Articles

Expression and Physicochemical Characterization of Human Proliferating Cell Nuclear Antigen†

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ABSTRACT: Human proliferating cell nuclear antigen (PCNA) was overexpressed in *Escherichia coli* as a soluble protein. Recombinant PCNA was purified to homogeneity by phosphocellulose, Q-Sepharose, Sephacryl S-200, and hydroxylapatite chromatography. Approximately 20 mg of PCNA was isolated from *E. coli* cells derived from 2 L of culture. Characterization of the recombinant protein showed that it was functionally active and that its properties were similar to those of purified human placental PCNA. Recombinant PCNA stimulated human DNA polymerase δ activity at least 25-fold with poly(dA)/oligo-(dT) as the template. Recombinant PCNA eluted with a M_r = 102 000 and a Stokes radius of 37 Å by high-performance gel-permeation chromatography. The sedimentation coefficient determined by glycerol gradient ultracentrifugation was 6.3 S. The molecular weight calculated from the Stokes radius and S value was 96 800. The behavior of PCNA was entirely consistent with its being a trimeric protein. Analytical ultracentrifugation and gel filtration revealed the existence of a dimeric species at low dilution. Cross-linking experiments revealed the presence of PCNA dimers which predominated, as well as a trimeric species. These studies provide biophysical evidence that PCNA is an oligomeric protein which behaves as a trimeric species at high protein concentrations but dissociates to a dimer at low protein concentrations.

Proliferating cell nuclear antigen (PCNA) was originally discovered as the antigen that reacted with sera from some patients with the autoimmune disorder systemic lupus erythematosus and whose appearance is restricted to proliferating cells (Miyachi et al., 1978). PCNA is abundant in the nuclei of proliferating cells and is identical to the protein cyclin, whose expression increases in the S phase of the cell cycle (Bravo et al., 1982; Mathews et al., 1984). PCNA synthesis increases in actively proliferating cells in a variety of tissues and species; its levels also increase severalfold immediately before the onset of S phase when quiescent cells are stimulated by serum and growth factors (Mathews et al., 1984). Reduction in the level of immunofluorescence to PCNA antibody by antisense oligonucleotides lowered the mitotic index and inhibited cellular DNA synthesis in Balb/c 3T3 cells (Jaskulski et al., 1988a). Platelet-derived growth factor was found to induce PCNA mRNA levels (Jaskulski et al., 1988b). These results suggested that PCNA was required for DNA synthesis and played a role in the regulation of cellular proliferation. Evidence that PCNA was involved in DNA replication came from studies which demonstrated that PCNA was required for the *in vitro* replication of SV40 DNA in extracts of human 293 (Prelich et al., 1987) and HeLa cells (Wold et al., 1988).

Studies of calf thymus polymerase (pol) δ had led to the discovery of a stimulating protein (Lee et al., 1984). This factor was shown to stimulate the processivity of pol δ and was in fact identical to PCNA (Tan et al., 1986; Prelich et al., 1987). These findings stimulated the study of the tandem roles of pol δ and PCNA in the replication of SV40 DNA, which has led to a significant appreciation of their roles in DNA replication. A current model based on these studies proposes a complex of pol δ, PCNA, and replication factor C (RFC) in leading-strand synthesis. An ordered formation of the complex has been proposed, with RFC binding to the primer terminus, followed by PCNA and pol δ. The function of PCNA is to act as a sliding clamp, while RFC acts to increase the efficiency of binding of PCNA to the primer termini (Tsurimoto & Stillman, 1991; Lee et al., 1991). This model is also supported by parallels with the T4 and *Escherichia coli* replication complexes, in which the T4 gene 45 protein and the *E. coli* holoenzyme β subunit have been shown to function as sliding clamps (Young et al., 1992). Current work also suggests that PCNA is involved in DNA excision repair in response to UV irradiation (Shivji et al., 1992; Nichols & Sancar, 1992; Zeng et al., 1994). Using immunoprecipitation and Western blot experiments, Xiong et al. (1992) showed potential combinatorial interactions of D-type cyclins, cyclin-dependent kinases, and p21 with PCNA. Thus the role of PCNA may be even more elaborate than is presently known.

The three-dimensional structure of the β subunit of the *E. coli* holoenzyme has been determined. It is a torus consisting of a dimeric arrangement of β subunit monomers that accommodates a DNA strand in the central cavity (Kong et al., 1992). The existence of a toroidal structure is entirely consistent with the function of the β subunit as a sliding

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DNA clamp. The same authors suggested a potential structural relationship between the β subunit, mammalian PCNA, and the T4 gene 45 protein on the basis of sequence alignment of the three proteins. In the β subunit each of the monomers possesses three structurally similar domains. Kong et al. (1992) postulated that PCNA is likely to have two of these domains per monomer, based on its smaller size, and that the native PCNA structure would have to be a trimer to form a toroidal structure similar to that of the β subunit.

The molecular mass of human PCNA is 29 261 Da, estimated from its deduced amino acid sequence (Almendral et al., 1987). It behaves on SDS−PAGE with an M_r of *ca.* 32 000 and on gel filtration as a protein of 102 000 (Lee et al., 1991). Thus it appears to be a trimer; this is in conflict with studies of calf thymus PCNA (Tan et al., 1986) and human PCNA (Nichols & Sancar, 1992), which are reported to be dimers. Studies of yeast PCNA have suggested that it may be a trimer or tetramer, although no detailed physicochemical characterization was performed (Bauer & Burgers, 1988). The question of the oligomeric structure of PCNA has not been rigorously examined and is of more than passing interest in view of the analogies made between PCNA, the β subunit of *E. coli*, and the gene 45 protein of T4 bacteriophage as DNA clamps whose function may be manifested in a toroidal structure (Kong et al. 1992; Kuriyan & O'Donnell, 1993). In this study we report the expression of PCNA, its functional characterization, and a study of its physicochemical properties.

