A Complex Consisting of Human Replication Factor C p40, p37, and p36 Subunits Is a DNA-dependent ATPase and an Intermediate in the Assembly of the Holoenzyme*

Jinsong Cai, Emma Gibbs, Frank Uhlmann†, Barbara Phillips, Nina Yao‡, Michael O’Donnell§, and Jerard Hurwitz¶

From the Program in Molecular Biology, Memorial Sloan-Kettering Cancer Center and §The Rockefeller University, New York, New York 10021

Human replication factor C (hRFC) is a multi-subunit protein complex capable of supporting proliferating cell nuclear antigen (PCNA)-dependent DNA synthesis by DNA polymerases δ and ε. The hRFC complex consists of five different subunits with apparent molecular masses of 140, 40, 38, 37, and 36 kDa. We have previously reported the expression of a three-subunit core complex, consisting of the p40, p37, and p36 subunits following coupled in vitro transcription-translation of the cDNAs encoding these proteins (Uhlmann, F., Cai, J., Flores-Rozas, H., Dean, F. B., Finkelstein, J., O’Donnell, M., and Hurwitz, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6521–6526). Here we describe the isolation of a stable complex composed of the p40, p37, and p36 subunits of hRFC from baculovirus-infected insect cells. The purified p40+p37+p36 complex, like the five-subunit RFC, contained DNA-dependent ATPase activity that was stimulated by PCNA, preferentially bound to primed DNA templates, interacted with PCNA, and was capable of unloading PCNA from singly-nicked circular DNA. In contrast to the five-subunit RFC, the three-subunit core complex did not load PCNA onto DNA. The p40+p37+p36 complex inhibited the elongation of primed DNA templates catalyzed by the DNA polymerase δ holoenzyme. Incubation of the p40+p37+p36 complex with the hRFC p140 and p38 subunits formed the five-subunit hRFC complex that supported PCNA-dependent DNA synthesis by DNA polymerase δ.

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† Student enrolled in the graduate program at the Physiologisch-Chemisches Institut, Universität Tübingen and is supported by the German Academic Exchange Service through funds of the "Zweites Hochschulsonderprogramm."
‡ Professor of the American Cancer Society. To whom correspondence should be addressed: Program in Molecular Biology, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., Box 97, New York, NY 10021.
§ These abbreviations used are: RFC, replication factor C; hRFC, human replication factor C; BRFC, baculovirus reconstituted replication factor C; pol, DNA polymerase; PCNA, proliferating cell nuclear antigen; ssDNA, single-stranded DNA; HSSB, human single-stranded DNA-binding protein, also called RPA; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; RU, response.

In this paper, we report that the purified p40-p37-p36 complex formed in a baculovirus overexpression system contains DNA-dependent ATPase activity that is stimulated by PCNA. This three-subunit complex binds preferentially to primed DNA, interacts with PCNA, and inhibits RFC/PCNA-dependent DNA elongation catalyzed by pol δ. High levels of the RFC/PCNA complex inhibited the elongation of primed DNA templates catalyzed by the DNA polymerase δ holoenzyme. Incubation of the p40+p37+p36 complex with the hRFC p140 and p38 subunits formed the five-subunit hRFC complex that supported PCNA-dependent DNA synthesis by DNA polymerase δ.

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p40p37-p36 complex can unfold PCNA from DNA but cannot load PCNA onto DNA. We also present evidence that the p40p37-p36 complex, when incubated with the p140 and p38 subunits, is converted to the five-subunit RFC active in supporting DNA elongation. This finding indicates that the p40p37-p36 complex is an intermediate in assembly of the RFC holoenzyme.

MATERIALS AND METHODS

Preparation of DNA and Proteins—Polyclonal antiserum to the p140 and oligo(dT)20-poly(dA)-tailed DNA was obtained from Pharmacia Biotech Inc. To prepare antibody coupled to protein A-agarose beads (50 μl containing 10 ml Tris-HCl, pH 8.0, and 0.1 M NaCl) the mixture was heated at 75 °C for 5 min and then cooled to room temperature and chilled on ice. pET16ap140 and 19bHis38 DNA were prepared as described previously (20). αX174 single-stranded circular (scs) viral DNA was obtained from New England Biolabs. Primed M13 ss cDNA and singly-nicked pBluescript (pBS) DNAs were prepared as described (21, 22) as were HSSB, poly δ, PCNA, and hRFC, purified from HeLa cytosolic extracts (Refs. 1, 3, 21, and 22, respectively). [32P]-Labeled PCNA (1500 cpm/μmol) was prepared using recombinant PCNA containing a CAP-dependent protein kinase consensus sequence at its N terminus as described previously (23).

Bio-PEGylation—Translation of RFC, p140, and p38 Subunits—Coupled in vitro transcription-translation reactions (12 or 24 μl containing pET16ap140 (37.5 ng/μl) and/or pET19bHis38 DNA (21 ng/μl) were carried out in the presence or absence of the p40-p37-p36 complex (83 fmol/μl) at 30 °C for 90 min as described (20).

