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Replication Factor C Interacts with the C-terminal Side of Proliferating Cell Nuclear Antigen*

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Replication factor C (RF-C) is a heteropentameric protein essential for DNA replication and repair. It is a molecular matchmaker required for loading of proliferating cell nuclear antigen (PCNA) onto double-stranded DNA and, thus, for PCNA-dependent DNA elongation by DNA polymerases δ and ϵ . To elucidate the mode of RF-C binding to the PCNA clamp, modified forms of human PCNA were used that could be ^{32}P -labeled *in vitro* either at the C or the N terminus. Using a kinase protection assay, we show that the heteropentameric calf thymus RF-C was able to protect the C-terminal region but not the N-terminal region of human PCNA from phosphorylation, suggesting that RF-C interacts with the PCNA face at which the C termini are located (C-side). A similar protection profile was obtained with the recently identified PCNA binding region (residues 478–712), but not with the DNA binding region (residues 366–477), of the human RF-C large subunit (Fotedar, R., Mossi, R., Fitzgerald, P., Rousselle, T., Maga, G., Brickner, H., Messner, H., Khastilba, S., Hübscher, U., and Fotedar, A., (1996) *EMBO J.*, 15, 4423–4433). Furthermore, we show that the RF-C 36 kDa subunit of human RF-C could interact independently with the C-side of PCNA. The RF-C large subunit from a third species, namely *Drosophila melanogaster*, interacted similarly with the modified human PCNA, indicating that the interaction between RF-C and PCNA is conserved through eukaryotic evolution.

To perform processive, accurate, and rapid DNA synthesis, DNA polymerases (pols)¹ require the aid of a set of proteins called DNA replication accessory proteins. The three best known eukaryotic accessory proteins are proliferating cell nuclear antigen (PCNA) (1), replication factor C (RF-C) (2), and replication protein A (3). These proteins are present in all eukaryotic cells examined and are mandatory for the function of replicative pols. Their tasks include the recruitment of pols when needed, the facilitation of pol binding to the primer

terminus, an increase in pol processivity, prevention of non-productive binding of pols to single-stranded DNA, the release of pols after DNA synthesis, and communication between the pols and other replication and cell cycle regulating proteins (for a review, see Ref. 4).

RF-C is essential in DNA replication since it is necessary for loading of PCNA onto DNA and for subsequent DNA synthesis of the leading strand by pol δ (5). The gross structure of the RF-C complex appears to be conserved through eukaryotic evolution since RF-C isolated from either yeast or human is composed of one large and four small subunits ($M_r = 140, 40, 38, 37,$ and 36 in human (6, 7) and $M_r = 94.9, 39.7, 38.2, 36.2,$ and 39.9 in yeast (8)). RF-C 140 contains the DNA binding activity (9) that has been further localized between amino acids (aa) 366–477 (10–12) to a region termed RF-C box I (8). The exact roles of the small subunits (RF-C 40, RF-C 38, RF-C 37, and RF-C 36) have not been determined; however, they share seven conserved regions with the large subunit, termed RF-C boxes II–VIII (8). Since the PCNA binding region within RF-C 140 (10, 13) contains the conserved RF-C boxes II–IV, all five subunits could be expected to bind PCNA. To date, only RF-C 40 has been shown to interact with PCNA (14).

The eukaryotic clamp loader, RF-C, appears to be structurally and functionally very similar to its prokaryotic and viral counterparts. The *Escherichia coli* γ complex consists, in analogy to human RF-C, of five subunits ($\gamma, \delta, \delta', \chi, \psi$) that cooperate to load the β clamp (the PCNA counterpart) onto the DNA (15). In bacteriophage T4, the clamp loader consists of two subunits (g44/62p) that cooperate to load the g45p clamp (16). The amino acid sequence similarity between some of the subunits of these clamp loaders from prokaryotic, eukaryotic, and viral systems (8, 17) suggests that their basic mechanism of action may be similar.

While a high resolution model for the sliding clamp formed by RF-C and PCNA is not yet available, an increasingly detailed picture of events occurring at the prokaryotic replication complex is emerging (18). It has been shown that both the γ complex and the core pol interact with the β ring (15). Prior to β clamp assembly on DNA, β shows higher affinity to the γ complex than to the core pol, but once β has been assembled on DNA, the core develops a stronger affinity for the ring and outcompetes the γ complex. After placing the β ring on the DNA, the γ clamp loader can dissociate from the complex and is ready for loading other β dimers. Naktinis *et al.* (19) were able to assign the β binding function to the δ subunit of the γ complex. In the uncharged state, δ is buried in the γ complex, preventing its binding to β , but upon ATP binding, the complex undergoes a conformational change and thus exposing δ for interaction with β .

In view of the analogy with the γ complex, we sought to shed some light on the structure of the RF-C-PCNA clamp. Specifically, how does RF-C interact with PCNA in complex forma-

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¹ The abbreviations used are: pol(s), DNA polymerase(s); RF-C, replication factor C; PCNA, proliferating cell nuclear antigen; aa, amino acids; wt, wild type; ct, calf thymus; eph, C-terminally phosphorylatable; nph, N-terminally phosphorylatable; KPA, kinase protection assay; PMSF, phenylmethylsulfonyl fluoride; C-side, C-terminal side; DTT, dithiothreitol; GST, glutathione S-transferase.

tion? The crystal structure of PCNA shows a homotrimeric, ring-shaped molecule with an overall negative charge but with a central hole surrounded by positive charges through which DNA slides (20). This structure is very similar to the three dimensional structure of the β clamp despite the lack of significant sequence similarities (21). The C termini of the β subunit, as well as those of PCNA, protrude from structurally homologous faces (C-side) of the ring and are likely candidates for interactions with other proteins. The C-terminal amino acids of the *E. coli* β ring have been shown to be important for the interaction with both the clamp loader and the pol (15). Studies by Fukuda *et al.* (22) suggested that RF-C binds to the C-side of human PCNA, specifically to Asp41 and aa 254–257, although the terminal four amino acids are dispensable. Asp41 is partially exposed close to the C terminus in the yeast PCNA structure, making the hypothesis plausible. Care, however, must be taken when interpreting the effects of deletion mutations in PCNA since it has been shown that deletion of C-terminal residues can have adverse effects on the PCNA structure (23), and the trimeric structure of PCNA has been shown to be necessary for the interaction with RF-C (24).

To study the interaction of RF-C with PCNA in more detail, we used modified PCNA carrying artificial phosphorylation sites at either their N or C termini (called nphPCNA and cphPCNA, respectively) (5). In kinase protection assay experiments (KPA) (18), nphPCNA or cphPCNA is incubated with the protein to be assayed for interaction. If the protein binds to PCNA close to either end, the artificial phosphorylation site is expected to become less accessible to the kinase so that a decrease in phosphorylation is observed. In this paper, we show that the pentameric calf thymus RF-C complex protects the C termini, but not the N termini, of PCNA from phosphorylation. We therefore conclude that RF-C interacts with the C-side of the PCNA ring. The RF-C 140 homologue of *Drosophila melanogaster* is also able to protect the C termini but not the N termini of PCNA, showing, besides its interaction with the C-side of PCNA, that the molecular interaction between these two auxiliary factors is conserved through evolution. More precisely, the interaction between PCNA and RF-C 140 is due to the PCNA binding region, located in human RF-C 140 between aa 478 and 712 (10), which also protects only the C-side of the ring. RF-C 140 is not the only RF-C subunit involved in PCNA binding as we could show that RF-C 36 also interacts with PCNA and is able to protect modified PCNA from phosphorylation.