EXPERIMENTAL PROCEDURES

Insertion of Human PCNA cDNA into pTACTAC Vector. Polymerase chain reaction (PCR) amplification was used for the insertion of the coding sequence of human PCNA into the pTACTAC vector (Zhang et al., 1992). The template used for PCR was a human PCNA cDNA clone, S14 (a generous gift from Dr. R. Bravo). The sense and antisense primers were based on the human PCNA cDNA sequence (Almendral et al., 1987). The primer 5′ TTTCATATGTTCGAGGCGCGC was used for the 5′ end of the coding sequence with an engineered *Nde*I site (underlined residues) at the initiating methionine codon. The second-strand primer at the 3′ end (5′ GAAAGCTTAAGATCCTTCTTC) had a *Hin*dIII site (underlined residues) after the termination codon (bold). The primers were phosphorylated with T4 polynucleotide kinase before use. The coding sequence for human PCNA was inserted into the pTACTAC vector (Zhang et al., 1992). The *Nde*I site at the initiating methionine codon and a unique *Hin*dIII site upstream of the *trpA* transcription terminator of the vector were used for the introduction of the coding sequence of human PCNA. The PCR conditions used were 94 °C, 1.5 min/55 °C, 2 min/72 °C, 3 min for 30 cycles. The product was a single band of about 0.8 kb which was subsequently purified on a Centricon 100 column followed by phenol/chloroform extraction. After digestion with *Nde*I and *Hin*dIII, the PCR product was heated at 55 °C for 30 min to inactive the restriction enzymes and was then ligated into the pTACTAC vector which had been previously digested with *Nde*I and *Hin*dIII and purified by agarose gel electrophoresis. The construct (pTTPC7) was then used to transform *E. coli* DH5α competent cells. The correctness of the inserted DNA was confirmed by DNA sequencing using the dideoxynucleotide termination method (Sanger et al., 1977). This was done by digestion of pTTPC7 with *Nde*I and *Hin*dIII and purification of the insert by agarose gel electrophoresis, followed by subcloning into the pGEM7z(−) vector (Promega Corp.).

Expression of Human PCNA from pTTPC7. Single colonies from *E. coli* DH5α cells harboring pTTPC7 were used to inoculate 5-mL cultures in Terrific media (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 0.017 M KH_2PO_4, and 0.072 M K_2HPO_4, containing ampicillin). After overnight growth at 37 °C these were used to inoculate 1-L cultures (Terrific media) and grown at 37 °C until the absorbance at 595 nm reached a value of about 0.3. Isopropyl β-thiogalactoside (IPTG) was then added to a concentration of 0.3 mM, and the culture was grown 16−18 h at 26−28 °C.

Purification of Recombinant PCNA. The cells from two 1-L cultures were harvested by centrifugation at 4000*g* at 4 °C for 30 min and resuspended in $^1/_{10}$ volume (200 mL) of buffer (25 mM Tris-HCl, 1 mM EDTA, 25 mM NaCl, 0.01% Nonidet P-40, 2 mM benzamidine, 2 mM pepstatin A, 1 mM phenylmethanesulfonyl fluoride, and 1 mM dithiothreitol, pH 7.4). The cells were disrupted by passage through a French press. The lysate was then centrifuged at 15000*g* for 1 h at 4 °C. The supernatant was then purified as described below.

Phosphocellulose Chromatography. The extract was adjusted to a conductivity equivalent to that of 60 mM NaCl and was loaded onto a phosphocellulose column (5.5 × 30 cm), equilibrated in TGEED buffer (50 mM Tris-HCl, 1 mM dithiothreitol, 0.5 mM EDTA, 0.1 mM EGTA, and 10% glycerol, pH 7.8). The same buffer was used to elute PCNA activity which was not bound to the column.

Q-Sepharose Chromatography. The flowthrough fractions from the phosphocellulose column were loaded onto a Q-Sepharose column (5 × 25 cm) equilibrated with TGEED buffer. The column was washed with the same buffer. This was followed by a linear salt gradient (1000 mL) from 0 to 0.8 M KCl in the equilibration buffer at a flow rate of 60 mL/h. Fractions of 12 mL were collected and the PCNA-containing fractions were identified by SDS−PAGE, Western blotting with a monoclonal antibody (19F4) against PCNA, and assay of the ability of the fractions to stimulate human pol δ activity. The active fractions were pooled and concentrated by ammonium sulfate precipitation and dialyzed against KGD buffer (10 mM potassium phosphate, 10% glycerol, and 1 mM dithiothreitol, pH 7.0).

Sephacryl S-200 Chromatography. The dialyzed material from the Q-Sepharose column (2 mL) was chromatographed on a Sephacryl S-200 column (2.6 × 90 cm) in KGD buffer/0.25 M NaCl. The fractions were analyzed by SDS−PAGE and Western blotting.

Hydroxylapatite Chromatography. The pooled fractions from the Sephacryl S-200 chromatography were loaded onto a hydroxylapatite column (3 × 10 cm) equilibrated in KGD buffer. The column was washed with 2 column volumes of the same buffer. The enzyme was then eluted with a linear salt gradient in a total volume of 400 mL from 0 to 0.3 M potassium phosphate in the equilibration buffer at a flow rate of 60 mL/h. Fractions of 4 mL were collected and determined by SDS−PAGE, Western blotting, and activity assay. Active fractions were dialyzed against KGEED buffer.

Assay of PCNA Activity. Sparsely primed poly(dA)/oligo(dT) was used as the template. The standard reaction (100

μL) contained 0.25 OD unit/mL poly(dA)/oligo(dT) (20:1), 200 μg/mL bovine serum albumin (BSA), 5% glycerol, 10 mM $MgCl_2$, 25 mM HEPES (pH 6.0), 5 μM TTP, and 16 μCi/mL [^{3}H]TTP and human placenta pol δ. Reactions were incubated for 30−60 min at 37 °C. The reactions were terminated by spotting on DE81 filter papers. Filters were washed four times with 0.3 M ammonium formate (pH 7.8) and once with 95% ethanol. The degree of stimulation of pol δ was determined and 1 unit of PCNA activity was defined as by Tan et al. (1986), *i.e.*, one unit caused the increase of 1 nmol of dTMP incorporated into poly(dA)/oligo(dT) at 37 °C per hour with 0.25 unit of pol δ.

Immunoblotting. This was performed using a PCNA monoclonal antibody (mAb19F4, American Biotechnology Inc., Plantation, FL). After electrophoresis in 5−15% gradient gels, the proteins were transferred to nitrocellulose membranes. Prestained protein standards (Sigma Chemical Co.) were used as molecular weight markers and also to provide for visual confirmation of efficient transfer. The nitrocellulose blot was incubated with 3% BSA in phosphate-buffered saline as a blocking agent. The blot was then incubated with mAb19F4 at a final concentration of 5 μg/mL for 12 h at 25 °C. After washing, the blot was then incubated with biotinylated sheep anti-mouse immunoglobulin, followed by incubation with streptavidin−biotinylated sheep anti-mouse immunoglobulin and finally with streptavidin−biotinylated peroxidase preformed complex. Color development was performed by incubation with 4-chloro-1-naphthol and hydrogen peroxide and was terminated with sodium azide.

Processivity Assay. The effect of PCNA on the processivity of pol δ was analyzed by polyacrylamide gel electrophoresis of the reaction products as described by Prelich et al. (1987). [^{32}P]-5′ end-labeled $(dT)_{16}$ was annealed to $(dA)_{2000}$ [poly(dA):oligo(dT) 20:1]. The reaction mixtures (60 μL) contained 0.33 OD unit/mL [^{32}P]poly(dA)/oligo(dT), 40 mM Tris-HCl, pH 6.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 10% glycerol, 0.1 mg/mL bovine serum albumin, 80 μM dTTP, 150 ng of PCNA, and 1 unit of pol δ activity. After incubation at 37 °C for 30 min, reactions were terminated by the addition of 10 μL of 20 mM EDTA and 100 μg/mL salmon testes DNA. The DNA was precipitated with ethanol and dissolved in deionized formamide, 10 mM EDTA, and 0.1% xylene cyanol. The samples were heated at 100 °C for 2 min, cooled on ice, and subjected to electrophoresis on 8% polyacrylamide 8 M urea gels.