Immunoprecipitation of RFC and Subunits—To precipitate in vitro translated products formed after incubation, the reaction mixture (12 μl) was incubated with polyclonal antisera (0.5 μl) against the RFC p37 subunit at 0 °C for 60 min. Immunocomplexes were then adsorbed to protein A-agarose beads (50 μl containing 0.5 M NaCl, 1.1 M Tris-HCl, or 1.5 M NaCl containing 10 ml Tris-HCl, pH 8.0, and 0.1 M NaCl. The mixture was mixed for 30 min at 4 °C, then placed on ice. Reactions were then filtered at 4 °C through a 0.45-μm filter and the radioactivity adsorbed to the filter was measured by liquid scintillation counting.

Protein Interaction Analyses—Protein-protein interactions were examined using surface plasmon resonance. The immobilization of PCNA on sensor chips was carried out using the carbodiimide coupling protocol specified in the manufacturer's instructions (Pharmacia Biosensor). The interaction between immobilized PCNA and hRFC or the p40-p37-p36 complex in solution was followed by monitoring changes in the surface concentration of proteins on sensor chips using the BIACore 2000 at room temperature.

DNA and PCNA Loading and Unloading Assays—The loading of [32P]-labeled PCNA onto DNA was carried out in reaction mixtures (50 μl), containing 0.5 pmol of singly nicked pBluescript DNA, 2.6 pmol of [32P]-labeled PCNA trimers (~1500 cpm/μmol) in 50 μl of incubation buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM NaCl, 8 mM MgCl2, 0.5 mM ATP, 4% glycerol, 5 mM DTT, and 200 mM bRFC. Reactions were incubated for 10 min at 37 °C, stopped on ice, and then applied at 4 °C to a Bio-Gel A15m column equilibrated with incubation buffer. Fractions of 170 μl were collected, and the radioactivity quantitated by Cerenkov counting. The release of [32P]-PCNA complexed to DNA (the unloading reaction) was carried out in reaction mixtures (50 μl) containing 83 fmol of PCNA (as trimer) loaded onto singly nicked DNA (isolated by gel filtration), incubation buffer (as described above), and bRFC or the p40-p37-p36 complex (in amounts as indicated). Mixtures were incubated at 37 °C for 10 min, and the reaction was halted by placing tubes on ice. Reactions were then filtered at 4 °C through a 5-ml gel filtration column (Bio-Gel, A15m, Bio-Rad) to resolve the [32P]-PCNA (eluting in the included volume) from [32P]-PCNA bound to DNA (eluting in the excluded volume). Fractions of 170 μl were collected and the [32P] quantitated by Cerenkov counting.

Preparation of Reconstituted Viruses—Reconstituted baculoviruses that produced the p40, p37, and p36 subunits of hRFC were as described previously (19).

Large Scale Infection of Insect Cells with hRFC-recombinant Baculoviruses and Preparation of Cell Extracts—S9 cells (Infrogen) were grown at 27 °C to a cell density of 2 × 107 cells/ml in Grace’s medium supplemented with 10% fetal bovine serum. S9 cells (2 × 107, 200 ml) were infected with recombinant viruses that produced the p40, p37, and p36 subunits of hRFC at a multiplicity of infection of 5 for each virus and were maintained in a 2-liter glass flask at 27 °C for 48 h with constant shaking (100 rpm). The cells were then harvested by centrifugation at 300 × g for 15 min. The cell pellet was washed with ice-cold phosphate-buffered saline, resuspended in 2 volumes of hypotonic buffer (50 mM Tris-HCl, pH 8.0, 10 mM KCl, 1.5 mM MgCl2, 20 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 μg/ml aprotinin, 0.2 μg/ml leupeptin, and 0.1 μg/ml antipain) per volume of packed cells, and lysed with 10 strokes of a Dounce homogenizer. After centrifugation at 2,400 × g for 30 min at 4 °C, the supernatant (cytosolic extract, 95 mg, 9 ml) was saved, and the nuclear pellet was resuspended in 2 volumes of extraction buffer (hypotonic buffer without 10 mM KCl) per volume of packed cells and the mixture adjusted to a final concentration of 0.42 μM NaCl. After centrifugation at 43,500 × g for 30 min at 4 °C, the supernatant (nuclear extract, 35 mg, 5 ml) was combined with the cytosolic extract, and the mixture was centrifuged at 44,000 × g for 30 min at 4 °C. The supernatant was used for the purification of the p40-p37-p36 complex as described below.

Purification of the p40p37p36 Complex—SDS-PAGE (9%) followed by Coomassie staining and Western blot analysis using antibodies specific for the p40, p37, and p36 subunits of RFC were employed to monitor all purification steps which were carried out at 4 °C.