EXPERIMENTAL PROCEDURES

Chemicals—Radioactive-labeled nucleotides were purchased from Amersham Corp. All other reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland).

Buffers—The following buffers were used. Buffer A contained 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.01% (v/v) Nonidet P-40, 1 mM DTT, 10 mM NaHSO₃, 1 mM PMSF, and 1 μ g/ml each pepstatin, leupeptin, and aprotinin. Buffer B contained buffer A plus 10% (v/v) glycerol. Buffer C contained 20 mM Tris-HCl (pH 7.5), 300 mM NaCl, 0.4 mM PMSF, and 1 μ g/ml each aprotinin, pepstatin, and leupeptin. Buffer D contained 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.4 mM PMSF, 1 mM DTT, 1 mM EDTA, and 0.5% Nonidet P-40. Buffer G contained 40 mM Tris-HCl (pH 7.5) and 10 mM MgCl₂. Buffer H contained 40 mM Tris-HCl (pH 7.5), 30 mM imidazole-HCl, 10 mM MgCl₂, 50 mM NaCl, and 0.1% Nonidet P-40. Buffer I contained 40 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 0.2 mg/ml bovine serum albumin. Buffer L contained 10 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1 mM PMSF, and 1 mM DTT. KPA buffer contained 20 mM Tris-HCl (pH 7.5), 12 mM MgCl₂, 200 μ g/ml bovine serum albumin, and 1 mM DTT. SDS-gel loading buffer contained 60 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 2% (v/v) glycerol, 0.005% (w/v) bromophenol blue, and 2% (v/v) β -mercaptoethanol.

Nucleic Acid Substrates—Poly(dA)_{1000–1500} and oligo(dT)_{12–18} were

purchased from Sigma and Pharmacia, respectively. Poly(dA)/oligo(dT) (base ratio 10:1) was prepared as described (25). Singly primed M13 single-stranded DNA was prepared according to (26). Oligonucleotides for modification and sequencing of the PCNA gene were from Microsynth (Balgach, Switzerland). The plasmid pT7/hPCNA carrying the cDNA of human PCNA was kindly provided by B. Stillman (Cold Spring Harbor Laboratory, NY). The pET19b/p36His, pET5a/40K, and pET16/p128 expression vectors containing the cDNA of RF-C 36, RF-C 40, and RF-C 128, respectively, of human RF-C were a gift of J. Hurwitz (New York). The cDNAs encoding the fragments of the human RF-C 140 were from A. Fotedar (La Jolla, CA).

Enzymes and Proteins—Restriction and modifying enzymes were purchased from U. S. Biochemical Corp., New England Biolabs (NEB), and Boehringer Mannheim. The catalytic subunit of cAMP-dependent protein kinase from bovine heart muscle was from Sigma. Human wtPCNA was produced in *E. coli* using the plasmid pT7/hPCNA and purified as described previously (27). Calf thymus pol δ and pentameric RF-C were isolated as described (25, 28). The three fragments of human RF-C 140, A, B, and A + B were purified according to (10). The N-terminal phosphorylatable PCNA (nphPCNA) was produced and purified as described in Podust *et al.* (5). Cloning and isolation of RF-C 140 of *D. melanogaster* will be described elsewhere.² Polyclonal antibodies against PCNA were raised in chicken and affinity purified with protein A-Sepharose. The alkaline phosphatase-conjugated anti-chicken antibody was from Sigma.

Replication Assays—The poly(dA)/oligo(dT) assay (RF-C-independent assay) was carried out according to (25). DNA replication on singly primed M13 DNA (RF-C-dependent assay) was performed as described previously (26).

Construction of PCNA with a C-terminal Phosphorylation Site—A construct encoding PCNA with the C-terminal consensus phosphorylation sequence for cAMP-dependent protein kinase (Arg-Ala-Ser-Val-Ala) was generated by PCR in analogy to a similar strategy on the N terminus (5). The original pT7/hPCNA expression vector (27) was used as a template for PCR with a 28-mer T7-promoter primer sequence and a 49-mer lower primer (5'-GCG CGG ATC CTA TGC AAC ACT TGC TCT TCT AGA TCC TTC TTC ATC CTC G-3') designed to introduce the phosphorylation site at the C terminus. The PCR product was digested with *Nde*I and *Bam*HI and recloned into the pT7 expression vector. The resulting clone (called pT7/cphPCNA) was sequenced to confirm the absence of mutations.

Purification of cphPCNA—*E. coli* BL21(DE3)pLysS cells transformed with the pT7/cphPCNA expression construct were grown at 37 °C in 200 ml of LB medium to A₆₀₀ = 0.4 and induced with 0.4 mM isopropyl thiogalactoside. After 3 h, cells were harvested by centrifugation and lysed by freezing/thawing in 10 ml of buffer A containing 25 mM NaCl. The mix was sonicated and centrifuged for 30 min at 25,000 \times g. The supernatant was loaded onto a 4-ml Q-Sepharose column and equilibrated in buffer B containing 100 mM NaCl. The column was washed with the same buffer, and proteins were eluted with a 50-ml linear gradient from 0.1 to 0.7 M NaCl in buffer B. The fractions containing cphPCNA were pooled and loaded on a 1.5-ml phenyl-Sepharose column equilibrated with buffer A containing 1.2 M NaCl. The column was washed with buffer A without Nonidet P-40, and cphPCNA was eluted with a 20-ml linear gradient from 1.2 to 0 M NaCl in buffer A without Nonidet P-40. cphPCNA containing fractions were pooled, brought to 50% (v/v) glycerol in buffer A and stored at –20 °C until use.

Purification of RF-C 36—*E. coli* BL21(DE3) cells were transformed with the pET19b plasmid containing RF-C 36 sequence linked to a His tag at its N terminus. The cells were grown in 1 liter of LB medium to A₆₀₀ = 0.6, and isopropyl thiogalactoside was added to a final concentration of 0.4 mM. The cells were further grown for 5 h at 37 °C, harvested by centrifugation at 5,000 \times g for 20 min, and then lysed in buffer C by three passages through a French press. The lysate was centrifuged for 20 min at 18,000 \times g, and the pellet was solubilized in 10 ml of buffer C containing 6 M guanidinium-HCl. After ultracentrifugation at 35,000 \times g for 40 min, the supernatant was dialyzed sequentially against buffer C containing urea (8, 4, 2, and finally 0 M). Most of the protein precipitated during the last dialysis step but could be solubilized by resuspending it in buffer C containing 0.05% (v/v) Tween 20 and 0.01% (v/v) Nonidet P-40.

Subcloning, Expression, and Purification of the His-tagged RF-C 40—The gene encoding RF-C 40 was cut out of the original pET5a

² L. K. Gaur, B. L. Allen, and S. H. Hardin, submitted for publication.

vector with *Eco*RI and cloned into the pET28a⁺ vector, to gain RF-C 40 with a His tag at its N terminus. The resulting clone was sequenced to confirm the reading frame and the presence of the His tag. RF-C 40 was expressed and purified essentially as RF-C 36 except that buffer D was used (instead of buffer C). Soluble protein was obtained by serial dialysis against buffer D containing urea (4, 3, 2, 1.5, 1, 0.5, 0.25, and finally 0 M), 10% glycerol, and 1 μ g/ml each of aprotinin, pepstatin, and leupeptin.