Nondenaturing Polyacrylamide Gel Electrophoresis for Determination of Relative Molecular Weights. The procedure used for nondenaturing protein electrophoresis was based on that of Bryan (1977). The nondenatured protein molecular weight markers were purchased from Sigma Chemical Co. A series of concentrations of polyacrylamide gel (from 4.5% to 10%) were used to determine native molecular weight of PCNA by the method of Hedrick and Smith (1968). Gels were stained for protein with Coomassie blue. The $100[\log(R_f \times 100)]$ values (ordinate) were plotted against the gel concentration as percent (abscissa) for each protein. The negative slopes from these graphs (ordinate) were plotted against the known molecular weights of standards (abscissa) on two-cycle log−log paper. PCNA molecular weight was determined from the graphs.

Protein Determinations. These were performed by the Bradford method (1976) using bovine serum albumin as a standard.

Chemical Cross-Linking. The chemical cross-linking of recombinant PCNA was performed with either EGS [ethylene glycol bis(succinimidyl succinate)] or DMS (dimethyl suberimidate, Pierce Chemical Co.). For the reactions with DMS, PCNA was dialyzed into 40 mM HEPES, pH 8.1, 100 mM KCl, 0.2 mM dithiothreitol, and 10% (w/v) glycerol. The DMS solutions (5 mg/mL) were prepared immediately before use in 100 mM HEPES, pH 8.1, and the pH was adjusted to approximately 8.5 by the addition of NaOH. The reaction mixtures (10 μL) for cross-linking with DMS contained 120 μg/mL PCNA and 2 mg/mL DMS in 40 mM HEPES, pH 8.5. The reactions were performed at room temperature and quenched by the addition of ethanolamine to a final concentration of 0.6 M. The EGS was dissolved in dry dimethyl sulfoxide (DMSO) at a concentration of 50 mM just before use. The reaction mixtures (10 μL) for cross-linking with EGS contained 60 μg/mL PCNA and 2.5 mM EGS in 20 mM sodium phosphate, pH 7.5, and 150 mM NaCl. The reactions were performed at room temperature and quenched by the addition of 5 μL of 150 mM Tris/glycine, pH 7.5. The products were analyzed by SDS−PAGE and Western blotting.

Glycerol Gradient Ultracentrifugation. The sedimentation coefficient of recombinant PCNA was determined by ultracentrifugation in 4.8 mL of a 10−30% glycerol gradient in HKED buffer (10 mM HEPES buffer, pH 7.4, 0.5 M KCl, 0.5 mM EDTA, and 1 mM dithiothreitol). The sample (200 μL, 2 mg/mL PCNA) was layered on top of the gradient. The contents were centrifuged at 35000g for 22 h at 4 °C. The tubes were punctured and 36 fractions of 5 drops each were collected. Catalase (11.3 S), aldolase (7.35 S), and BSA (4.4 S) at concentrations of 4 mg/mL were used as the standards. The fractions were assayed for PCNA by stimulation of pol δ activity and the positions of the standards were determined by protein assay.

Sedimentation Equilibrium Analysis. The PCNA sample was dialyzed against reference buffer (20 mM potassium phosphate, pH 7.0, 5% glycerol, and 1 mM DTT). Protein concentrations of 1, 0.5, and 0.25 mg/mL were placed in the outer, middle, and inner cells, respectively, of the centerpiece. Reference buffer was placed in the opposite cell of each pair. The centerpiece was enclosed with sapphire windows and interference window holders. Equilibrium ultracentrifugation was conducted by the meniscus depletion method of Yphantis (1964) in the Beckman Model E ultracentrifuge. The speed was 20 100 rpm, the temperature was 20 °C, and the time for equilibration was 21 h. The interference fringe photos were recorded on Kodak metallographic photographic plates and measured with a Nikon 6C profile projector. Meniscus depletion and attainment of equilibrium were ascertained by examination at several time points. Approximately 15 fringes were read across each image and plotted as ln fringe displacement (centimeters) vs r_2 (distance from the center of rotation in centimeters). The best-fit straight line was used to calculate the apparent (weight-average) molecular weight at each protein concentration, assuming $\bar{v} = 0.74$.

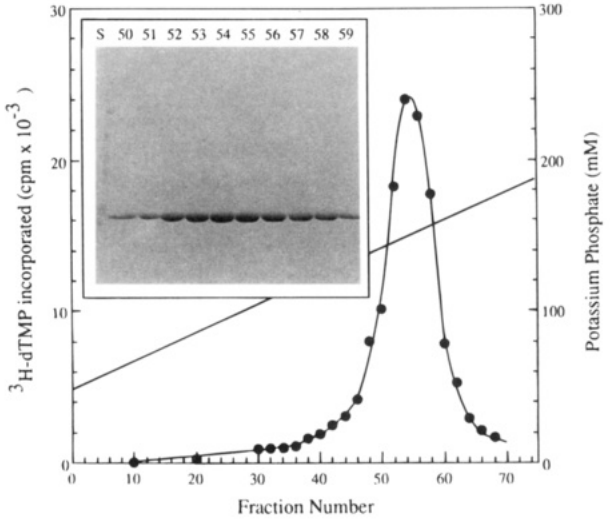

FIGURE 1: Hydroxylapatite chromatograpihy of recombinant PCNA. The active fractions from the Sephacryl S-200 chromatography step were pooled and chromatographed on a hydroxylapatite column (3 × 10 cm) and eluted with a linear potassium phosphate gradient as described in Experimental Procedures. Fractions of 4 mL each were collected and assayed for the amount of [^{3}H]dTMP incorporated into poly(dA)/oligo(dT) template in the presence of pol δ. The inset in the upper left corner shows the Coomassie blue stain of the SDS−PAGE of the fractions across the peak of activity.