Extracts (120 mg of protein, 13 ml), prepared as described above, were adjusted to 0.1 μM NaCl and chromatographed on a SP-Sepharose column (1.5 × 9.9 cm) equilibrated with buffer A (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM aprotinin, 0.2 mM leupeptin, 0.1 mM antipain, and 10% glycerol) plus 0.1 μM NaCl. After washing the column with 3-bed volumes of equilibration buffer, bound proteins were eluted using a 50-ml gradient from 0.1 to 0.4 mM NaCl in buffer A. Fractions containing the p40, p37, and p36 subunits eluted at 0.25 mM NaCl and were pooled (14 mg protein, 15 ml). After adjusting the NaCl concentration to 0.05 M, the pooled fractions were loaded onto a Q-Sepharose Hepes-NaOH, pH 7.5, 2 mM MgCl2, 1 mM DTT, 100 μg/ml BSA, and 20 μM NaCl), the p40-p37-p36 complex (in amounts as indicated), and 40 fmol of 5′-[32P]-labeled poly(dA)-tailed DNA (700–1500 cpm/μmol), or 5′-[32P]-labeled oligo(dT)12 (2000–4000 cpm/μmol), or 5′-[32P]-labeled poly(dA)12 hybridized to unlabeled oligo(dT)12 at various molar ratios as indicated. After incubation for 30 min at 37 °C, the mixtures were adsorbed through alkaline-washed nitrocellulose filters (Millipore, HA 0.45 μm) which were then washed three times with 0.5 ml of binding buffer. The radioactivity adsorbed to the filter was measured by liquid scintillation counting.

Purification of the p40p37p36 Complex—SDS-PAGE (9%) followed by Coomassie staining and Western blot analysis using antibodies specific for the p40, p37, and p36 subunits of RFC were employed to monitor all purification steps which were carried out at 4 °C.
Characterization of hRFC p40-p37-p36 Complex

RESULTS

Isolation of the p40-p37-p36 Complex from Baculovirus-infected Insect Cells—The p40-p37-p36 complex was assembled in vivo by coexpressing the p40, p37, and p36 subunits in baculovirus-infected insect cells. After harvesting the cells, cytosolic and nuclear extracts were prepared as described under “Materials and Methods.” The combined cytosolic and nuclear extracts were prepared as described under “Materials and Methods.” Following glycerol gradient centrifugation, three gradient fractions (100 µg, 0.66 mg, 114 mg units/mg) were stored at -80 °C and showed no loss of activity over a 2-month period with repeated cycles of freezing and thawing. The yield of the p40-p37-p36 complex obtained from 200 ml of infected Sf9 cells was ~0.3 mg, representing a total recovery of 10% of the protein (as estimated by Western blot analysis). Denstometry analysis of Coomassie-stained gels was performed using the Molecular Imager and Imaging Densitometer (Bio-Rad).

Fig. 1. Purification of the RFC p40-p37-p36 complex from baculovirus-infected Sf9 cells. A, expression and purification of the RFC p40-p37-p36 complex. The p40-p37-p36 complex expressed and purified from Sf9 cells was analyzed by 9% SDS-PAGE followed by staining with Coomassie Brilliant Blue (lanes 1–3) or by Western blotting (lanes 4–6). The additions to each lane were as follows: lane 1, 30 µg of uninfected Sf9 cell extract; lanes 2, 30 µg of extract from cells infected with three baculoviruses expressing the p40, p37, and p36 subunits; lane 3, 0.8 µg of the pooled glycerol gradient isolated p40-p37-p36 complex, prepared as described under “Materials and Methods”; lanes 4–6, immunoblots of the glycerol gradient purified complex used in lane 3 probed with antibodies specific for the p40 (lane 4), p37 (lane 5), or p36 (lane 6) subunit. Molecular mass markers are indicated at the top of the figure, and the position of each RFC subunit is indicated at the right. B, SDS-PAGE analysis of glycerol gradient fractions. The p40-p37-p36 complex, eluted from the ATP-agarose column (see “Materials and Methods”), was subjected to 15–35% glycerol gradient centrifugation (Fig. 1A, lanes 1 and 2). The overproduced p40, p37, and p36 subunits were not visible on SDS-PAGE after Coomassie staining. All three subunits were detected by Western blot analyses using antibodies specific for each subunit, and the amount of p36, p37, and p40 protein formed was estimated to be 0.5–1% total protein (data not shown). The p40-p37-p36 complex was purified by a number of chromatographic steps and glycerol gradient centrifugation as described under “Materials and Methods.” Following glycerol gradient centrifugation, three protein bands were observed that migrated through SDS-polyacrylamide gels at positions corresponding to those of the hRFC p40, p37, and p36 subunits (lane 3). The identity of each subunit was confirmed by Western blot analysis using polyclonal antibodies specific for each subunit (lanes 4–6). Denstometry analysis of the Coomassie-stained gels indicated that the p40, p37, and p36 subunits were present at a molar ratio of 1.0:1.1:1.0. The RFC p40, p37, and p36 subunits cosedimented through
the glycerol gradient (Fig. 2A), peaking in fraction 13, between aldolase (158 kDa, fractions 9 and 10) and BSA (66 kDa, fraction 14) with a sedimentation coefficient of 4.8, indicating that these subunits exist as a stable complex. When individual subunits were subjected to glycerol gradient centrifugation, the p40 and p37 subunits peaked at fraction 16, whereas the p36 subunit was detected in lower fractions of the gradient most likely due to protein aggregation (data not shown). The cosedimentation of the three subunits is consistent with our previous finding that in vitro transcribed-translated hRFC p40, p37, and p36 subunits form a stable complex consisting of equimolar amounts of each subunit (20).