Subcloning, Expression, and Purification of the His-tagged Human RF-C 140—A linker encoding a 6-His tag was cloned into the single *Bst*BI restriction site at the end of the gene encoding RF-C 140 in pET16b. This resulted in the expression of the human RF-C 140 His-tagged at its C terminus. RF-C 140 was expressed and purified as described above for RF-C 40.

KPA—250 ng (3 pmol) of cphPCNA were incubated with an excess of the protein to be tested and with 3 μ Ci [γ -³²P]ATP in a 10- μ l total volume of KPA buffer. After 2 min at 37 °C, the phosphorylation reaction was started by the addition of 0.75 units of cAMP-dependent protein kinase. The reaction mix was further incubated at 37 °C. After 1, 2, 5, and 10 min, 2- μ l aliquots were withdrawn, and the reactions were stopped with 8 μ l of 2% SDS. The samples were then analyzed by electrophoresis on a 12% SDS-polyacrylamide gel. Gels were fixed in 10% trichloroacetic acid, soaked in 10% acetic acid/12% methanol, dried, and autoradiographed. Gels with labeled products were exposed on a special screen for PhosphorImager (Molecular Dynamics, Inc.). After exposure, the screen was scanned into the PhosphorImager and quantitation was performed with the Image Quant program of Molecular Dynamics. To exclude false-positive results due to intrinsic ATPase activity, samples were tested for the presence of free phosphate by thin layer chromatography.

PCNA Pull-down Assay—80 pmol of the His-tagged proteins to be assayed were mixed with 20 μ l of Ni-NTA resin in buffer G. The mixture was incubated by rocking for 1 h at 4 °C. After 3 min of centrifugation at 2,000 \times g, the beads were washed 3 times with 1 ml of buffer G. Then 80 pmol of human wtPCNA were added to the beads in a 100- μ l total volume of buffer I. In order to allow binding of wtPCNA to the Ni-NTA coupled proteins, the suspension was incubated at room temperature for 1 h. After centrifugation as above, the resin was washed 4 times with buffer H, and the proteins were eluted by boiling the beads in 30 μ l of SDS-gel loading buffer. The proteins were separated by electrophoresis on a 12% SDS-polyacrylamide gel, and PCNA was detected by immunoblotting with polyclonal antibodies.

RESULTS

PCNA Containing an Artificial Phosphorylation Site Either at the C Terminus or at the N Terminus Can Be Phosphorylated *In Vitro* by cAMP-dependent Protein Kinase and Is Functionally Active in DNA Synthesis—The C termini of PCNA, as well as those of the β clamp in prokaryotes, protrude from one face of the ring, making them candidates for interaction with other proteins. The C termini of the β clamp in *E. coli* have been shown to be involved both in the recognition of β by the γ clamp loader and in the binding of the pol (15). Considering the structural and functional similarities of the prokaryotic and eukaryotic pol sliding clamps, we designed experiments to test the hypothesis that PCNA and RF-C interact in a way similar to that of the β clamp and its clamp loader. For this purpose, we used plasmids containing the human PCNA open reading frame with an artificial phosphorylation site for cAMP-dependent protein kinase fused either to its 3'- or 5'-end (5). Predictably, these modified forms of PCNA (called cphPCNA and nphPCNA, respectively) run slightly slower than unmodified PCNA on an SDS-polyacrylamide gel due to the presence of the phosphorylation sites (Fig. 1A, cphPCNA). Unlike the unmodified PCNA, cphPCNA and nphPCNA (see Ref. 5) can be efficiently phosphorylated *in vitro* (Fig. 1B, cphPCNA). Moreover, these PCNA forms have full activity in replication assays on primed single-stranded M13 DNA (RF-C-dependent assay; Fig. 1C, cphPCNA) and on poly(dA)/oligo(dT) (RF-C-independent assay, data not shown) (see also Ref. 5).

The Calf Thymus RF-C Complex Protects the C Termini, But Not the N Termini, of PCNA from Phosphorylation—First, we studied with KPA the interaction of the calf thymus RF-C

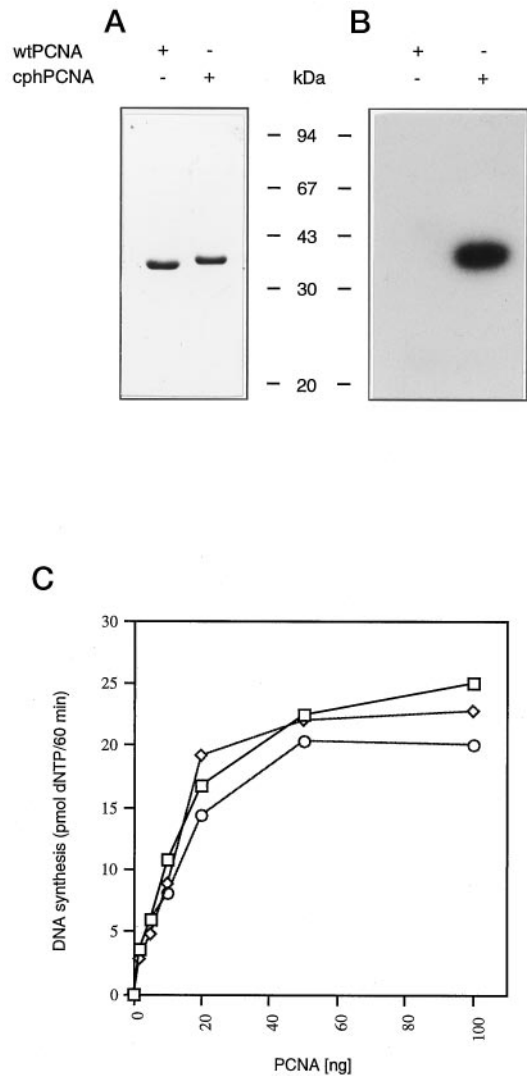


FIG. 1. cphPCNA can be phosphorylated *in vitro* and is functionally active. A, wtPCNA and cphPCNA (1.5 μ g each) were loaded onto a 12% SDS-polyacrylamide gel, electrophoresed, and stained with Coomassie Blue. B, 0.4 μ g of wtPCNA and cphPCNA each were incubated with [γ -³²P]ATP and 0.75 units of cAMP-dependent protein kinase for 10 min at 37 °C as described under "Experimental Procedures." The samples were electrophoresed (12% SDS-polyacrylamide gel), and the gel was fixed in 10% acetic acid containing 12% methanol, dried, and autoradiographed. C, modification of PCNA does not change its functional activity *in vitro*. cphPCNA was first phosphorylated by incubation for 10 min with cAMP-dependent protein kinase and 1 mM ATP. DNA synthesis was then performed in an assay containing singly primed M13DNA (RF-C-dependent assay) as described under "Experimental Procedures." Squares, wtPCNA; diamonds, cphPCNA, unphosphorylated; circles, cphPCNA, phosphorylated.

complex with human PCNA. For this purpose, cphPCNA or nphPCNA and a 2-fold molar excess of the calf thymus RF-C complex were preincubated with [γ -³²P]ATP for 2 min at 37 °C to allow interaction between the proteins. After addition of the cAMP-dependent protein kinase, the kinetics of the phosphorylation reaction were assayed. If RF-C interacts with PCNA close to either terminus, the accessibility of the respective artificial phosphorylation sites to the kinase is expected to be reduced. This results in a reduction of the amount of radioactivity incorporated in the PCNA ring. In Fig. 2, we show that a 2-fold molar excess of RF-C inhibited phosphorylation of cphPCNA by approximately 50% after 10 min but did not inhibit the phosphorylation of nphPCNA. This experiment provided evidence that RF-C interacts with PCNA close enough to the C