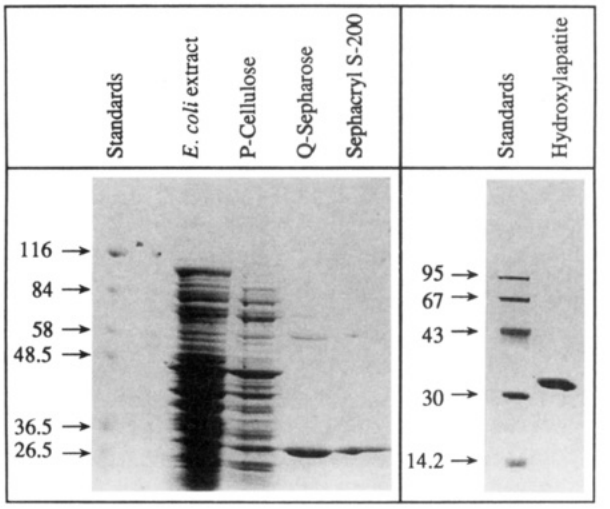

FIGURE 2: SDS−PAGE of PCNA at different steps of purification. Samples (25 μL) of the combined fractions obtained at different steps of purification (Experimental Procedures) were analyzed by SDS−PAGE on a 5−15% gradient gel and stained with Coomassie blue. Left panel: Samples were run on an SDS 5−15% gradient polyacrylamide gel. S refers to the prestained protein standards (α_2-macroglobulin, 180 kDa; β-galactosidase, 116 kDa; fructose-6-phosphate kinase, 84 kDa; pyruvate kinase, 58 kDa; fumarase, 48.5 kDa; lactate dehydrogenase, 36.5 kDa; triosephosphate isomerase, 26.5 kDa). Right panel: Samples were run on an SDS−7% polyacrylamide gel. S refers to the protein standards (Bio-Rad, phosphorylase, 95 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; α-lactalbumin, 14.2 kDa).

RESULTS

Expression and Purification of Recombinant Human PCNA. The coding sequence of human PCNA was isolated by PCR amplification from its cDNA (Almandral et al., 1987) and inserted into the pTACTAC vector (Experimental Procedures). Subsequent reisolation and DNA sequencing showed that the insert contained the correct sequence encoding an open reading frame of 261 amino acid residues as expected (Almendral et al., 1987). The insertion of the coding sequence for human PCNA into pTACTAC vector allowed its expression as a soluble protein. After optimization of the growth conditions, it was estimated that the levels of expression were about 4% of the soluble *E. coli* protein.

Purification of Human PCNA. Recombinant PCNA was purified to homogeneity by chromatography on phosphocellulose, Q-Sepharose, Sephacryl S-200, and hydroxylapatite (Experimental Procedures). The elution behavior of the protein at the final purification step on hydroxylapatite is shown in Figure 1. The purification of PCNA was monitored by assay of the stimulation of purified human placental pol δ activity and by SDS−PAGE and Western blotting. The progress of the purification as monitored by SDS−PAGE is shown in Figure 2. The molecular mass of the purified PCNA on SDS−PAGE was estimated to be 32.5 kDa (Figure 2, right panel). This is consistent with our previously observed behavior of PCNA isolated from human placenta (Lee et al., 1991; Yang et al., 1991) and slightly higher than the calculated molecular mass of 29 261 (Alemandral et al., 1987). The yields of PCNA from a typical preparation are summarized in Table 1. About 20 mg of purified protein could be obtained from 2 L of cell culture with about 40% recovery. The final specific activity was about 15 000 units/mg of protein, which is comparable to that of the calf thymus PCNA (Tan et al., 1986). Purified recombinant PCNA was stable on storage at −70 °C over periods of half a year with no loss of activity.

Table 1: Purification of Recombinant Human PCNA

step	protein (mg)	activity (units)	specific activity (units/mg)	recovery (%)
extract	4190			
phosphocellulose	3280	806 880	246	100
Q-Sepharose	195	580 000	2970	72
Sephacryl S200	47	399 870	8470	50
hydroxylapatite	21.5	314 340	14600	39

Functional Comparison of Recombinant and Human Placental PCNA. The activities of human placental and recombinant PCNA were compared by their effects on purified human placental pol δ. The results show that the degrees of stimulation of pol δ by placental and recombinant PCNA were almost identical (Figure 3). Recombinant PCNA, like the protein isolated from human placenta, stimulated the processivity of human placental pol δ on sparsely primed templates when the reaction products were examined by gel electrophoresis (Figure 4). Thus, recombinant PCNA is functionally similar to the wild-type protein.

Native M_r of Recombinant Human PCNA As Determined by HPLC Gel Filtration and Glycerol Gradient Ultracentrifugation. Purified recombinant PCNA was chromatographed on a Protein Pak Glass 300SW HPLC column which had been calibrated with appropriate protein standards. PCNA was assayed by its stimulatory activity on pol δ and eluted as a single peak with an estimated molecular weight of 102 000 (Figure 5A). This is consistent with our previous findings of PCNA isolated from human placenta (Lee et al., 1991). SDS−PAGE of the active fractions confirmed the presence of the PCNA as a single polypeptide band of 32.5 kDa (not shown). The Stokes radius was determined to be 37.2 Å in the same experiment by a plot of $[-\log K_{av}]^{1/2}$ vs Stokes radius. This value is similar to the that of 36.5 Å

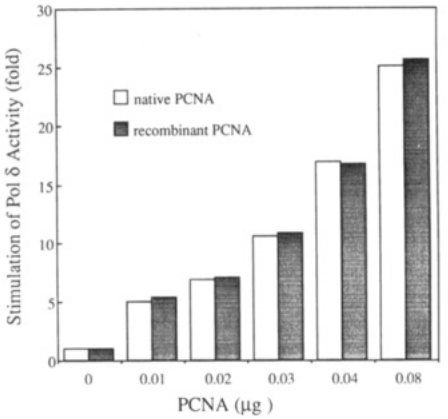

FIGURE 3: Stimulation of pol δ activity by native and recombinant PCNA. Human pol δ and PCNA were purified from human placenta as described by Lee et al. (1991). Recombinant PCNA was purified from 2 L of *E. coli* cells harboring the pTTPC7 plasmid after IPTG induction (Experimental Procedures). The diagram shows the stimulation of pol δ activity on poly(dA)/oligo(dT) by native and recombinant PCNA.

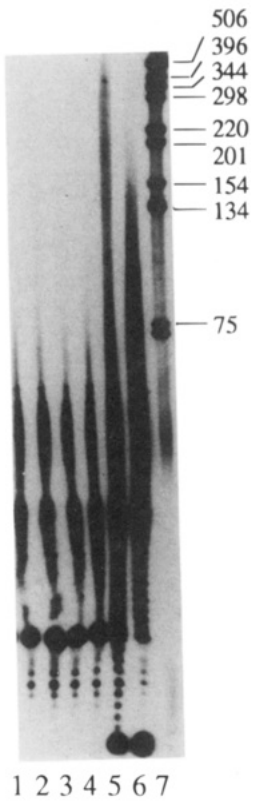

FIGURE 4: Effects of native and recombinant PCNA on the processivity of pol δ. Reactions were performed as described in Experimental Procedures and contained 1 unit of pol δ/assay and, where added, 150 ng of purified human PCNA or recombinant PCNA. Reaction mixtures were analyzed on polyacrylamide gels as described in Experimental Procedures. Lane 1, no additions; lane 2, pol δ alone; lane 3, human PCNA alone; lane 4, recombinant human PCNA; lane 5, pol δ + human PCNA; lane 6, pol δ + human recombinant PCNA; lane 7, 1-kb DNA ladder.

determined for calf thymus PCNA by Tan et al. (1986). The sedimentation coefficient of native recombinant PCNA was determined to be 6.3 S by glycerol gradient ultracentrifugation (Figure 5B). The molecular weight, calculated by the method of Siegel and Monty (1966) from the sedimentation coefficient and Stokes radius, was 96 800. This is higher than the value of 75 000 calculated for the calf thymus PCNA by Tan et al. (1986), based on a lower S value of 5.0 for the calf thymus protein.