We have also constructed a baculovirus vector that produces the RFC p36 subunit with an additional 6-histidine residue at the N terminus. The p40-p37-p36 complex was assembled by coexpressing the His-tagged p36 subunit with the p40 and p37 subunits in baculovirus-infected insect cells, and the complex was purified using a Ni\(^{2+}\) affinity column followed by glycerol gradient centrifugation. The p40-p37-p36 complex that eluted from the Ni\(^{2+}\) column with imidazole contained excess levels of the uncomplexed His-tagged p36 subunit in addition to the p40-p37-p36 complex. The excess p36 subunit was not totally removed by the glycerol gradient centrifugation. The purified p40-p37-p36 complex containing either the untagged or His-tagged p36 subunit had essentially identical properties (data not shown).

The RFC p40-p37-p36 Complex Contains DNA-dependent ATPase Activity—ATP hydrolysis is required in order for RFC to load PCNA onto primed DNA templates and recruit pol δ. However, none of the hRFC subunits isolated from E. coli or baculovirus overexpression systems has been shown to exhibit ATPase activity (17; data not shown) suggesting that multiple subunits may be required for this activity. Therefore, we examined the purified p40-p37-p36 complex for its ability to hydrolyze ATP, and as shown in Fig. 1C this DNA-dependent activity peaked in fraction 13 of the glycerol gradient, coincidental with the sedimentation of the p40-p37-p36 protein complex.

The effects of various DNA effectors on the ATPase activity of the complex were examined (Fig. 2A). The p40-p37-p36 complex possessed weak ATPase activity that was stimulated maximally (34-fold) by φX174 ss DNA, 10-fold by poly(dA)\(_{300}\)-oligo(dT)\(_{30}\), 6-fold by poly(dA)\(_{300}\), and 4-fold by oligo(dT)\(_{30}\).

These properties are similar to those observed for the five-subunit RFC purified from HeLa cells (3) or from baculovirus-infected insect cells (19). Each molecule of the p40-p37-p36 complex hydrolyzed 4.5 and 1.4 molecules of ATP/min at 37 °C in the presence of φX174 ss DNA and poly(dA)\(_{300}\)-oligo(dT)\(_{30}\), respectively. These values are 2–3 times lower than those observed with the five-subunit hRFC purified from HeLa cells (3) and 4–7 times lower than that of the hRFC (19). The Hissagged p40-p37-p36 complex contained DNA-dependent ATPase activity that was 2-fold higher than the untagged three-subunit complex. This shows that the purification steps required to isolate the untagged p40-p37-p36 complex leads to some inactivation of this complex.

We also examined the effects of PCNA and HSSB, previously shown to enhance hRFC DNA-dependent ATPase activity (3, 19). As shown in Fig. 2B, PCNA stimulated the ATPase activity of the three-subunit complex 2- to 3-fold in the presence of poly(dA)\(_{300}\)-oligo(dT)\(_{30}\) but not poly(dA)\(_{300}\). This property is identical to that of hRFC and hRFC, suggesting that optimal ATP hydrolyzing activity is achieved when the p40-p37-p36 complex is associated with PCNA on primed DNA. However, the ATPase activity of the p40-p37-p36 complex was unaffected by HSSB (data not shown) in contrast to results observed with hRFC and hRFC. It is possible that the stimulatory effects of HSSB depend on its interaction with the p140 and/or p38 subunits.

