Replication factor C (RF-C) and proliferating cell nuclear antigen (PCNA) assemble a complex, called sliding clamp, onto DNA. The clamp in turn loads DNA polymerases (pol) δ and ε to form the corresponding holoenzyme, which plays an essential role in replication of eukaryotic chromosomal DNA and in several DNA repair pathways. To determine the fate of RF-C after loading of PCNA onto DNA, we tagged the RF-C subunit p37 with a protein kinase A recognition motif, so that the recombinant five-subunit RF-C complex could be 32P-labeled and quantitatively detected in femtomolar amounts. Nonspecific binding of RF-C to DNA was minimized by replacing the p140 subunit with an N-terminally truncated p140 subunit lacking the previously identified nonspecific DNA binding domain. Neither of these modifications impaired the clamp loading activity of the recombinant RF-C. Using gel filtration techniques, we demonstrated that RF-C dissociated from the DNA after clamp loading or pol δ holoenzyme assembly, while PCNA or PCNAPol δ complex remained bound to DNA. PCNA catalytically loaded onto the template-primer was sufficient by itself to tether pol δ and stimulate DNA replication. The readaddition of RF-C to the isolated PCNA-DNA complex did not further stimulate pol δ DNA synthesis. We conclude that pol δ holoenzyme consists of PCNA and pol δ core and that RF-C serves only to load PCNA clamp.

A number of eukaryotic proteins have been found to have a dual role in chromosomal DNA replication and DNA repair. Pol1 δ, a replicative DNA polymerase (reviewed in Refs. 1 and 2), is also involved in various DNA repair pathways (3–6). Pol ε has been implicated in both DNA replication and DNA repair.
analyze the function of this protein in eukaryotic DNA synthesis. Two modifications of recombinant human RF-C have been designed that enable us to track the fate of RF-C after loading of PCNA onto DNA. First, the RF-C subunit p37 was tagged with a protein kinase A recognition motif (55), so that the recombinant five-subunit RF-C complex could be 32P-labeled and quantitatively detected in fentomolar amounts. Second, the RF-C complex was assembled with a truncated p140 subunit lacking the N-terminal DNA binding domain to reveal whether specific protein-protein interactions retain RF-C on DNA. The results presented below demonstrate that RF-C dissociates from PCNA after loading and is not required to tether pol δ to the clamp.

MATERIALS AND METHODS

**Proteins and Nucleic Acid**—Purification procedures for calf thymus pol δ (56), topoisomerase I (57), recombinant human PCNA and PCNA-ph (58), recombinant human pol ε-primase (59), recombinant human RF-A (60), recombinant SV40 T antigen (61), and Escherichia coli SSB (62) were described. The catalytic subunit of cAMP-dependent protein kinase from bovine heart muscle (PK) was from Sigma. Pwo DNA polymerase was from Boehringer Mannheim; other enzymes were from Promega. M13(mp19) ssDNA and singly primed ssDNA (53) were from Promega. M13(mp19) ssDNA and singly primed ssDNA (53) and SV40 origin-containing plasmid pUC-HS (61) were prepared as described (53).

**Baculovirus Encoding the Truncated p140 Subunit**—An N-terminal truncation of p140 was generated by polymerase chain reaction using truncated p140 was generated by polymerase chain reaction using and SV40 origin-containing plasmid pUC-HS (61) were prepared as described (53).

**Baculovirus Encoding the Phosphorylatable p37 Subunit**—An artificial sequence phosphorylatable by protein kinase A was introduced at the N-terminus of p37 using the same strategy as for PCNA-ph (58). The plasmid pVL1393/RFC37 was partially digested with NdeI and dephosphorylated, and the linearized DNA was isolated by agarose gel electrophoresis. Two oligonucleotides, dTATGGAGAACGAGCTTGCAGGG, and dRSKFRC140 as a template. The polymerase chain reaction product was digested by *Bam*H and EcoRI. The resulting 550-base pair fragment was ligated into pVL1393 plasmid, followed by the insertion of the 2056-base pair EcoRI/PstI fragment of RFC140. The resulting plasmid encoded the C-terminal p140 open reading frame, beginning with an engineered Met start codon immediately preceding the codon for Asp554 of the p140 subunit. Baculovirus derived from this plasmid was called v140-Δ.