Table 2: Estimated Subunit Ratios for PCNA[a]

procedure	mol wt	ratio SDS−PAGE	ratio mol mass
gel filtration	102 000	3.1	3.5
nondenaturing gel electrophoresis	96 000	3.0	3.2
calculated from S and Stokes radius	96 800	3.0	3.3
sedimentation equilibrium	68 000	2.1	2.3

[a] The ratios were calculated on the basis of the SDS−PAGE monomer weight (32 500) and the estimated molecular mass from the protein sequence (29 261).

Analysis of the Native Molecular Weight of Recombinant PCNA by Nondenaturing Polyacrylamide Gel Electrophoresis. The molecular weight of recombinant PCNA was determined by the method of Hedrick and Smith (1968). This method involves electrophoresis in gels of different acrylamide compositions under nondenaturing conditions. The relative mobilities were plotted against gel concentration, and the slopes of the lines were determined. Estimation of the molecular weight was made by a secondary plot of the negative slopes against the molecular weights for the protein standards. This gave an estimated molecular weight of 96 000 for PCNA (Figure 5C). This is consistent with the molecular weight obtained by gel filtration, as expected since both methods depend on the migration of the protein through a porous matrix.

Determination of Molecular Weight by Analytical Ultracentrifugation. The molecular weight of recombinant PCNA was determined by sedimentation equilibrium in the analytical ultracentrifuge as described in Experimental Procedures. The weight-average M_r was found to vary depending on the protein concentration, from 83 300 at 1 mg/mL to 71 700 at 0.25 mg/mL. The results are shown in Figure 5D. It is seen that extrapolation to zero protein concentration yields a M_r of 68 000. These results are consistent with the formation of a PCNA trimer at high concentrations of protein, with dissociation to a dimer at lower protein concentrations. No evidence for a monomeric form was apparent. These results should be taken in combination with those from other procedures, particularly gel filtration (Table 2). It is concluded that PCNA behaves as a trimer in solution at protein concentrations above 1 mg/mL but is capable of dissociation to a dimer at low protein concentrations.

Demonstration of the Oligomeric Nature of PCNA by Chemical Cross-Linking. From the previous experiments it is apparent that the native molecular weight of PCNA, determined by several procedures, is in the range of 90 000−100 000, consistent with its being a trimer. The sedimentation equilibrium analysis confirmed that PCNA is a trimer in solution but also indicated that it can dissociate to a dimer at low dilution. In order to obtain physical evidence for the existence of oligomeric species, the effects of several cross-linking agents on PCNA were examined. The two that were tested were DMS and EGS. DMS, under the conditions tested, failed to cross-link PCNA (Figure 6, top). EGS, with a longer cross-linking distance (16.1 Å) than DMS (11 Å), was able to cross-link PCNA (Figure 6). EGS rapidly cross-linked PCNA at a concentration of 0.06 mg/mL to a dimeric species within 1 min; after 15 min, a trimeric species was apparent but never became the predominant species (Figure 6, top). The lack of effect of DMS also provides additional evidence demonstrating that the cross-linking seen with EGS