The RFC p40-p37-p36 Complex Binds Specifically to Primed DNA—Previous experiments indicated that the interaction between RFC and DNA is mediated by the p140 subunit (16). However, the observation that the p40-p37-p36 complex contained DNA-dependent ATPase activity suggests that it should also interact with DNA. We examined the DNA-binding properties of this complex using a nitrocellulose filter binding assay. High ionic strength (175 mM NaCl) was required to observe selective binding of hRFC to primed DNA and not to single-stranded DNA (19). However, under these conditions the p40-p37-p36 complex did not bind to either type of DNA. When the salt concentration was lowered, selective binding of the core complex to primed DNA was observed at 20 mM NaCl. Thus the DNA binding assay with the p40-p37-p36 complex was carried out at this salt concentration. As shown in Fig. 3, the p40-p37-p36 complex bound poly(dA)\(_{300}\) or oligo(dT)\(_{30}\), inefficiently. However, the complex bound poly(dA)\(_{300}\) and poly(dT)\(_{30}\), and the binding efficiency was markedly increased as the molar ratio of oligo(dT)\(_{30}\) to poly(dA)\(_{300}\) increased, suggesting that the p40-p37-p36 complex specifically recognized
oligo(dT)30 at a molar ratio of 5:1, 1:1, and 1:5, and the interaction inhibits PCNA-dependent DNA elongation catalyzed by pol δ—The p40 and p140 subunits of hRFC have been previously shown to interact with PCNA (17, 25). To explore further the interaction between RFC subunits and PCNA, we determined the direct interaction between PCNA and the p40-p37-p36 complex using the surface plasmon resonance technique as described under “Materials and Methods.” As shown in Fig. 4, when solutions of bRFC or the p40-p37-p36 complex were passed over a sensor surface on which 33 fmol of PCNA (3,000 RU) was immobilized, an increase in mass on the sensor surface was detected. In this experiment, 27 fmol of bRFC (8,104 RU) was retained on the PCNA-coated chip, corresponding to a stoichiometry of -1 molecule of bRFC bound per molecule of PCNA trimer (Fig. 4A). The p40-p37-p36 complex (4 fmol, 440 RU) also bound a sensor chip to which an equivalent amount of PCNA had been coupled, corresponding to a stoichiometry of -1 molecule of the p40-p37-p36 complex bound per 8 molecules of PCNA trimer (Fig. 4B). Consistent with this result, about 5 times more PCNA was co-immunoprecipitated with RFC than with the p40-p37-p36 complex using polyclonal antibodies against the p37 subunit (data not shown).

The p40-p37-p36 complex did not support the PCNA-dependent DNA elongation reaction catalyzed by pol δ (see below, Fig. 7B). However, the three-subunit complex inhibited the elongation of singly primed M13 DNA catalyzed by the pol δ holoenzyme. DNA elongation reactions were carried out as described under “Materials and Methods” using 4.4 fmol of singly primed M13 DNA. Reactions shown in lanes 1–7 were carried out in the presence of 10 fmol of pol δ, 20 fmol of bRFC, 11.3 fmol of PCNA trimer, and the p40-p37-p36 complex in amounts as follows: lane 1, none; lane 2, 1.12 pmol; lane 3, 0.56 pmol; lane 4, 0.28 pmol; lane 5, 0.14 pmol. The reactions shown in lanes 6 and 7 were carried out in the presence of 10 fmol of pol δ, 20 fmol of bRFC, 560 fmol of PCNA trimer in the presence and absence of 0.28 pmol of the p40-p37-p36 complex, respectively. Total nucleotide incorporation (pmol), measured following acid precipitation and liquid scintillation counting, was as follows: lane 1, 16.8; lane 2, 8.64; lane 3, 12; lane 4, 12; lane 5, 19.1; lane 6, 30.4; lane 7, 29.6.

Fig. 3. The RFC p40-p37-p36 complex preferentially binds to primed DNA. A nitrocellulose filter binding assay was used to examine the DNA-binding properties of the p40-p37-p36 complex as described under “Materials and Methods.” The complex, in amounts as indicated, was incubated with 32P-labeled poly(dA)300, oligo(dT)30, or poly(dA)300-oligo(dT)30 at a molar ratio of 5:1, 1:1, and 1:5, and the interaction monitored as described under “Materials and Methods.” The 100% value represented 40 fmol of input DNA.

DNA primer ends. The selective binding of the p40-p37-p36 complex to primed DNA is similar to that of the five-subunit RFC. Selective binding of primed DNA templates under low ionic condition was also observed with each purified subunit of the three-subunit complex. However, the efficiency of binding was lower (2–10-fold). The requirement for low ionic strength to observe DNA binding by the p40-p37-p36 complex suggests that this three-subunit complex may not be a major factor influencing the binding of RFC to primed DNA.

The RFC p40-p37-p36 Complex Interacts with PCNA and Inhibits PCNA-dependent DNA Elongation Catalyzed by pol δ—The p40 and p140 subunits of hRFC have been previously shown to interact with PCNA (17, 25). To explore further the interaction between RFC subunits and PCNA, we determined the direct interaction between PCNA and the p40-p37-p36 complex using the surface plasmon resonance technique as described under “Materials and Methods.” As shown in Fig. 4, when solutions of bRFC or the p40-p37-p36 complex were passed over a sensor surface on which 33 fmol of PCNA (3,000 RU) had been coupled, an increase in mass on the sensor surface was detected. In this experiment, 27 fmol of bRFC (8,104 RU) was retained on the PCNA-coated chip, corresponding to a stoichiometry of -1 molecule of bRFC bound per molecule of PCNA trimer (Fig. 4A). The p40-p37-p36 complex (4 fmol, 440 RU) also bound a sensor chip to which an equivalent amount of PCNA had been coupled, corresponding to a stoichiometry of -1 molecule of the p40-p37-p36 complex bound per 8 molecules of PCNA trimer (Fig. 4B). Consistent with this result, about 5 times more PCNA was co-immunoprecipitated with RFC than with the p40-p37-p36 complex using polyclonal antibodies against the p37 subunit (data not shown).