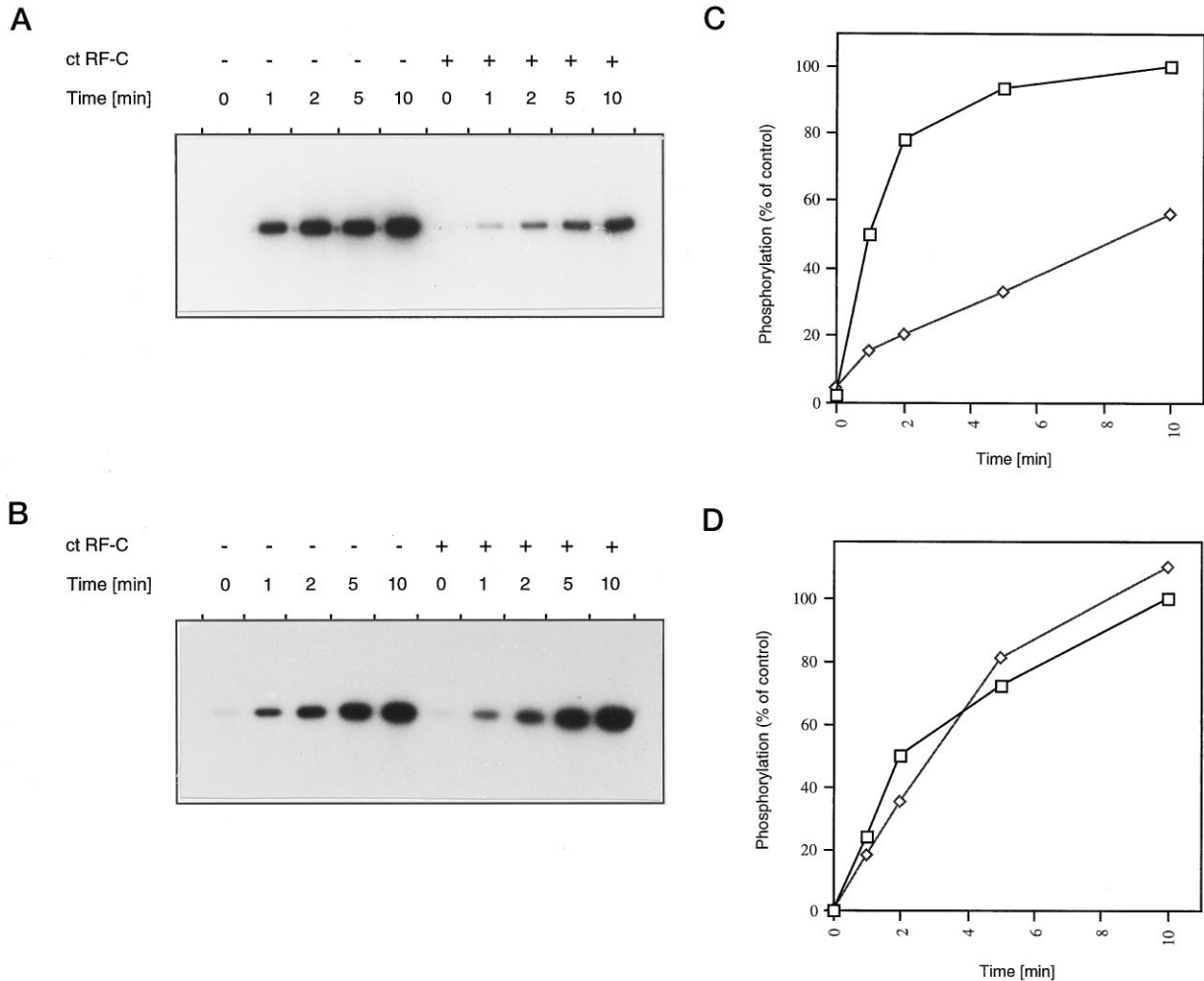


FIG. 2. Pentameric calf thymus RF-C can protect the C termini of PCNA from phosphorylation. cphPCNA (A) or nphPCNA (B) were treated with cAMP-dependent protein kinase in the absence or in the presence of a 2-fold molar excess of pentameric calf thymus RF-C as described under "Experimental Procedures." Time points were analyzed in a 12% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The first five lanes represent the reaction without ctRF-C and the last five lanes with ctRF-C. C and D, quantitation of autoradiographs A and B, respectively. Squares, no ctRF-C; diamonds, ctRF-C. % of control, phosphorylation of cphPCNA alone after 10 min was taken as 100%.

termini to protect them from phosphorylation.

The PCNA Binding Region of Human RF-C 140 Is Able to Protect PCNA from Phosphorylation, the DNA Binding Region Is Not—Having shown that the pentameric calf thymus RF-C complex interacts with the C-side of human PCNA, we next wanted to investigate the role of RF-C 140 in this interaction. Two functional regions of human RF-C 140 have recently been identified (10), the DNA binding (fragment A) and the PCNA binding regions (fragment B). The DNA binding activity was localized between aa 366 and 477, where the conserved box I (restricted to the large subunit) is situated (8). This region shows striking similarity to the N-terminal regions of all bacterial DNA ligases sequenced to date (11, 29–31) and somewhat less, but still significant, similarity to the automodification domain of eukaryotic poly(ADP-ribose) polys. The PCNA binding region, which is adjacent to but does not overlap with the DNA binding region, includes aa 478–712. This region of the protein has been shown to inhibit SV40 DNA replication *in vitro*, as well as RF-C-dependent loading of PCNA onto DNA and RF-C-dependent DNA synthesis. It also showed a dominant negative phenotype when expressed in mammalian cells (10). Since the pentameric RF-C complex could protect cphPCNA from phosphorylation (see Fig. 2), the effect of the PCNA binding region of RF-C 140 (fragment B) alone was investigated in the same assay. Using a 6-fold molar excess of RF-C

140 fragment B, 80% less phosphorylation of cphPCNA was observed after 10 min of incubation (Fig. 3A). The RF-C 140 fragment B, however, did not significantly protect human PCNA containing an artificial phosphorylation site at its N terminus (data not shown). This result further supported the notion that RF-C binds to PCNA on the side where its C termini are located rather than to the other face of the ring. To exclude the possibility that the protection seen is an artifact caused by the intrinsic ATPase activity of RF-C 140 fragment B, we separated 2 μ l of the KPA samples on polyethyleneimine-thin layer chromatography after different incubation times at 37 °C. In all cases tested, the amount of free [³²P]phosphate produced was negligible (data not shown). Consistent with its lack of interaction with PCNA, a 6-fold molar excess of fragment A produced no inhibition of phosphorylation (Fig. 3B). RF-C 140 fragment A + B, a fragment containing both regions A and B, produced 60% inhibition of phosphorylation. In summary, these results supported the hypothesis that the PCNA binding region of RF-C 140 interacts with sites close to the C termini of PCNA.