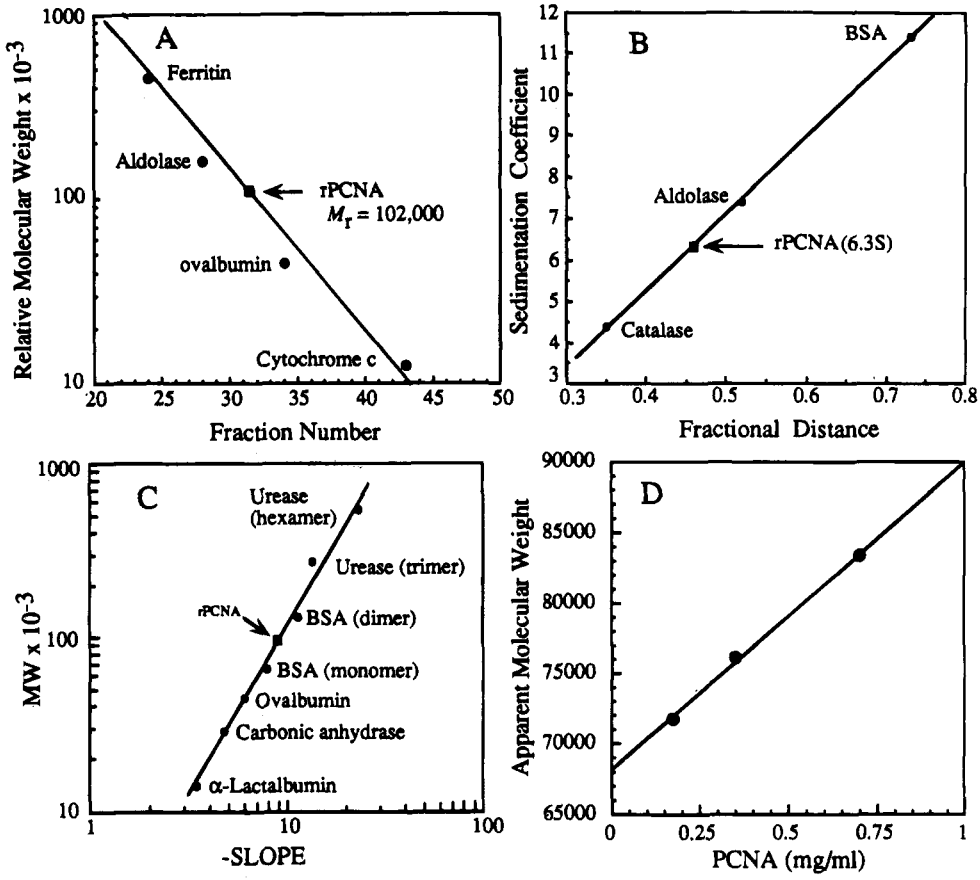

FIGURE 5: Determination of the molecular weight of recombinant PCNA. (A) Determination of size by gel filtration. Purified recombinant PCNA (0.2 mg in 0.25 mL) was chromatographed on a Protein Pak Glass 300SW column on a Waters 650 HPLC system which had been calibrated with appropriate protein standards (ferritin, 450 kDa; aldolase, 158 kDa; ovalbumin, 45 kDa; cytochrome *c*, 12.5 kDa). PCNA was assayed by its stimulatory activity on pol δ. The elution position of recombinant PCNA, assayed by its ability to stimulate pol δ activity, is indicated with an arrow. (B) Glycerol gradient ultracentrifugation. The sedimentation coefficient of recombinant PCNA was determined by ultracentrifugation in a 10−30% glycerol gradient (Experimental Procedures). Catalase (11.3 S), aldolase (7.35 S), and BSA (4.4 S) at concentrations of 4 mg/mL were used as the standards. The fractions were assayed for PCNA by stimulation of pol δ activity and the positions of the standards were determined by protein assay. (C) Determination of size by nondenaturing polyacrylamide gel electrophoresis. Purified recombinant PCNA (3 μg/lane) was electrophoresed with protein standards in nondenaturing polyacrylamide gels of differing polyacrylamide concentrations (Experimental Procedures). The migration distances for each protein were plotted against the gel concentrations, and the negative slopes of the plots were replotted against molecular weights as shown above. The protein standards (Sigma Chemical Co.) were α-lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA monomer (66 kDa), BSA dimer (132 kDa), urease trimer (272 kDa), and urease hexamer (545 kDa). The position of recombinant PCNA is indicated by an arrow. (D) Determination of size by analytical ultracentrifugation. The weight-average values of M_r were determined by the Yphantis meniscus depletion method (see Experimental Procedures). Data are plotted as M_r versus protein concentration of PCNA. Extrapolation to zero protein concentration gives M_r 68 000 for PCNA.

is intramolecular. Little increase in the relative amount of trimeric species was observed under conditions where much higher concentrations of EGS (up to a 10-fold increase) or prolonged reaction times (up to 1 h) were used; rather, formation of a tetrameric species was observed under conditions where intermolecular cross-linking could be expected (not shown). When PCNA concentrations were increased up to 0.4 mg/mL, the dimer remained the predominant cross-linked species (Figure 6, bottom). These findings are consistent with two possibilities: first, that after initial cross-linking of the trimeric species of PCNA a rapid dissociation to the dimeric cross-linked form had occurred, or second, that there is a significant amount of the dimeric species present. In studies of the T4 gene 45 protein, which is thought to be toroidal, cross-linking resulted in appearance of linear trimeric species as well as an additional slower migrating form which represented the cross-linked toroidal species. The latter exhibited a slower migration rate, similar to what is observed with internally cross-linked polypeptides (Jarvis et al., 1989). Such a species was not observed in our experiments.

In the case of the β subunit of *E. coli* holoenzyme, the intersubunit contacts have been shown to involve the N- and C-terminal regions (Kong et al., 1992). Examination of the primary sequence of PCNA indicates that it possesses a number of lysyl residues in the N- and C-terminal regions which could potentially be involved in cross-linking by EGS, *i.e.*, if PCNA indeed is analogous to the β subunit, "head-to-tail" cross-linking by EGS is possible. We have expressed a mutant of PCNA in which the C-terminal 19 residues have been deleted. The truncated PCNA elutes on gel filtration much later than the dimeric form of PCNA, consistent with its being monomeric (Figure 7). In addition, it no longer undergoes cross-linking with EGS (inset, Figure 7) and is functionally incompetent in the activation of pol δ (not shown).

Effect of Protein Concentration and Mg^{2+} on the Oligomeric State of PCNA. Because the analytical ultracentrifugation data and the cross-linking experiments indicated that PCNA can dissociate to a dimer, we reexamined its behavior

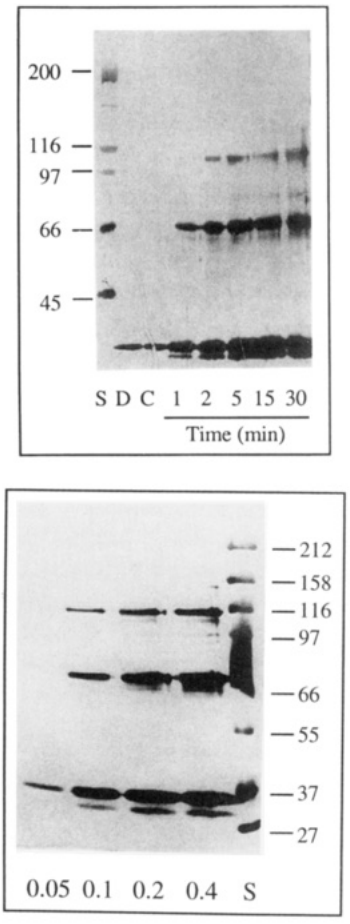

FIGURE 6: Cross-linking of PCNA by ethylene glycol bis-(succinimidyl succinate). (Top) PCNA was reacted with EGS for the indicated times and the samples were examined by SDS−PAGE on 5−15% gradient gels followed by silver staining as described in Experimental Procedures. S refers to the protein standards (Bio-Rad; myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase, 97 kDa; BSA, 66 kDa; ovalbumin, 45 kDa). D refers to the reaction of PCNA with dimethyl suberimidate (DMS) for 30 min. C refers to a control mixture with no added EGS. (Bottom) PCNA reacted with EGS at the final concentrations shown in milligrams per milliliter with EGS for 30 min as described in Experimental Procedures. S refers to the protein standards (New England Biolabs; myosin, 212 kDa; maltose binding protein−β-galactosidase fusion protein, 158 kDa; β-galactosidase, 116 kDa; phosphorylase, 97 kDa; serum albumin, 66 kDa; glutamic dehydrogenase, 55 kDa; lactate dehydrogenase, 37 kDa; triosephosphate isomerase, 27 kDa.

during gel filtration as a function of protein concentration (Figure 8). The results show that, at 0.4 mg/mL, PCNA elutes as a single peak as previously observed (*cf.* Figure 5A). At 0.1 mg/mL and at 0.04 mg/mL, a biphasic size distribution is observed. The apparent molecular weights of the two peaks were in a ratio of 3.0:2.2, so that the two peaks represent trimer and dimer forms. Mg^{2+} (10 mM) was found to affect the dimer−trimer equilibrium. As can be seen (Figure 8), Mg^{2+} favors the dissociation of the trimeric species; this is particularly evident for the experiments at 0.04 mg/mL PCNA, where complete dissociation to the dimer is observed. At a starting sample concentration of 0.1 mg/mL, complete dissociation to the dimeric species was observed on gel filtration when the Mg^{2+} concentration was increased to 50 mM (not shown).