The p40-p37-p36 complex did not support the PCNA-dependent DNA elongation reaction catalyzed by pol δ (see below, Fig. 7B). However, the three-subunit complex inhibited the elongation of singly primed M13 DNA catalyzed by pol δ, PCNA, and bRFC. As shown in Fig. 5, the addition of increasing amounts of the p40-p37-p36 complex to this DNA elongation system resulted in an increased inhibition of DNA synthesis, as evidenced by the accumulation of DNA products shorter than full-length (lanes 1–6). The inhibitory effects of the three-subunit complex were pronounced at low levels of PCNA (Fig. 5). As shown in Fig. 5 (lane 7), the addition of high levels of PCNA reversed the inhibition, whereas high levels of pol δ or RFC was less effective (data not presented). These observations suggest that the p40-p37-p36 complex inhibited DNA elongation by competing with the five-subunit RFC for the interaction with PCNA.

Inhibition of the elongation reaction with each of the core
subunits alone was examined. Only the p40 subunit significantly affected the DNA elongation reaction, and its effect was qualitatively different than that observed with the three-subunit complex. The p40 subunit alone markedly decreased the synthesis of full-length M13 DNA without reduction of nucleotide incorporation, and its effect was evident at high concentrations of PCNA, similar to those used in Fig. 5, lane 7 (data not presented).

The RFC p40-p37-p36 Complex Unloads PCNA from DNA—The p40-p37-p36 complex did not load PCNA onto singly nicked circular duplex DNA (data not shown). However, as shown in Fig. 6A, when the three-subunit complex was incubated with P-PCNA complexed with DNA in the absence of ATP, PCNA was displaced from the DNA as shown by the decrease in P-PCNA isolated from the excluded region and the concomitant increase of PCNA detected in the included volume. The amount of PCNA (23, 43, and 55%) displaced from the PCNA-DNA complex increased with the addition of increasing amounts of the p40-p37-p36 complex (6, 12, and 24 pmol, respectively) (Fig. 6, A and B). However, compared with the ATP-dependent bRFC-catalyzed unloading reactions in which 5, 20, and 50 fmol of bRFC protein removed 23, 68, and 88% of the PCNA from DNA, respectively, the efficiency of the three-subunit complex was low (~10-fold less), suggesting that the p38 and/or p140 subunits of hRFC play important roles in the unloading reaction. High levels (11 pmol) of the RFC p40 subunit also unloaded PCNA from DNA (35%) in the absence of ATP but neither the p37 nor p36 subunits unloaded PCNA from DNA under the same conditions (data not shown). These findings suggest that p40 subunit is responsible for the unloading activity of the p40-p37-p36 complex. It should be emphasized that while RFC unloaded PCNA from DNA in an ATP-dependent manner, the unloading of PCNA by the p40-p37-p36 complex or the p40 subunit alone did not require ATP, and the efficiency of the reaction was unaffected by ATP (data not presented).

The RFC p40-p37-p36 Complex Is an Intermediate in the Assembly of the 5-Subunit RFC—Previous studies in which the five cloned human genes of RFC were expressed in an in vitro coupled transcription-translation system showed that these gene products formed a stable five-subunit complex containing approximately equimolar levels of each subunit (20). An examination of the interactions between the RFC subunits indicated that a stable three-subunit core complex containing the p40, p37, and p36 subunits formed that interacted with p38 and p140 only when both subunits were present. These findings suggested a model in which the p38 and p140 subunits bind cooperatively to the core complex in assembling the holoenzyme (20).

The availability of the homogeneous p40-p37-p36 core complex permitted us to examine whether the isolated complex acted as an RFC holoenzyme assembly intermediate. For this purpose we examined whether RFC activity could be reconstituted upon mixing the isolated baculovirus expressed p40-p37-p36 complex with the p140 and p38 subunits. As shown in Fig. 7A, when the p40-p37-p36 complex was incubated with in vitro translated p140 and p38 subunits and immunoprecipitated with antibodies specific for the p37 subunit, a complex containing both P-PCNA-p140 and p38 subunits was formed (lane 5). Consistent with previous findings, the large subunit did not interact with the p40-p37-p36 complex in the absence of the p38 subunit (Fig. 7A, lane 3). However, the p38 subunit was co-immunoprecipitated with the p40-p37-p36 complex in the absence of the p140 subunit (lane 4), although the amount precipitated was about eight times lower than that precipitated when the p140 subunit was also present in the reaction (Fig. 7A, lane 5). This result suggests that hRFC p38 subunit plays an important role in the interaction between the p140 subunit and the p40-p37-p36 core complex.