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**Role of RF-C in Pol δ Holoenzyme Assembly**

**SV40 Polymerase Assay**—Reaction mixtures (final volume of 20 μl) contained 40 mM Tris-HCl (pH 7.5), 0.2 mg/ml BSA; 1 mM DTT; 10 mM MgCl₂; 1 mM ATP; a 50 μM concentration each of dATP, dGTP, and dTTP; 15 μM (α-32P)dCTP (5000 cpm/μmol); 100 ng of primed ssDNA; 100 ng of PCNA; 1.2 μg of *E. coli* SSB; 0.3 units of pol δ; and RF-C as indicated in the figures. Samples were incubated for 30 min at 37 °C, the reactions were terminated by adding 1 ml of ice-cold 10% (v/v) trichloroacetic acid, and acid-insoluble material was analyzed by scintillation counting. For product length analysis, the reactions were terminated by treating them with proteinase K (60 μg/ml) for 30 min at 37 °C in the presence of 1% (v/v) SDS and 20 mM EDTA (pH 8.0). The DNA was then precipitated with ethanol and analyzed by electrophoresis on an alkaline 1.5% agarose gel, followed by autoradiography.

**Phosphorylation of RF-C-ph and PCNA-ph**—For nonradioactive phosphorylation, reaction mixtures (20 μl) contained 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 12 mM MgCl₂, 0.1 mM NaCl, 20 μM ATP, 10 units of PK, and 800 fmol of RF-C-Δ-ph. For 32P labeling of proteins, reaction mixtures (10 μl) contained 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 12 mM MgCl₂, 0.1 mM NaCl, 15 units of PK, 10 μCi of [γ-32P]ATP (3000 Ci/μmol), and 4.2 pmol of PCNA-ph or 800 fmol of RF-C-ph. The mixture with PCNA-ph was incubated for 20 min at 30 °C, while the mixture with RF-C-ph was incubated for 5 min at 25 °C. 5 μl (2.1 pmol) of [γ-32P]RF-C-ph or [γ-32P]PCNA-ph and 1 μl (80 fmol) of [γ-32P]RF-C-ph were immediately used for gel filtration experiments. The rest of the reaction mixture was treated by the addition of SDS to a final concentration of 1% and heating at 95 °C. This material, containing known concentrations of PCNA-ph and RF-C-ph, was used to determine the specific radioactivity of these proteins.

**Gel Filtration of the [γ-32P]PCNA-ph/[γ-32P]RF-C-ph DNA Complexes**—The clamp-loading reaction mixture (100 μl) contained 40 mM Tris-HCl (pH 7.5), 0.2 mg/ml BSA; 1 mM DTT, 10 mM MgCl₂, 1 mM ATP, 186 fmol (400 ng) of primed ssDNA, 4.8 μg of *E. coli* SSB, 2.1 pmol of [32P]PCNA-ph, and 80 fmol of [32P]RF-C-ph. Reaction mixtures were incubated for 4 min at 37 °C and immediately loaded onto a Bio-Gel A-15 m column (0.5 × 10 cm) equilibrated in buffer G (40 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 1 mM DTT). Chromatography was performed at room temperature (23–25 °C) with buffer G, and three-drop fractions (~180 μl) were collected. Aliquots of these fractions and standard amounts of [32P]PCNA-ph and [32P]RF-C-ph were analyzed by SDS-PAGE and quantified using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

**Pol δ DNA Synthesis in Gel Filtration Fractions**—Reaction mixtures (final volume of 100 μl) contained 40 mM Tris-HCl (pH 7.5), 0.2 mg/ml BSA, 1 mM DTT, 10 mM MgCl₂, 1 mM ATP, 400 ng of primed ssDNA, 4.8 μg of *E. coli* SSB, 4.9 pmol of PCNA, and 80 fmol of RF-C-ph. Samples were incubated for 4 min at 37 °C and then loaded onto a Bio-Gel A-15 m column (0.5 × 10 cm) equilibrated in buffer G. Collected fractions were immediately placed on ice. 100-μl aliquots of these fractions were supplemented to give a final concentration of 0.2 μg/ml BSA; a 50 μM concentration each of dATP, dGTP, and dTTP, 15 μM (α-32P)dCTP (5000 cpm/μmol); and 1.2 units of pol δ. Reaction mixtures were incubated for 30 min at 37 °C, and products were analyzed by scintillation counting or alkaline agarose gel electrophoresis.