RF-C 140 Homologue of D. melanogaster Protects the C Termini of PCNA from Phosphorylation—Since it has been observed that PCNA from distantly related species can be interchanged *in vitro* and *in vivo*, we next tested RF-C 140 of *D. melanogaster*. Although human and *Schizosaccharomyces*

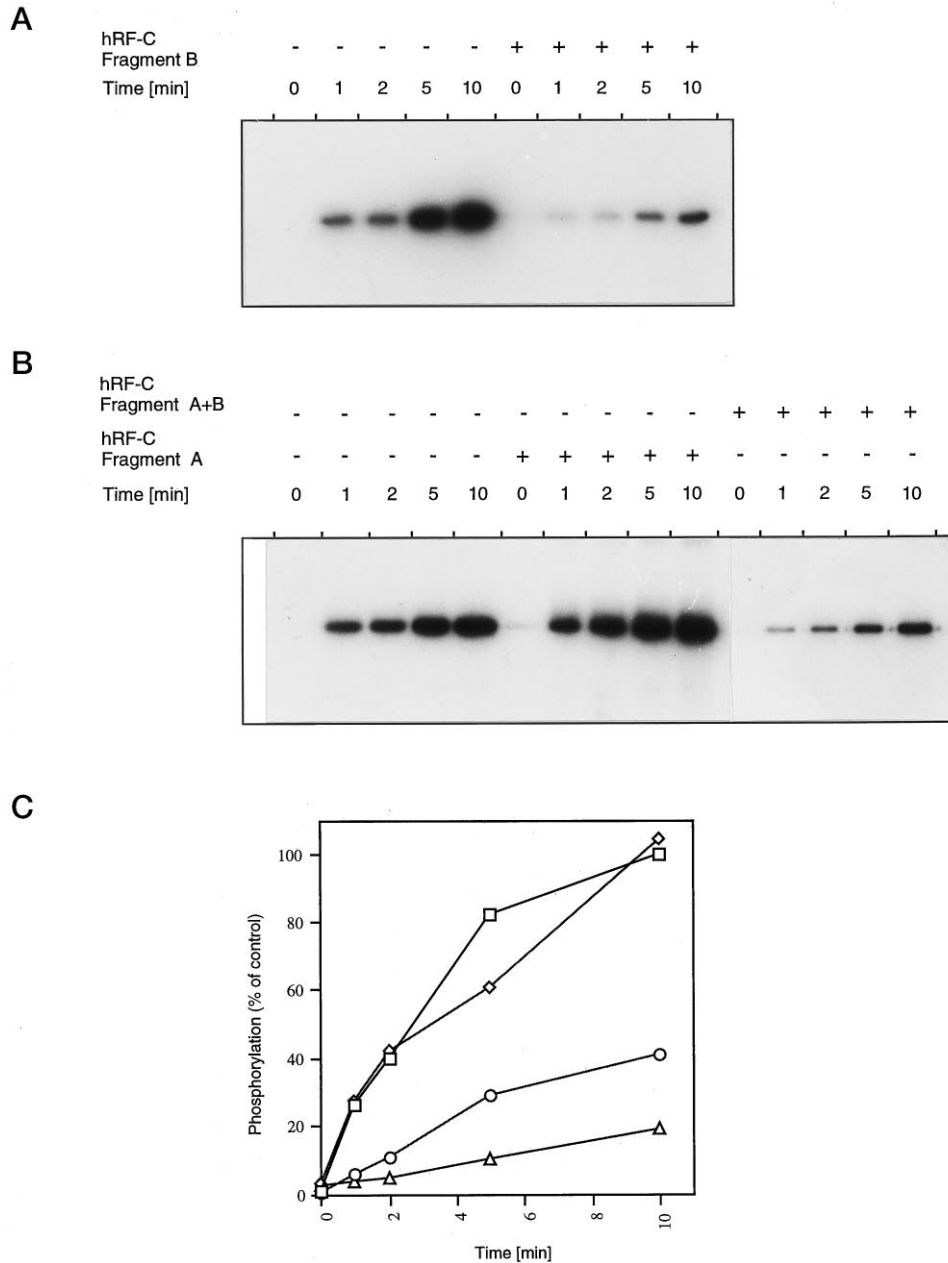


FIG. 3. **The PCNA binding domain but not the DNA binding domain of RF-C can protect cphPCNA from phosphorylation.** *A*, cphPCNA was incubated with a 6-fold molar excess of RF-C 140 fragment B (aa 378–712; the PCNA binding region) as described under “Experimental Procedures.” For further details, see the legend to Fig. 2. *B*, incubation of cphPCNA with a 6-fold excess of RF-C 140 fragment A (aa 366–477; the DNA binding region) and of fragment A + B (aa 366–712; both regions). *C*, quantitation of the autoradiograph. *Squares*, no RF-C; *diamonds*, fragment A; *triangles*, fragment B; *circles*, fragment A + B. % of control, phosphorylation of cphPCNA alone after 10 min was taken as 100%.

pombe PCNA are only 51% identical, the human protein can complement a *Schizosaccharomyces* PCNA deletion mutation (32). Similarly, *D. melanogaster* PCNA could substitute for mammalian PCNA in SV40 *in vitro* replication although the proteins are only 71% identical (33). We used the cloned RF-C 140 of *D. melanogaster* fused with maltose binding protein² and tested the purified fusion protein in KPA. A 3-fold molar excess of *D. melanogaster* RF-C 140 led to a 45% inhibition of cphPCNA phosphorylation by cAMP-dependent kinase (Fig. 4). A 7-fold excess of the *D. melanogaster* RF-C 140 did not cause any protection of the N-terminally phosphorylatable PCNA (data not shown), further supporting the involvement of the PCNA region close to the C termini in the interaction with RF-C. The DNA binding region of *D. melanogaster* RF-C 140 did not protect cphPCNA from phosphorylation (data not shown), consist-

ent with our results with human RF-C 140 fragment A (see above). These results confirmed that RF-C interacts with PCNA at the C-side, and not at the N-side, and that the large subunit is involved in this interaction. Moreover, the molecular interactions between PCNA and RF-C 140 have been conserved through eukaryotic evolution.

Human RF-C 36 Interacts Independently with PCNA Close to the C Termini, whereas RF-C 40 Is Not Able to Protect PCNA in KPA or to Inhibit DNA Replication in Vitro—An interaction between the four small RF-C subunits and PCNA is plausible because all of them show sequence similarity to the PCNA binding region (10) of RF-C 140 (Fig. 5). Of the four individual small subunits, only RF-C 36 and RF-C 40 could be produced as soluble single subunits. This is not surprising in view of the work published by Uhlmann *et al.* (34) where the coexpression