The products formed in the reactions described in Fig. 7A were examined for their ability to support the elongation of a singly primed M13 DNA in a replication reaction containing HSSB, pol d, and PCNA. As shown in Fig. 7B, the complex containing all five subunits (lane 7) supported DNA synthesis. No replication activity was observed upon omission of RFC (lane 2), p140 and p38 (lane 3), the p40-p37-p36 complex (lane 4), the p38 (lane 5), or the p140 subunits (lane 6). These results are in keeping with the requirement for all five RFC subunits in the DNA elongation reaction. It should be noted that the adsorption of RFC to protein A beads containing antibodies to the p37 subunit reduced the activity of RFC by approximately 70% (compare lanes 1 and 8). Most of this inhibition was due to the presence of protein A beads which reduced incorporation by approximately 50% (compare lanes 1 and 9). Since the p40-p37-p36 complex immunoprecipitated on the beads was in molar excess over the reconstituted five-subunit RFC and could potentially inhibit DNA elongation (see Fig. 5), high levels of PCNA (750 fmol) were used to obviate this problem.

It should also be noted that the above experiments were carried out using in vitro translated RFC p140 and p38 subunits. We have also carried out these experiments using purified RFC p140 and p38 subunits isolated from baculovirus.
in vitro translated p38; lane 7, the p40, p37, p36 complex and in vitro translated p38 was carried out with 50 fmol of bRFC and protein A-agarose beads devoid of any antibodies. Total nucleotide incorporation (pmol), determined following acid precipitation and liquid scintillation counting, was as follows: lane 1, 16.2; lane 2, <0.2; lane 3, <0.2; lane 4, <0.2; lane 5, <0.2; lane 6, <0.2; lane 7, 9.18; lane 8, 8.58; lane 9, 7.92.

**DISCUSSION**

The mechanism of elongation of primed DNA templates during DNA replication is functionally conserved in prokaryotes and eukaryotes. DNA polymerases use a circular “sliding clamp” and a multi-subunit “clamp loader” as accessory factors to achieve their processivity. The clamp loaders isolated from *E. coli*, phage T4, and human all consist of five subunits, capable of hydrolyzing ATP in a DNA-dependent manner, binding to primed DNA templates, and interacting with their corresponding clamp proteins and SSBs (26, 27). The clamp loaders isolated from *E. coli*, phage T4, and human all consist of five subunits, capable of hydrolyzing ATP in a DNA-dependent manner, binding to primed DNA templates, and interacting with their corresponding clamp proteins and SSBs (26, 27). The *E. coli* and human clamp loaders are also clamp unloaders while the T4 clamp dissociates spontaneously from DNA in the absence of the T4 DNA polymerase (9, 28, 29) and thus may not require the product 44/62 to function as a clamp loader. Significant progress has been made in all three systems in assigning functions to the individual subunits of the clamp loaders (summarized in Table 1).

The intrinsic DNA-dependent ATPase activity of RFC is essential for both the catalytic loading of PCNA onto primed DNA and subsequent recruitment of pol ε. We report here that a sub-complex consisting of hRFC p40, p37, and p36 subunits hydrolyzes ATP in a DNA-dependent manner with close to 50% of the efficiency observed with the five-subunit hRFC. The contributions of the p140 and p38 subunits to the hRFC ATPase activity remain to be determined. The finding that the hRFC ATPase activity resides in a core p40-p37-p36 heterotrimer but not with these subunits alone is surprising since the *Saccharomyces cerevisiae* Rfc3 subunit has been reported to contain DNA-dependent ATPase activity (18). Whether this reflects different functions of these evolutionary related subunits or a unique structural problem with the human p36 subunit is presently unclear.

**TABLE I**

| Activity | E. coli (γ complex) | Phage T4 (gp44/62) | Human (hRFC) |
|----------|-------------------|-------------------|-------------|
| Subunits | γ,δ,ε,κ,ψ | gp44,62 | p140, p40, p38, p37, p36 |
| DNA-dependent ATPase | γ,δ,ε,κ,ψ | gp44 | p40, p37, p36 |
| Clamp binding | δ | gp62 | p140, p40, p38, p36 |
| SSB binding | ? | gp44 | ? |
| DNA binding | ? | gp44 | p140, p40, p37, p36 |
| Clamp unloading | δ | NA | p40, p37, p36, p40 |

*a The phage T4 clamp loader contains four gp44 subunits complexed with one gp62 subunit.