DISCUSSION

Human PCNA was expressed as a recombinant protein in the pTACTAC vector as a soluble protein. The properties

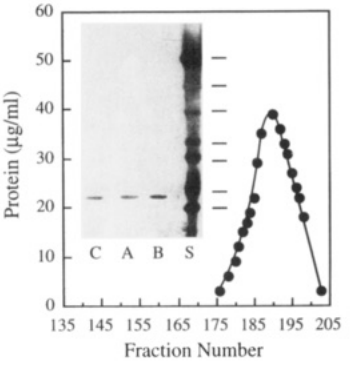

FIGURE 7: Truncated PCNA behaves as a monomeric species. The purified truncated mutant of PCNA (residues 1−242, 1.2 mg/mL, 0.4 mL) was subjected to gel filtration on a Protein Pak Glass 300SW HPLC column (Experimental Procedures). Fractions of 60 μL were collected and the protein content of each fraction was determined (solid circles). The elution of the protein was consistent with its being a monomer (cf. Figure 8). The insert shows an SDS−polyacrylamide gel of truncated PCNA after cross-linking with EGS as for the experiment in Figure 6. The protein concentration was 0.2 mg/mL, and 0.5 μg was loaded on each lane. C refers to a control without added EGS; A and B are reactions of the mutant PCNA with 2 mg/mL EGS for 30 min at protein concentrations of 0.1 and 0.2 mg/mL, respectively; S refers to protein standards (α_2-macroglobulin, 180 kDa; β-galactosidase, 116 kDa; fructose-6-phosphate kinase, 84 kDa; pyruvate kinase, 58 kDa; fumarase, 48.5 kDa; lactate dehydrogenase, 36.5 kDa; triosephosphate isomerase, 26.5 kDa).

of recombinant PCNA were found to be indistinguishable from those of native human PCNA (Lee et al., 1991) in terms of its behavior on SDS−PAGE, immunoreactivity to a monoclonal antibody against human PCNA, and functional ability to stimulate human pol δ. The yields (*ca.* 10 mg/L of cell culture) make this a useful system for the preparation of PCNA; by comparison, yields from human placenta are generally about 100 μg/placenta (Lee et al., 1991). Thus, this system is useful for the preparation of PCNA and is suitable for structure−function studies of the protein by mutagenesis. Yeast and human PCNA have been expressed using a T7 promoter-driven vector with yields of 15 mg/2-L culture, but the physicochemical properties of the protein were not reported (Tsurimoto & Stillman, 1991; Fien & Stillman, 1992).

A significant aspect of the present work is the characterization of the native molecular weight of human PCNA and the determination that it exists as an equilibrium mixture of dimeric and trimeric oligomers. We had previously observed that purified human placental PCNA eluted on gel filtration with an apparent molecular weight of 102 000 (Lee et al., 1991), suggesting that human PCNA was a trimer, in apparent contradiction to the report that calf thymus PCNA is a dimer (Tan et al., 1986). However, because of the small amounts of material available, it could not be eliminated that this might be due to the association of PCNA with other proteins or to an asymmetric nature of the protein which could result in a disproportionately larger apparent M_r on gel filtration. Such discrepancies between molecular mass and relative molecular weights can be extremely large. For example, the phosphatase inhibitor 2 protein with a molecular mass of 23 kDa behaves on gel filtration with a M_r of 80 000, while the phosphatase inhibitor 1 protein with a molecular mass of 16 kDa behaves as a protein of 60 000 (Shenolikar & Nairn, 1990). Thus, the oligomeric nature of PCNA could not be taken for granted on the basis of comparison of

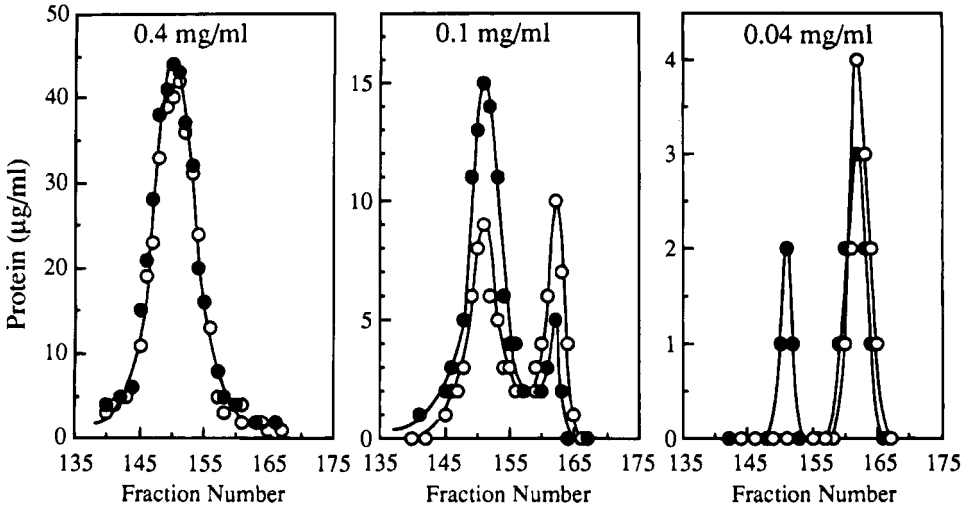

FIGURE 8: Concentration dependence of the oligomeric state of PCNA on gel filtration. Purified PCNA was dialyzed against 50 mM HEPES, 80 mM NaCl, 0.1 mM EDTA, 0.02% sodium azide, and 5% glycerol, with and without 10 mM $MgCl_2$. The protein concentrations of the solutions were adjusted to 0.4, 0.1, and 0.04 mg/mL. Each sample (0.4 mL) was chromatographed on a Protein Pak Glass 300SW HPLC column; fractions (60 μL) were collected at a flow rate of 0.6 mL/min. The protein content of each fraction was determined. The column was then calibrated with protein standards as for Figure 5A. The results obtained for the PCNA solutions with starting concentrations of 0.4, 0.1, and 0.04 mg/mL are shown from left to right. Data in the absence of Mg^{2+} are shown as solid circles, and data in the presence of Mg^{2+} are shown as open circles.

molecular weights determined by SDS−PAGE and gel filtration.

In the studies reported here we have used a number of procedures to examine the oligomeric nature of PCNA. Our results obtained using several procedures for molecular weight determination (summarized in Table 2), as well by chemical cross-linking data, establish beyond a reasonable doubt that PCNA is a homotrimer. This confirms the hypothesis of Kong et al. (1992) that PCNA is a trimer. Our studies show that the behavior of PCNA is more complex in that it does not behave simply as a trimer but is capable of existing as a dimer at low protein concentrations. Taken together, our findings provide evidence that there may be a significant equilibrium between dimeric and trimeric forms of PCNA. These findings would also resolve differences between our data and that for calf thymus PCNA, which has been reported to be a dimer (Tan et al., 1986), since the predominant species observed depends on the protein concentrations used in the analysis.

As already noted, our data do not provide evidence for the existence of a toroidal form of PCNA in solution on its own, but they do not preclude the possibility that both linear and toroidal trimers could exist. This could have emerged from cross-linking studies, since cross-linking could also have revealed the presence of species that migrated more slowly expected for a trimer, *i.e.*, a cross-linked toroid (rather than a linear trimer), as has been shown by cross-linking of the gene 45 protein (Jarvis et al., 1989). The formation of such a species is predictable, based on the symmetry of subunit interactions required for the formation of a torus. The failure to observe a toroidal cross-linked form may be due to the dissociation of the trimer after cross-linking, or the linear and toroidal trimer forms may exist in an equilibrium that strongly favors the linear trimer. Alternatively, it may be that interaction with RFC (see below) is needed for formation of the toroidal form.