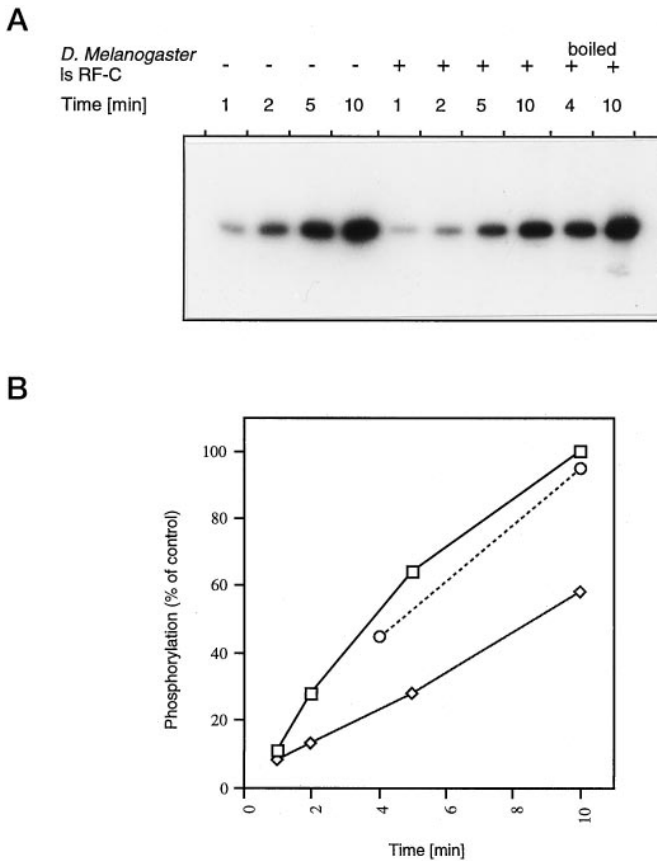


FIG. 4. The large subunit of *D. melanogaster* RF-C can protect the C termini of human PCNA from phosphorylation. A, cph-PCNA was incubated as described for the previous experiments with a 3-fold excess of *D. melanogaster* RF-C 140. The last two lanes show incubation of PCNA with RF-C 140 after boiling it for 2 min. B, quantification of the cphPCNA phosphorylation. Squares, no *D. melanogaster* RF-C; diamonds, *D. melanogaster* RF-C; circles, *D. melanogaster* RF-C, boiled. % of control, phosphorylation of cphPCNA alone after 10 min was taken as 100%.

of at least a subset of small subunits was required to get soluble proteins. Nevertheless, we managed to solubilize RF-C 36 and RF-C 40, and tested their ability to protect cphPCNA and nphPCNA in the KPA. Incubation of RF-C 36 with cphPCNA for 10 min resulted in a 60% inhibition of phosphorylation of PCNA (Fig. 6, A and B), providing evidence that RF-C 36 interacts with PCNA at the C-side. On the other hand, no protection was observed when RF-C 36 was incubated with nphPCNA (data not shown). Further evidence for an interaction between PCNA and RF-C 36 was obtained with RF-C-dependent replication assays on singly primed M13 DNA. RF-C 36 inhibited DNA replication (Fig. 6C), most likely by interfering with the function of RF-C. These results were confirmed by the inability of RF-C 36 to inhibit RF-C-independent DNA synthesis (Fig. 6D). On the other hand, we were unable to show any inhibition of DNA synthesis by RF-C 40 (both RF-C-dependent and -independent) (Fig. 6, C and D) even by using very low amounts (0.1 and 0.05 units) of pol δ and PCNA (data not shown), nor could we detect protection of either cph- or nph-PCNA from phosphorylation in the KPA (data not shown). However, we found weak ATP-dependent PCNA binding activity in RF-C 40 by direct pull-down assays (see below). Fig. 6, C and D, also shows the effect of His-RF-C 140 fragment B on DNA replication, confirming the results already obtained with the GST-RF-C 140 fragment B (10). His-RF-C 140 fragment B

inhibited RF-C-dependent DNA synthesis by about 90% and, therefore, appears to interfere strongly with the RF-C-PCNA interaction. This strong interaction with PCNA was even reflected in RF-C-independent DNA replication where RF-C 140 fragment B, unlike the RF-C 36 and RF-C 40, caused inhibition of nucleotide incorporation and thus likely trapping PCNA from pol δ .

A Pull-down Assay Supports the Interaction Data Obtained with KPA and DNA Replication—To support the data obtained with KPA and with the *in vitro* DNA replication experiments, we took advantage of the fact that RF-C 140, RF-C 140 fragment B, RF-C 36, and RF-C 40 are all His tagged so that they could be bound to Ni-NTA-coupled resin, allowing detection of PCNA binding with pull-down assays (see “Experimental Procedures”). We found that RF-C 140, RF-C 140 fragment B, and RF-C 36 were able to bind to PCNA in the absence of ATP, whereas RF-C 40, under the conditions used, was not (Fig. 7). This was consistent with the results obtained in the KPA and in the DNA replication experiments. Inclusion of 1 mM ATP did not significantly alter the amount of PCNA pulled down by RF-C 140, RF-C 140 fragment B or RF-C 36, but a weak PCNA binding activity (less than 1% compared to RF-C 140, RF-C 140 fragment B, and RF-C 36) appeared with RF-C 40 (data not shown), thereby indicating that RF-C 40 was properly folded.

DISCUSSION

Since its first isolation from human cells (2), RF-C has been the subject of several studies aimed at revealing its structure, function and properties. Several functions of this replication factor have been identified, but the precise mechanisms hidden behind its function remain a subject of speculation. It appears that many features of RF-C action in DNA replication are comparable with the γ complex of *E. coli*, a prokaryotic counterpart. Indeed, in spite of great evolutionary distance, sequences and functions are similar between these two protein complexes. There are, however, details specific to the eukaryotic replication machinery. A main goal of this work was to begin characterizing the molecular interactions between RF-C and PCNA by first determining the region within PCNA that is bound by RF-C.

The subunits comprising the RF-C complex carry out a variety of activities. Human RF-C 40 has been shown to bind ATP (9, 35). RF-C 37 was proposed to take part in the binding of RF-C to the primer termini of both leading and lagging strands (14). RF-C is a DNA- and PCNA-activated ATPase, an activity that has been linked to the RF-C 3 subunit in yeast (36), which corresponds to human RF-C 36. Recently, it was shown that RF-C 140 specifically binds to PCNA, and a PCNA binding region was located between aa 478–712 (10). This PCNA binding region contains the boxes II-IV that are conserved in all RF-C subunits. This region also shows significant similarities to the *E. coli* pol III subunits γ/τ (37, 38) and δ' (39) and to the bacteriophage T4 gp44 protein (40).

In the crystal structure of PCNA, the C termini protrude from one face of the ring (20), appearing as candidates for interaction with other proteins, including RF-C. A modified form of PCNA, the C termini of which could be artificially phosphorylated with ^{32}P , and different subunits and fragments of RF-C have been used to screen for possible interactions. We have shown that PCNA and calf thymus RF-C can interact in a way that makes the C termini of PCNA less accessible to cAMP-dependent kinase. A 2-fold molar excess of RF-C over PCNA caused a 50% inhibition of phosphorylation. This suggests that RF-C binds to PCNA close to the C termini of the latter. In this interaction, the large subunit is certainly involved. In fact, incubation of cphPCNA with the PCNA binding region of human RF-C 140 resulted in a consistent inhibition of

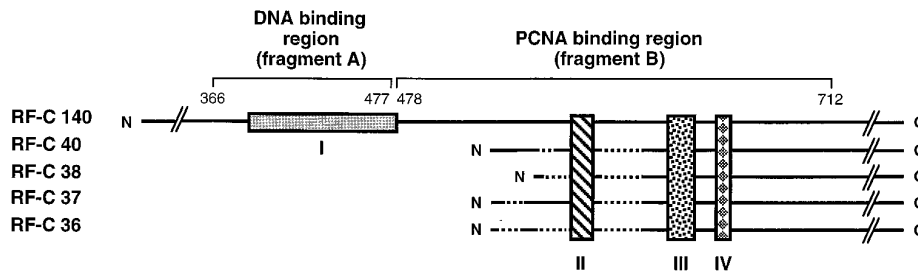


FIG. 5. Conserved regions II, III, and IV in the four small RF-C subunits are located in the PCNA binding region of RF-C 140. A schematic alignment of the five RF-C subunits show that the small subunits share three conserved boxes (indicated as II, III, and IV) with the PCNA binding region (aa 478–712) of RF-C 140. The DNA binding region (aa 366–477) of RF-C 140 contains RF-C box I (indicated as I). Thick lines represent the amino acid sequence, whereas dotted lines mark breaks in the small subunit sequences for alignment with RF-C 140.