**Protein Concentration**—Protein concentration was determined by densitometric scanning of Coomassie-stained protein bands in denaturing polyacrylamide gels using Image Store 7500 (Ultra-Violet Products, Inc.). As protein standards, known amounts of BSA were loaded onto the same gel.
RESULTS

Rationale for the Recombinant Five-subunit RF-C Complex Modifications—We demonstrated previously that the N termini of three RF-C subunits, p40, p37, and p36, could be His-tagged without interfering detectably with assembly and activity of RF-C complexes (53, 63). Fusion of a His tag to the p40 subunit was found to be the most convenient for analytical purposes. The RF-C complex assembled with p40-his and purified using Ni-NTA resin and Mono Q chromatography could be resolved by SDS-PAGE into five distinct equimolar bands (Fig. 1A, lanes 1 and 3). Assembly of RF-C with p37-his or p36-his resulted in a minor analytical disadvantage, in that the small RF-C subunits overlapped on SDS-PAGE (Ref. 53 and data not shown). Therefore, we prepared p140-p40-his/p38/p37-p36 complex as a minimally modified RF-C (Fig. 1A, lane 1).

Radiolabeling of proteins bearing an artificial phosphorylatable tag has been shown to be an efficient technique for biochemical studies (58, 64). We designed an RF-C complex that could be 32P-labeled in vitro, as well as isolated by affinity chromatography. For this purpose, a protein kinase A recognition motif (55) was fused to the N terminus of the p37 subunit (p37-ph). By testing various combinations of baculoviruses, we found that the best yield of the five-subunit RF-C upon expression in insect cells and purification on Ni-NTA resin was obtained with full-length p140 subunit (panel A), RF-C complexes assembled with N-terminally deleted p140 subunit (panel B), RF-C complexes assembled with p37 wild type (filled circles), RF-C complexes assembled with p37-ph (open triangles).

"Materials and Methods"). As predicted from the data of Uhlmann et al. (49), p140-Δ efficiently assembled into stoichiometric RF-C-Δ and RF-C-Δ-ph complexes in the baculovirus expression system (Fig. 1A, lanes 3 and 4).

Characterization of RF-C Complexes—First, we tested whether p37-ph subunit assembled into the five-subunit complex is accessible to the protein kinase. Incubation of RF-C complexes with [γ-32P]ATP and PK resulted in efficient and specific labeling of p37-ph subunit (Fig. 1B). No phosphorylation was observed in any other RF-C subunit, confirming that the protein labeling was specific to the phosphorylation tag.

Next, we tested whether modification of small RF-C subunits might adversely affect the enzymatic activity of RF-C complexes. Analysis of RF-C-dependent pol δ holoenzyme DNA synthesis showed that the unphosphorylated tagged p37-ph in RF-C-ph did not impair the activity of RF-C (Fig. 2A, compare RF-C-ph with RF-C; Fig. 2B, compare RF-C-Δ-ph with RF-C-Δ). To test for possible effects of the phosphorylated tag on the activity of RF-C, 800 fmol of RF-C-Δ-ph was incubated with 10 units of PK in the presence of 20 μM [γ-32P]ATP at 25°C as described under “Materials and Methods.” Aliquots of labeled protein were analyzed by SDS-PAGE, and [32P]p37-ph was quantified in parallel with standard amounts of [γ-32P]ATP. Under these conditions, 60% of the RF-C molecules were phosphorylated in 12 min. Parallel reactions were performed with unlabeled ATP, and aliquots containing 5 fmol of phosphorylated RF-C-Δ-ph were assayed in the pol δ DNA synthesis reaction. No loss of activity of RF-C was detected despite 60% phosphorylation of the protein (data not shown), suggesting that phosphorylation of the tag on p37-ph did not affect RF-C activity.