The behavior of PCNA in free solution also needs to be considered in the light of its interactions with the primer−template, pol δ, and RFC. Studies of yeast pol δ/PCNA interactions with singly primed linear and circular template−primers (Burgers & Yoder, 1993) have shown that PCNA-dependent pol δ activity was only observed for the linear species, leading to a proposal that the mechanism of PCNA binding (in the absence of ATP and RFC) must involve sliding of a preformed torus onto the DNA strand. While indirect, this provides attractive evidence for the existence of a toroidal form. It also suggests that linear trimers of PCNA either are not present or are incapable of functional interaction with pol δ in the absence of other factors. Other studies, using gel retardation assays with model oligonucleotide template−primers, have shown that pol δ and PCNA interact with and bind strongly to the template−primer (Tsurimoto & Stillman, 1991; Ng et al., 1993). The view that only the preformed toroidal form is functional is seemingly at odds with our findings that the only demonstrable forms are linear dimer and trimeric species; however, it should be borne in mind that in these experiments with yeast PCNA a large excess of PCNA was used (Burgers & Yoder 1993). Thus, it is possible that under these conditions a sufficient amount of the toroidal species was present to provide for the results observed even if it is present in low amounts.

Our data provide evidence for a hitherto unsuspected behavior of PCNA, namely, that it is readily dissociated to a dimer at low protein concentrations. The concentrations at which significant dimer formation was observed are in the micromolar range. The levels of PCNA are reported to be about 0.015% of the total protein in HeLa cells (Morris & Mathews, 1989), so that the estimated cellular concentration would also be in the micromolar range. This suggests that free PCNA may be a dimer *in vivo*, although the physiologically relevant concentration within the nucleus could be much higher. Certainly, at the concentrations where PCNA is normally used for in vitro assays of pol δ activity, it would be expected to be largely in the dimeric form. The association−dissociation behavior of PCNA is reminiscent of that of the *E. coli* DNA polymerase III holoenzyme β subunit, which is dissociated from a dimeric to monomeric

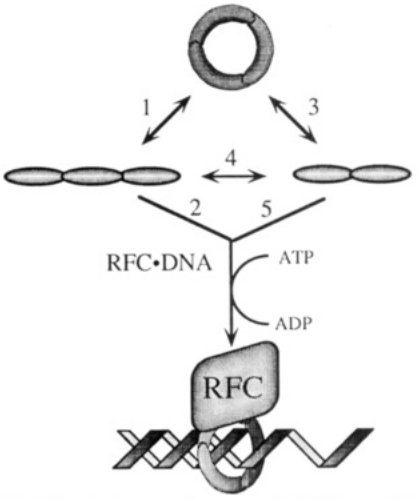

FIGURE 9: Model of the recruitment of PCNA to DNA by RFC that takes into account the potential interconversion of PCNA oligomers. The model takes into account the findings that PCNA exists as an equilibrium between linear dimers and trimers, and possibly also a toroidal trimer form. For explanation see text.

form by Mg^{2+} (Griep & McHenry, 1988). The discrete separation of dimer and trimer peaks of PCNA observed on gel filtration, rather than a continuous shift in molecular weight, was somewhat surprising since it suggests that the dimer−trimer equilibrium is relatively slow. Since it is the trimeric form that is functional, this provides for the possibility that control of the rate and direction of the equilibrium could possibly underlie the regulation of PCNA function by other proteins. Recent studies of the recombinant 40-kDa subunit of RFC have shown that it is capable of binding to PCNA (Pan et al., 1993), and it will be of interest to determine the effects of this subunit on the association−dissociation behavior of PCNA. Thus, our findings that there is a dimer−trimer equilibrium in which the dimer predominates at low protein concentrations, and the failure to observe the toroidal species, has implications in that these oligomeric interconversions may provide insights into the potential mechanism by which PCNA is loaded onto the DNA primer terminus in a functional toroidal form by RFC. Extensive studies have detailed the functions of RFC, also known as A1, a five-subunit protein complex that is required for the loading of PCNA at the primer−template site and is then followed by interaction with pol δ. RFC possesses DNA-stimulated ATPase activity, and PCNA further stimulates ATPase activity of the RFC•DNA complex (Lee & Hurwitz, 1990; Lee et al., 1991; Tsurimoto & Stillman, 1991; Burgers, 1991). RFC exhibits functional analogies to the T4 gene 44/62 proteins (Tsurimoto & Stillman 1990; Young et al., 1992; Jarvis et al., 1989) and the *E. coli* γ complex (O'Donnell et al., 1993) in being involved in the formation of the replication complex by the "loading" of the sliding clamp onto DNA and the facilitation of the binding of the DNA polymerase.

Our findings can be accommodated in this view of RFC function if it is proposed that the recruitment of PCNA to the primer−template terminus by RFC involves the conversion of the linear PCNA to the toroidal form *in situ*. This is shown diagrammatically in Figure 9. In this model, RFC binds to the primer terminus and serves to recruit PCNA. The mechanism by which PCNA, largely present as a dimer−linear trimer mixture, engages the RFC•DNA strand as the toroidal form involves the following possibilities. The RFC•DNA complex may interact with the linear PCNA trimer and cause a conformational change that results in a linear trimer to torus interconversion (2). If PCNA preexists in the toroidal form in the free state, then the effect of RFC may be to drive the opening and closing of the toroid (1 and 2). Since PCNA may exist in equilibrium with a dimeric form (3, 4), a third possibility is that RFC may interact with the dimeric species (bearing in mind that the cellular concentration of PCNA may be low enough that this may be the predominant species) and cause a conformation change that drives the assembly of the torus (5). It has been found that ATP binding to RFC is required for formation of the complex of RFC, DNA, and PCNA and that ATP hydrolysis is required for efficient functional loading of PCNA (Burgers & Yoder 1993), although it is noted that PCNA binding can be achieved with a nonhydrolyzable ATP analog (Lee & Hurwitz, 1990). The hypothesis shown in Figure 9 is attractive since it provides an additional rationale for the role of RFC, which might involve not only the binding of PCNA but also the induction of conformational changes that drive the formation of a PCNA torus, with the participation of ATP hydrolysis providing the energy. In this context, Sancar and Hearst (1993) have proposed that proteins that promote stable complexes between the two proteins, using ATP hydrolysis to drive the conformational changes involved, represent examples of what they have termed "molecular matchmakers". The properties of RFC are largely consistent with the definition of a molecular matchmaker as described by Sancar and Hearst (1993).

ADDED IN PROOF

The crystal structure of yeast pCNA has been recently determined by Krishna et al. (1994). These authors have shown that pCNA does indeed form a trimeric ring.

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