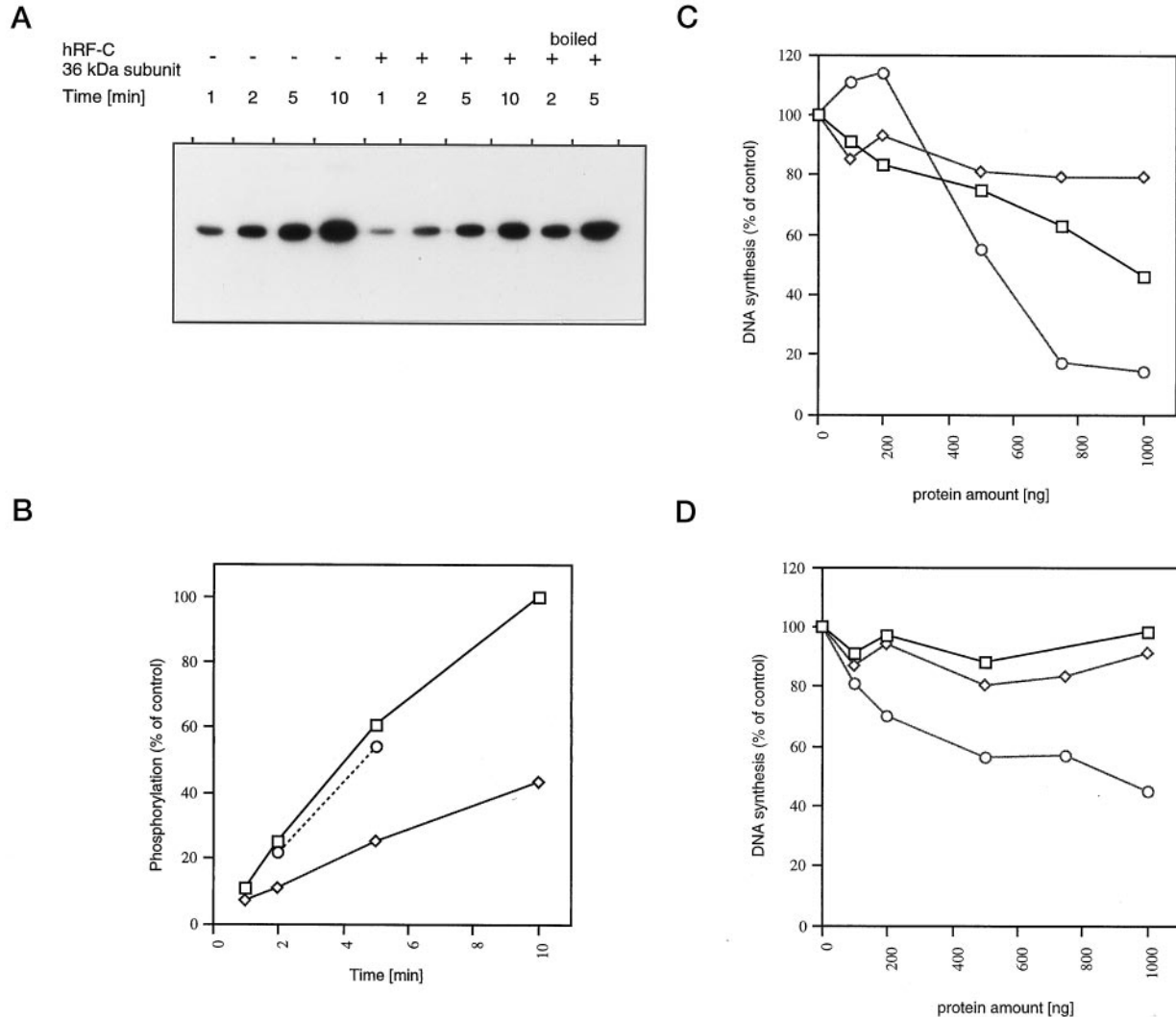


FIG. 6. The 36-kDa small subunit of human RF-C can protect the C termini of PCNA. A, cphPCNA was incubated with a 6-fold excess of RF-C 36 for different time periods as described under "Experimental Procedures." In the last two lanes, RF-C 36 was boiled for 2 min before incubation with PCNA. B, quantitation of the autoradiograph. Squares, no RF-C; diamonds, RF-C 36; circles, RF-C 36, boiled. % of control, phosphorylation of cphPCNA alone after 10 min was taken as 100%. C, effect of RF-C 36 and RF-C 40, as well as of the His-RF-C 140 fragment B, on RF-C-dependent DNA synthesis. The effect of the addition of 100, 200, 500, and 1000 ng of the above mentioned proteins was tested in an RF-C-dependent assay as described under "Experimental Procedures." D, effect of human RF-C 36 and RF-C 40, as well as of the His-RF-C 140 fragment B, on RF-C-independent DNA synthesis. Diamonds, RF-C 40; squares, RF-C 36; circles, His-RF-C 140 fragment B.

PCNA phosphorylation, while the DNA binding region, in agreement with its inability to bind PCNA, did not have the same effect. A high degree of protection of the C termini from phosphorylation was also obtained with RF-C 36, suggesting that this subunit interacts with the same region of PCNA. The lower phosphorylation inhibition (50%) reached with the RF-C 36 compared with the 80% inhibition obtained with the RF-C

140 fragment B could be explained with a lower affinity of the small subunit to the same site on PCNA. It is unclear why RF-C 140 fragment B and fragment A + B do not behave in the same way, but a similar effect has been observed in other studies (10). It may be that the DNA binding region exerts some negative steric effect on the PCNA binding region. The fact that the native pentameric RF-C complex, as well as *D. melano-*

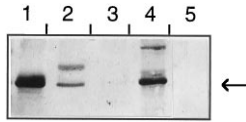


FIG. 7. A pull-down assay supports the interaction data obtained with KPA and DNA replication. The His-tagged proteins to be tested for interaction with PCNA were bound to Ni-NTA beads: lane 1, RF-C 140 fragment B; lane 2, RF-C 36; lane 3, RF-C 40; lane 4, human RF-C 140; lane 5, no protein. PCNA was then added to the beads and binding to the different His-proteins detected as described under "Experimental Procedures." The arrow indicates the PCNA binding to the RF-C 140 fragment B, to RF-C 36, and to RF-C 140 (lanes 1, 2, and 4, respectively).

gaster RF-C 140 and the PCNA binding region of human RF-C 140, could not significantly protect the N termini of PCNA from phosphorylation are evidence for an interaction of RF-C at the C-side of the PCNA ring. The binding of *D. melanogaster* RF-C 140 to human PCNA gives rise to an important observation, namely the strong conservation in the mode of interaction of PCNA and RF-C throughout eukaryotic evolution. In fact, the RF-C complex from *Saccharomyces cerevisiae*, mouse, human, and *D. melanogaster* are all able to interact with human PCNA, as expected from the high degree of sequence similarity observed in the PCNA binding region.