In contrast, the N-terminal truncation of p140 did affect the activity of the RF-C complex. More than 100 fmol (30 ng) of RF-C or RF-C-ph was required for maximal pol δ DNA synthesis in the standard reaction mixture containing 100 ng of singly primed ssDNA (42 fmol of DNA molecules) (Fig. 2A). These
amounts of recombinant RF-C corresponded closely to the amounts of natural RF-C (20–60 ng) used for pol δ DNA synthesis on 100 ng of singly primed ssDNA (34, 36, 37). This requirement for RF-C in greater than stoichiometric amounts may indicate that RF-C binds to the DNA template not only at the template-primer junction but also elsewhere. The amount of SSB necessary for optimal support of pol δ holoenzyme DNA synthesis was determined empirically (1.2 μg of SSB per 100 ng of primed M13 ssDNA) and was expected to be sufficient to cover all of the template. On the other hand, it is difficult to exclude the possibility that some stretches of ssDNA or hairpins still remained accessible for RF-C under these conditions.

A distinctly different dependence of pol δ DNA synthesis on the concentration of RF-C was observed with RF-C and RF-C-ph (Fig. 2B). As little as 20 fmol of RF-C was enough to obtain maximal DNA synthesis on 42 fmol of DNA template. 2 fmol of RF-C resulted in 50% of maximal DNA synthesis, indicating that RF-C may cycle up to 10 times in loading PCNA onto DNA. This efficient DNA synthesis with substoichiometric amounts of RF-C-ph implies that RF-C is not an obligatory stable component of the pol δ holoenzyme for DNA synthesis. However, these data do not rule out the possibility that RF-C may transiently chaperone the tethering of pol δ core to the loaded PCNA molecule.

Both RF-C and RF-C-ph were tested in an SV40 diploymerase replication system, which included SV40 origin-containing plasmid DNA and the following purified proteins: T antigen, pol α-primase, topoisomerase I, RP-A, PCNA, pol δ, and RF-C as described under "Materials and Methods." DNA synthesis products were analyzed by denaturing agarose gel electrophoresis.

To test whether RF-C remains as a part of the sliding clamp on the DNA, we used the phosphorylated tagged PCNA-ph and RF-C-ph to measure the stoichiometry of both proteins bound to DNA after clamp loading. First, we analyzed the assembly of PCNA clamp on singly primed ssDNA using phosphorylatable RF-C derivative containing full-length p140 subunit. Protein-DNA complexes were assembled with 168 fmol of primed ssDNA, 2.1 pmol of [32P]PCNA-ph, and 500 fmol of [32P]RF-C-ph, and subjected to gel filtration through Bio-Gel A-15m, as described under "Materials and Methods." A, 12-μl aliquots of the eluted fractions were analyzed by SDS-PAGE. Radioactive PCNA-ph and p37-ph bands were separately quantified using a PhosphorImager. B, increasing amounts of [32P]RF-C-ph (open triangles) and [32P]PCNA-ph (filled circles) were analyzed by SDS-PAGE in parallel with samples of the eluted fractions to calculate the amounts of RF-C and PCNA in the gel filtration fractions. C, quantification of PCNA-ph (circles) and RF-C-ph (triangles) in the void volume fractions. Proteins were bound to singly primed DNA (filled symbols) or unprimed DNA (open symbols).

(Fig. 3, compare lanes 2–4 and 5–7).

Analysis of Protein-DNA Complexes Assembled with [32P]PCNA-ph and [32P]RF-C-ph—To test whether RF-C remains as a part of the sliding clamp on the DNA, we used the phosphorylated tagged PCNA-ph and RF-C-ph to measure the stoichiometry of both proteins bound to DNA after clamp loading. First, we analyzed the assembly of PCNA clamp on singly primed ssDNA using phosphorylatable RF-C derivative containing full-length p140 subunit. Protein-DNA complexes were assembled with 168 fmol of primed ssDNA, 2.1 pmol of [32P]PCNA-ph, and 500 fmol of [32P]RF-C-ph and subjected to gel filtration through Bio-Gel A-15m. Analysis of void volume fractions revealed that both RF-C and PCNA coeluted with DNA (Fig. 4A). Although PCNA-ph was routinely labeled for 20 min at 30 °C, while RF-C-ph was labeled for 5 min at 25 °C, the specific radioactivity of RF-C-ph was severalfold higher than that of PCNA-ph (Fig. 4B). Therefore, while the radioactive band of [32P]p37-ph was more intense than that of [32P]PCNA-ph (Fig. 4A), the molar amount of PCNA was greater than that of RF-C in the DNA-bound material (Fig. 4C). Assuming that 100% of DNA molecules were eluted in fractions 9–11, the stoichiometry of primed DNA/PCNA/RF-C in the void volume fractions was determined to be 1:0.96:0.40. When unprimed ssDNA covered with SSB was used in a control experiment, much less PCNA was recovered in the DNA-containing fractions, while the amount of DNA-bound RF-C was still significant (Fig. 4C). In this experiment, the molar ratio of unprimed...
DNA/PCNA/RF-C was determined to be 1:0.08:0.30. Additional control experiments showed that the appearance of RF-C-ph in the void volume fractions was dependent on DNA and therefore was not the result of protein aggregation (data not shown). Comparison between the experiments with primed versus unprimed DNA (Fig. 4C) suggested that the clamp specifically loaded onto the template-primer junction coexisted solely of PCNA, while some RF-C bound unspecifically to DNA independently of the clamp. However, this interpretation is open to criticism, and other possibilities cannot be excluded. In an attempt to decrease nonspecific binding of RF-C to DNA, gel filtration experiments were also carried out in buffer G containing 100 mM NaCl. However, again, a significant amount of RF-C-ph eluted in the void volume fractions (Fig. 5B). RF-C was previously shown to catalytically remove PCNA when used for the clamp loading reaction.