**FIG. 7.** The p40-p37-p36 complex is an intermediate in the assembly of the five-subunit RFC. **A**, complex formation after incubation of the p40-p37-p36 complex and the p140 and p38 subunits. Coupled in vitro transcription-translation reactions (12 μl) with plasmid DNAs containing either the coding sequence for hRFC p140 or the p38 subunit were carried out in the presence or absence of the p40-p37-p36 complex (1 pmol) followed by immunoprecipitation (IP) using a polyclonal antibody against the hRFC p37 subunit. The immunoprecipitated products were analyzed by 9% SDS-PAGE followed by autoradiography to visualize the [35S]-labeled p140 and p38 protein products. Shown in lane 1 is 10% of the reaction mixture containing the p40-p37-p36 complex and in vitro translated p140 and p38 subunits prior to immunoprecipitation. Lanes 2–6 represent immunoprecipitation reactions containing various RFC subunits as follows: lane 2, in vitro translated [35S]p140 and [35S]p38; lane 3, the p40-p37-p36 complex and [35S]p140; lane 4, the p40-p37-p36 complex and [35S]p38; lane 5, the p40-p37-p36 complex and the [35S]p140 and [35S]p38 subunits. B, reconstitution of RFC activity. RFC subunits immunoprecipitated on protein A-agarose beads were assayed for their ability to support the elongation of singly primed M13 DNA as described under “Materials and Methods.” Products were analyzed by alkaline agarose gel electrophoresis followed by autoradiography. Reactions shown in lanes 1 and 2 were carried out in the presence and absence of 50 fmol of hRFC, respectively; reactions shown in lanes 3–8 were carried out after immunoprecipitation of RFC subunits as follows: lane 3, the p40-p37-p36 complex; lane 4, in vitro translated p140 and p38; lane 5, the p40-p37-p36 complex and in vitro translated p140; lane 6, the p40-p37-p36 complex and in vitro translated p38; lane 7, the p40-p37-p36 complex and in vitro translated p140 and p38; lane 8, 100 fmol of hRFC. The reaction shown in lane 9 was carried out with 50 fmol of hRFC and protein A-agarose beads devoid of any antibodies. Total nucleotide incorporation (pmol), detected following acid precipitation and liquid scintillation counting, was as follows: lane 1, 16.2; lane 2, <0.2; lane 3, <0.2; lane 4, <0.2; lane 5, <0.2; lane 6, <0.2; lane 7, 9.18; lane 8, 8.58; lane 9, 7.92.
little DNA-dependent ATPase activity, whereas the hetero
dimers γδ and γ′δ′ and the heterotrimer γδδ′ all contain
significant DNA-dependent ATPase activity (30–33). These ob-
ervations are similar to those found with hRFC subunits as
reported here. The hRFC p40 subunit can bind ATP (16, 17)
and thus by analogy with the γ subunit in E. coli may be the
critical site for the ATPase activity of the clamp loader. It is
possible that p40 when expressed alone folds into an aberrant
conformation incapable of hydrolyzing ATP but when coex-
pressed with the p36 and p37 subunits becomes folded into an
active conformation capable of hydrolyzing ATP. In support of
this, the stable p40–p37–p36 complex could not be formed in
vitro by mixing purified p40, p37, and p36 subunits (data not
shown). At present, we cannot rule out the possibility that the
p36 or p37 may act as the catalytic subunit of hRFC ATPase,
since both contain ATP-binding sequences, although neither
purified p36 nor p37 binds ATP (17, data not shown). RFC
complexes reconstituted with subunits individually mutated in
their putative ATP binding sites should define the subunit(s)
essential for ATPase activity.

It was previously reported that RFC bound to DNA through
the large subunit (16) and that the p37 subunit had weak
DNA-binding activity (17). In this report, we provide evidence
that p37, together with the p36 and p40 subunits, possess
DNA-binding activity essential for the DNA dependence of
the ATPase activity. Based on these observations, we suggest
that the p140 subunit mediates the initial RFC DNA binding step
followed by a DNA–p40–p37–p36 interaction required for the
stimulation of ATP hydrolysis.

The p40–p37–p36 complex is not a clamp loader nor does it
support PCNA-dependent DNA synthesis by pol δ, indicating
an essential role for the p140 and/or p38 subunit in the loading
reaction. The unloading of PCNA from DNA may also require multiple
interactions between the different RFC subunits and PCNA.

Following incubation with the p38 and p140 subunits, the
p40–p37–p36 sub-complex can be converted to the five-subunit
complex active in supporting DNA replication. Thus the five-
subunit RFC complex may be assembled in two steps: the
formation of the p40–p37–p36 complex, and the subsequent
recruitment of the p140 and p38 subunits. Since p38 interacts
with both the p140 subunit and the p40–p37–p36 complex, it
may act as a bridge between the large subunit and the three-
subunit core complex. However, the four-subunit complex (p40,
p37, p36, and p38) isolated by immunoprecipitation after mix-
ing the p40–p37–p36 complex with p38 could not be converted to
the five-subunit complex following incubation with the p140
subunit (data not shown), suggesting that p38 and p140 sub-
units bind cooperatively to the core complex. Complexes that
contain less than five subunits could not support PCNA-de-
pendent DNA synthesis, indicating that all five subunits are
required to constitute functional RFC.

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Holoenzyme

A Complex Consisting of Human Replication Factor C p40, p37, and p36 Subunits Is a DNA-dependent ATPase and an Intermediate in the Assembly of the Holoenzyme

Jinsong Cai, Emma Gibbs, Frank Uhlmann, Barbara Phillips, Nina Yao, Michael O’Donnell and Jerard Hurwitz

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