In RF-C 140, the DNA binding region and the PCNA binding region are adjacent (10) so that formation of a DNA/RF-C complex could lead to a higher affinity of RF-C to PCNA and to a subsequent formation of the DNA/PCNA/RF-C complex. RF-C 140 could bind first to DNA and PCNA and facilitate, by a concerted action, the binding of the small subunits that alone would have a lower affinity for the ring. Small subunits other than RF-C 36 might also be involved in the PCNA interaction since, as mentioned before, they show significant sequence similarity to each other and, more importantly, with the PCNA binding region of RF-C 140 (Fig. 5).

In summary, our results show that RF-C interacts with a distinct face of the PCNA ring, namely with the C-side. This interaction certainly involves the large subunit; among the small subunits, RF-C 36 also interacts with PCNA at the C-side. Our attempts to determine a site of interaction between RF-C 40 were not successful. Although we could detect ATP-dependent binding of His-RF-C 40 to PCNA, the interaction seen was too weak to have an effect in KPA or *in vitro* DNA replication assays. Indeed, preliminary experiments with the yeast two-hybrid system failed to show an interaction between RF-C 40 and PCNA,³ further suggesting that RF-C 40 may not interact strongly with PCNA when removed from the RF-C complex. If RF-C covers the C-side of PCNA (as implied by the protection from phosphorylation of cphPCNA but not of nphPCNA), it seems plausible that the other ring side would be free for interactions with other proteins, e.g. with pol δ . Another possibility would be that pol δ competes for PCNA interaction with RF-C and, thus, also binds to the C-side of the ring. That this is the case for the prokaryotic replication complex was shown by Naktinis *et al.* (15). However, addition of pol δ to the KPA did not show any difference in protection between cph- and nphPCNA (data not shown). It is, therefore, not yet possi-

ble to speculate about the structure of the possible PCNA/RF-C/pol δ complex.

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REFERENCES

- Prelich, G., Kostura, M., Marshak, D. R., Mathews, M. B., and Stillman, B. (1987) *Nature* **326**, 471–475
- Tsurimoto, T., and Stillman, B. (1989) *Mol. Cell. Biol.* **9**, 609–619
- Wold, M. S., and Kelly, T. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 2523–2527
- Hübscher, U., Maga, G., and Podust, V. N. (1996) *DNA Replication in Eukaryotic Cells* (De Pamphilis, M. L., ed) pp. 525–543, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Podust, L. M., Podust, V. N., Sogo, J. M., and Hübscher, U. (1995) *Mol. Cell. Biol.* **15**, 3072–3081
- Lee, S. H., Kwong, A. D., Pan, Z.-Q., and Hurwitz, J. (1991) *J. Biol. Chem.* **266**, 594–602
- Tsurimoto, T., Fairman, M. P., and Stillman, B. (1989) *Mol. Cell. Biol.* **9**, 3839–3849
- Cullmann, G., Fien, K., Kobayashi, R., and Stillman, B. (1995) *Mol. Cell. Biol.* **15**, 4661–4671
- Tsurimoto, T., and Stillman, B. (1991) *J. Biol. Chem.* **266**, 1961–1968
- Fotedar, R., Mossi, R., Fitzgerald, P., Rousselle, T., Maga, G., Brickner, H., Messier, H., Khashtilba, S., Hübscher, U., and Fotedar, A. (1996) *EMBO J.* **15**, 4423–4433
- Burbelo, P. D., Utani, A., Pan, Z. Q., and Yamada, Y. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 11543–11547
- Lu, Y., Zeff, A. S., and Riegel, A. T. (1993) *Biochem. Biophys. Res. Commun.* **193**, 779–786
- McAlear, M. A., Howell, E. A., Espenshade, K. K., and Holm, C. (1994) *Mol. Cell. Biol.* **14**, 4390–4397
- Pan, Z. Q., Chen, M., and Hurwitz, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 6–10
- Naktinis, V., Turner, J., and O'Donnell, M. (1996) *Cell* **84**, 137–145
- Kaboord, B. F., and Benkovic, S. J. (1996) *Biochemistry* **35**, 1084–1092
- O'Donnell, M., Onrust, R., Dean, F. B., Chen, M., and Hurwitz, J. (1993) *Nucleic Acids Res.* **21**, 1–3
- Stukenberg, P. T., Turner, J., and O'Donnell, M. (1994) *Cell* **78**, 877–887
- Naktinis, V., Onrust, R., Fang, L., and O'Donnell, M. (1995) *J. Biol. Chem.* **270**, 13358–13365
- Krishna, T. S., Kong, X. P., Gary, S., Burgers, P. M., and Kuriyan, J. (1994) *Cell* **79**, 1233–1243
- Kelman, Z., and O'Donnell, M. (1995) *Nucleic Acids Res.* **23**, 3613–3620
- Fukuda, K., Morioka, H., Imajou, S., Ikeda, S., Ohtsuka, E., and Tsurimoto, T. (1995) *J. Biol. Chem.* **270**, 22527–22534
- Brand, S. R., Bernstein, R. M., and Mathews, M. B. (1994) *J. Immunol.* **153**, 3070–3078
- Jónsson, Z. O., Podust, V. N., Podust, L. M., and Hübscher, U. (1995) *EMBO J.* **14**, 5745–5751
- Weiser, T., Gassmann, M., Thömmes, P., Ferrari, E., Hafkemeyer, P., and Hübscher, U. (1991) *J. Biol. Chem.* **266**, 10420–10428
- Podust, V., Mikhailov, V., Georgaki, A., and Hübscher, U. (1992) *Chromosoma (Berl.)* **102**, 133–141
- Fien, K., and Stillman, B. (1992) *Mol. Cell. Biol.* **12**, 155–163
- Podust, V. N., Georgaki, A., Strack, B., and Hübscher, U. (1992) *Nucleic Acids Res.* **20**, 4159–4165
- Thorbjarnardottir, S. H., Jónsson, Z. O., Andresson, O. S., Kristjánsson, J. K., Eggertsson, G., and Palsdóttir, A. (1995) *Gene (Amst.)* **161**, 1–6
- Bunz, F., Kobayashi, R., and Stillman, B. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 11014–11018
- Luckow, B., Bunz, F., Stillman, B., Lichter, P., and Schutz, G. (1994) *Mol. Cell. Biol.* **14**, 1626–1634
- Waseem, N. H., Labib, K., Nurse, P., and Lane, D. P. (1992) *EMBO J.* **11**, 5111–5120
- Ng, L., Prelich, G., Anderson, C. W., Stillman, B., and Fisher, P. A. (1990) *J. Biol. Chem.* **265**, 11948–11954
- Uhlmann, F., Cai, J. S., Floresroz, H., Dean, F. B., Finkelstein, J., O'Donnell, M., and Hurwitz, J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 6521–6526
- Chen, M., Pan, Z. Q., and Hurwitz, J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 2516–2520
- Li, X., and Burgers, P. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 868–872
- Flower, A. M., and McHenry, C. S. (1986) *Nucleic Acids Res.* **14**, 8091–8101
- Yin, K. C., Blinkowa, A., and Walker, J. R. (1986) *Nucleic Acids Res.* **14**, 6541–6549
- Dong, Z., Onrust, R., Skangalis, M., and O'Donnell, M. (1993) *J. Biol. Chem.* **268**, 11758–11765
- Spicer, E. K., Nossal, N. G., and Williams, K. R. (1984) *J. Biol. Chem.* **259**, 17790–17795

³ Z. O. Jónsson, R. Mossi, R. Hindges, and U. Hübscher, in preparation.