Complete removal of [32P]PCNA-ph from the assembled PCNA-DNA complex allowed us to test whether readaptation of RF-C to the gel filtration fractions had any effect on pol δ DNA synthesis. PCNA was loaded onto DNA by RF-C-Δ, and the PCNA-DNA complex was isolated by gel filtration as described above. Three 210-μl aliquots, each containing 100 fmol of PCNA-bound DNA were taken from the void volume fraction (0.7 ml), and supplemented with BSA, dNTPs, ATP, pol δ, and, where indicated, 200 fmol of RF-C or RF-C-Δ. DNA synthesis in the first 6 min of the reaction was identical regardless of whether RF-C, RF-C-Δ, or no protein was added back to the DNA synthesis reaction mixture (Fig. 6A). In another experiment, the void volume fraction containing PCNA-bound DNA was aliquoted in 50-μl reaction mixtures, supplemented with BSA, dNTPs, ATP, pol δ, and increasing amounts of RF-C or RF-C-Δ. RF-C-Δ added in amounts up to 300 fmol did not affect pol δ DNA synthesis, while RF-C inhibited the reaction about 2-fold (Fig. 6B).

RF-C was previously shown to catalytically remove PCNA clamp(s) from nicked circular DNA (45, 51). Since the readaptation of RF-C apparently inhibited DNA synthesis to some extent, we tested whether clamp unloading might contribute to the inhibitory effect of RF-C. [32P]PCNA-ph was loaded onto singly primed ssDNA (168 fmol) by RF-C-Δ (80 fmol), and PCNA-DNA complex was isolated by gel filtration. The two void volume fractions with the most radioactivity were combined, and 200-μl aliquots from this pool were supplemented with BSA, ATP, and 500 fmol of RF-C or RF-C-Δ. The reaction mixture was incubated for 10 min at 37 °C, cooled on ice, and immediately rechromatographed on a second Bio-Gel A-15 m column at 4 °C. As expected, PCNA-DNA complex partially dissociated during the second incubation, and PCNA was recovered in both the DNA-bound form (Fig. 6C, fractions 6–8) and the free form (fractions 11–19). Incubation of isolated PCNA-DNA complex with RF-C or RF-C-Δ increased dissociation of PCNA from DNA; however, RF-C-dependent unloading was not significant in comparison with spontaneous clamp disassembly (Fig. 6C).

