to be associated with the fork, the MCM helicase, three DNA polymerases and their associated proteins, the Okazaki fragment maturation machinery, and perhaps also the mismatch repair machinery, already make up a complex 3 to 5 MDa in size. Although the physical size of such a complex if globular would only be 12 to 15 nm in diameter and should therefore be able to fit through the cohesin ring, there are other considerations that greatly increase the physical size of the replisome.

In order to achieve coordinate replication of the leading and lagging strands, the lagging strand is proposed to fold back onto itself, forming an extruded loop, the trombone model (42). The core of the small T4 replisome already has an estimated size of ~20 nm, without even taking the looped-out lagging-strand DNA into consideration. Assuming that cellular replisomes also adopt a trombone model, it is evident that the much larger eukaryotic replisome with its associated ssDNA loop coated with RPA would be very unlikely to fit through the 30- to 40-nm-diameter hole of cohesin. However, if the trombone structure could be relaxed and a linear arrangement of the replication components achieved, the replication machinery could easily pass through the cohesin ring. Since PCNA rings situated on both the leading and the lagging strand are central structural organizing elements of the replisome, unloading of PCNA by Ctf18-RFC from only one of the strands would be expected to cause a collapse of the trombone superstructure, without necessarily displacing the DNA polymerases. Continued replication would mediate passage of the fork through the cohesin ring. After replicational passage through the cohesin ring is completed, reloading of PCNA would reestablish the superstructure of the replisome. This reloading could either be accomplished by the Ctf18-RFC complex that still has the PCNA bound to it, at novel DNA structures, or at normal template-primer locations free of RPA or, alternatively, by normal RFC.

_CTF8_ deletion mutants and similarly _CTF8_ and _DCC1_ mutants show synthetic lethality not only with other mutants in cohesion and chromosome segregation but also with mutations in the lagging-strand DNA replication apparatus (45). Cohesion establishment gene _CTF7_ shows synthetic lethality with_ POL30_ (PCNA) mutations, and overexpression of PCNA suppresses the temperature sensitivity of a _ctf7_ mutant (44). Ctf7 also interacts with the Ctf18-RFC complex (21). Since interaction was also detected with RFC and with the DNA damage checkpoint Rad24-RFC complex, it is likely that this interaction with Ctf7 is mediated through the small subunits of RFC common to all these complexes. Ctf7 is a lysine acetylase, although the importance of the acetylation of Ctf7 for establishment sister chromatid cohesion has been called into question (3, 20). Therefore, it remains unclear how the demonstrated genetic link between Ctf7 and PCNA can be put into a mechanistic framework to further clarify the role of Ctf18-RFC in the establishment of chromatid cohesion through loading/unloading of PCNA. It should be stressed that besides the Ctf18-RFC complex, establishment of sister chromatid cohesion depends on several other proteins whose functions can be envisaged at the replication fork. Among those, Chl1 is a putative DNA helicase and Ctf4 is a Pol α-interacting protein (12, 19, 31, 36, 43). Deletions in each of these genes show phenotypes very analogous to those in the _CTF18, DCC1_, and _CTF8_ subunit genes of the Ctf18-RFC clamp loader; failure to establish efficient sister chromatid cohesion in haploids and severe defects in chromosome disjunction during meiosis, with resulting poor spore viability in homozygous diploid mutants. Of these various establishment-associated proteins, only Chl1 has a putative PCNA-binding motif (NNHLLFQFF, 643 to 650 [conserved amino acids are in boldface]), although interaction of an establishment factor with PCNA through a nonconsensus interaction domain also remains a possibility (reviewed in reference 13). How each of these proteins, perhaps in a complex, could mediate the passage of the replication fork past or through the cohesin ring remains to be determined.

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REFERENCES