These experiments provide evidence that RF-C plays no role in DNA synthesis, once PCNA clamp has been loaded and pol δ has bound to the PCNA-DNA complex. Loaded PCNA clamp does not seem to retain RF-C on DNA. Yet the possibility remains that pol δ core, when it is bound to the clamp, may stabilize RF-C within the complex. To test this possibility, protein-DNA complexes assembled with [32P]PCNA-ph, [32P]RF-C-Δ-ph, and pol δ were analyzed by gel filtration (Fig. 7A). Again, [32P]PCNA-ph, but not [32P]RF-C-Δ-ph, was recovered in the void volume fractions. The stoichiometry of primed DNA/PCNA/RF-C complex (1:1.53:0.02) was essentially the natural RF-C and isolated by gel filtration, RF-C was detected in DNA-bound form (35, 37, 45), but no conclusion could be made about the role of RF-C in DNA synthesis. PCNA loaded onto linear single-stranded DNA template via a double-stranded end was able to stimulate pol δ DNA synthesis in the absence of RF-C (67). However, this reaction was inefficient, requiring a large excess of PCNA and the presence of a macromolecular crowding agent. The addition of RF-C to such reaction mixtures greatly increased DNA synthesis, again leaving the role of RF-C in DNA synthesis unresolved (67). The use of [32P]RF-C-Δ-ph for the PCNA clamp loading reaction allowed us to isolate enzymatically formed PCNA-DNA complexes depleted of RF-C (Fig. 5B). These stable PCNA-DNA complexes were tested for pol δ holoenzyme DNA synthesis without any additional RF-C, crowding agents, or excess of unbound PCNA. The addition of dNTPs and pol δ core enzyme to the gel filtration fractions was sufficient to support DNA synthesis (Fig. 5C).

Analysis of RF-C Requirements for DNA Synthesis after Clamp Loading—When the PCNA clamp was assembled with

![Graphical representation of DNA synthesis](image-url)
The DNA products synthesized by pol δ holoenzyme with RF-C continuously present in the reaction mixture were compared with those synthesized after RF-C was removed by gel filtration (Fig. 8). When all components were added to the reaction mixtures prior to incubation at 37 °C, DNA synthesis was more efficient with RF-C-Δ than with RF-C (Fig. 8, lanes 3 and 4), in agreement with the results above (Fig. 2). In the next assay, the PCNA clamp was loaded by RF-C-Δ. PCNA-DNA complexes were separated from the clamp loader by gel filtration (see Fig. 4, A and B), and replication was carried out after the addition of pol δ and dNTPs (Fig. 8, lane 5, gel filtration 1). Products synthesized in the absence of RF-C and, therefore, within 1 lifetime of the PCNA clamp, were comparable in length with those synthesized using an excess of RF-C and PCNA (Fig. 8, compare lane 5 with lanes 3 and 4). Last, pol δ holoenzyme was assembled on the primed ssDNA in the presence of RF-C-Δ, protein-DNA complexes were isolated by gel filtration (see Fig. 7), and replication was carried out after the addition of dNTPs (Fig. 8, lane 6, gel filtration 2). Again, PCNA and pol δ recovered in complexes with DNA in the absence of RF-C were sufficient for DNA synthesis. The products synthesized after gel filtration 2 were shorter than after gel filtration 1 (compare lanes 5 and 6). The length of DNA products synthesized by pol δ holoenzyme was previously shown to be dependent on the concentration of pol δ core enzyme (34, 65, 68). Therefore, DNA synthesis was more efficient in the reaction mixture containing saturating amounts of pol δ core enzyme (gel filtration 1) than in the reaction mixture containing only those pol δ molecules recovered in the DNA-bound fractions (gel filtration 2).

**DISCUSSION**

RF-C catalytically loads PCNA onto primed ssDNA. PCNA is involved in multiple DNA metabolic processes (23–25), which require RF-C to load it onto DNA. Biochemical studies so far have not resolved conclusively whether RF-C and PCNA form a functional entity on DNA, or whether RF-C, like its prokaryotic functional counterparts E. coli γ-complex (69) and gp44-gp62 of bacteriophage T4 (70), dissociates once PCNA encircles the
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DNA. Unlike the γ-complex and gp44-gp62, RF-C displays a significant affinity for DNA. As a result, the concentrations of RF-C required for pol δ and pol ε DNA synthesis were greater than stoichiometric relative to the concentrations of template-primer (Refs. 34, 36, and 37; see also Fig. 2A). Moreover, when PCNA clamp or pol holoenzyme were assembled by RF-C on the DNA, and protein-DNA complexes were isolated by gel filtration, RF-C was recovered in DNA-bound fractions (Refs. 35–38 and 45; see also Fig. 4). On the other hand, recent studies demonstrated that one molecule of RF-C could load five molecules of PCNA onto nicked circular dsDNA (51), and one molecule of RF-C derivative containing truncated p140 subunit could load more than 20 molecules of PCNA onto DNA (49). These data suggested that RF-C may act catalytically in the clamp loading reaction, assembling the PCNA trimer on the DNA and then dissociating. However, due to the limitation of the nicked dsDNA template, the above studies (49, 51) could not address the question whether PCNA trimer by itself is able to tether pol δ to DNA and whether RF-C has any role in DNA synthesis after loading of the clamp.

In this study, we have detected and quantified complexes formed by RF-C, PCNA, and pol δ on primed M13 ssDNA. Complexes of known composition were then assayed in pol δ DNA synthesis on the same template. When the sliding clamp was assembled with 32P-labeled PCNA and RF-C, both proteins co-eluted with the template during gel filtration (Fig. 4). Quantification of complexes bound to primed and unprimed templates showed that 0.88 mol of PCNA and 0.10 mol of RF-C bound specifically to 1 mol of primer, suggesting that after clamp loading, RF-C dissociates, while PCNA remains tightly bound to the template-primer. This interpretation was confirmed by demonstrating that RF-CΔ could be completely removed from PCNA-DNA and PCNA-pol δ-DNA complexes by gel filtration (Figs. 5 and 7). Thus, protein-protein interactions did not retain RF-C stably in the loaded clamp or in pol δ holoenzyme. PCNA clamp by itself on DNA was entirely sufficient to tether pol δ and stimulate DNA replication (Fig. 8). No stimulatory effect on pol δ DNA synthesis was found when RF-C was added back to the isolated PCNA-DNA complex (Fig. 6). Furthermore, the readdition of increasing amounts of RF-C, but not RF-CΔ, even partially inhibited pol δ DNA synthesis (Fig. 6B). We did not observe efficient unloading of PCNA by RF-C from primed ssDNA (Fig. 6C). Under the same conditions, PCNA clamps were removed more efficiently from circular dsDNA than from primed ssDNA by both RF-C and RF-CΔ (data not shown). It is possible that the structure of the template modulates the clamp-unloading reaction, favoring removal of the clamp from continuous dsDNA and protecting the clamps positioned at a template-primer junction. Since RF-C did not perceptibly remove PCNA clamps from primed ssDNA under our experimental conditions, inhibition of DNA synthesis by RF-C (Fig. 6B) occurred by some other mechanism. One possibility is that RF-C inhibited pol δ DNA synthesis unspecifically by binding to DNA. Consistent with this idea, RF-CΔ, lacking the domain with strong affinity to DNA, caused no adverse effect on DNA synthesis. In summary, we conclude that PCNA by itself is sufficient to serve as a sliding clamp and that pol δ holoenzyme consists of PCNA and pol δ core.

RF-C and the Protein Competition for PCNA Clamp—Analysis of physical and functional interactions of PCNA with a growing number of proteins suggests that many of these proteins bind to PCNA in a competitive and mutually exclusive fashion (25, 33, 71). RF-C may be independent of such a competition, although it binds the same “front” side of PCNA bound by other proteins (32, 33). For example, p21 bound to PCNA had little effect on RF-C-catalyzed PCNA loading onto DNA (68, 72). DNA ligase I bound to PCNA and inhibited pol δ DNA synthesis but did not inhibit and even stimulated RF-C-catalyzed loading of PCNA onto DNA (73). We show in this report that pol δ had no influence on the RF-C-dependent assembly of the PCNA-DNA complex (compare Figs. 5 and 7). RF-C forms a very tight complex with PCNA in the presence of ATP (52), and the affinity of other proteins for PCNA might be not sufficient to compete with this RF-C-PCNA interaction. On the other hand, RF-C loses its affinity for PCNA once it is loaded on the DNA, and no additional protein seems to be necessary to displace RF-C from the clamp.

If neither PCNA nor pol δ retains RF-C on DNA (Figs. 4, 5, and 7), does this mean that RF-C dissociates from the site of DNA synthesis? The basic components required for DNA replication and nucleotide excision repair machineries have been determined (19, 74). However, whether and how these proteins are co-assembled to form efficient machineries is not understood. Partially purified multiprotein complexes containing pol α, pol δ, and RF-C have been reported (75, 76), but it is not known exactly how RF-C is integrated in these structures. A search for RF-C-interacting proteins may show whether RF-C is solely devoted to PCNA or is retained in a network of protein-protein interactions during DNA replication and repair.

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