The diverse function of proliferating cell nuclear antigen (PCNA) may be regulated by interactions with different protein partners. Interestingly, the binding sites for all known PCNA-associating proteins are on the outer surface of the C termini (“front”) sides of the PCNA trimer. Using cell extracts and purified human PCNA protein, we show here that two PCNA homotrimers form a back-to-back doublet. Mutation analysis suggests that the Arg-5 and Lys-110 residues on the PCNA back side are the contact points of the two homotrimers in the doublet. Furthermore, short synthetic peptides encompassing either Arg-5 or Lys-110 inhibit double trimer formation. We also found that a PCNA double trimer, but not a homotrimer alone, can simultaneously accommodate chromatin assembly factor-1 and polymerase δ. Together, our data supports a model that chromatin remodeling by chromatin assembly factor-1 (and, possibly, many other cellular activities) are tightly coupled with DNA replication (and repair) through a PCNA double trimer complex.

Proliferating cell nuclear antigen (PCNA) is involved in the regulation of a wide spectrum of biological functions, including DNA replication, repair, cell-cycle control, and chromatin remodeling (1–5). PCNA may exist in the cell as a homotrimer, but not a homotrimer alone, can simultaneously accommodate chromatin assembly factor-1 and polymerase δ. Together, our data supports a model that chromatin remodeling by chromatin assembly factor-1 (and, possibly, many other cellular activities) are tightly coupled with DNA replication (and repair) through a PCNA double trimer complex.

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The abbreviations used are: PCNA, proliferating cell nuclear antigen; CAF-1, chromatin assembly factor; CHO, Chinese hamster ovary cell; pol, polymerase; GFP, green fluorescent protein; PBS, phosphate-buffered saline.
Fig. 1. PCNA exists as a double-trimer complex in mammalian cells. A, CHO cells were cross-linked using formaldehyde for 30, 90, or 180 min (lanes 2, 3 and 4, respectively) prior to extraction. Proteins were separated on 8% SDS-PAGE, followed by immunostaining with anti-PCNA antibody (PC10). Lane 1, no cross-link; DT, T, and Mono denote double trimer, single trimer, and monomer PCNA molecules, respectively. B and C, two-dimensional PAGE analysis indicated that the PCNA monomer, homotrimer, and double trimer all have the same pI (4.57). Cell extracts were treated with 1.5% formaldehyde for 20 (B) or 90 min (C). Immunostaining was carried out with PC10. D, bacterially expressed human PCNA was purified with a combination of Sephacryl and UnoQ chromatography, followed by further purification using Superdex 200 10/300 GL. The x- and y-axes are fraction numbers and integral density (IOD) of PCNA detected by Western blotting, respectively. Arrowheads 1–4 are the positions of β amylase (molecular weight, 200,000), alcohol dehydrogenase (molecular weight, 150,000), bovine serum albumin (molecular weight, 68,000), and carbonic anhydrase (molecular weight, 29,000), respectively. E, samples taken from fractions 62 (lanes 1 and 2), 64 (lanes 3 and 4), 76 (lanes 5 and 6), 78 (lanes 7 and 8), or purified from SDS-PAGE (lanes 9 and 10) were either not (−) cross-linked (X-link) (lanes 1, 3, 5, 7, and 9) or cross-linked (+) (lanes 2, 4, 6, 8, and 10). Note that samples isolated from SDS-PAGE were renatured prior to this experiment. The samples were analyzed by PAGE-Western blotting using a PC10 antibody. D denotes the PCNA dimer.

1 mM EDTA, 10% glycerol, 0.5% Triton X-100, 1.0 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture. The extracts (1 mg/ml protein) were then incubated at 4 °C overnight with agarose-conjugated PC10 monoclonal anti-PCNA antibody. The immunoprecipitate was washed four times with PBS after which PCNA complexes were dissociated from antibodies by treatment with elution buffer (0.1 M glycine, 0.125 M NaCl, pH 2.8). The pH of the supernatant was immediately adjusted to 7.0 using 1.0 M Tris, followed by a second immunoprecipitation, using 10 μl of a monoclonal antibody against the 150-kDa catalytic subunit of pol δ (C-2, Santa Cruz Biotechnology). Subsequently, the immunoprecipitate was washed four times with PBS, eluted with LDS sample buffer, and boiled for 30 s. The proteins were then analyzed by SDS-PAGE and Western blot analysis. For a negative control, normal mouse IgG (Santa Cruz) was used.

For the co-immunoprecipitation and subsequent Western blot analysis described in Fig. 4, D–F, total cell extracts of CHO cells transfected with either wild type or the R5A/K110A mutant PCNA construct were prepared as described previously (25). Briefly, cell extracts prepared in 800 μl of radioimmune precipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and the mixture of protease inhibitors) were centrifuged at 8,000 × g for 10 min. The supernatant was collected (first extract), and the pellet was resuspended in 50 μl of DNA digestion buffer (25 mM Tris, pH 7.4, 5 mM MgCl₂), and treated with DNase I (10 units) for 10 h at 4 °C. The DNA digestion was stopped by adding 100 μl of 9.0 M urea and 2% Nonidet P-40. The supernatant (second extract) was collected after the solution was centrifuged for 10 min at 10,000 × g. The first and the second extracts were then combined (−1.5 mg/ml protein) and subjected to immunoprecipitation, using 10 μl of a monoclonal antibody against the 150-kDa subunit of CAF-1 (NB 500-207, Novus Biologicals, Littleton, CO), followed by incubation overnight with 40 μl of protein A/G-agarose (Santa Cruz Biotechnology). The immunoprecipitate was washed four times with PBS and boiled for 2 min in 150 μl of LDS sample buffer. The protein complexes were then subjected to SDS-PAGE and Western blot analysis using a goat polyclonal anti-CAF-1 (p150 subunit) antibody (C-21, Santa Cruz Biotechnology), a mouse monoclonal anti-pol δ (p125 subunit) antibody, or a PC10 anti-PCNA antibody.

Peptide-based Inhibition of PCNA Double Trimer Formation—Peptides were synthesized by Genemed Synthesis (San Francisco, CA) at >90% purity. Cell extracts prepared using hypotonic buffer (2.7 mM KCl, 1.0 mM KH₂PO₄, 10 mM Na₂HPO₄, 15 mM NaCl, pH 7.2) were incubated with 0.1, 0.3, or 1.0 mg/ml peptide for 10 min on ice followed by cross-linking by treatment with 1.5% formaldehyde for 15 min at room temperature.

PCNA Structural Analysis—The following web-based programs were used to analyze PCNA structure: Deep View/Swiss-pdb Viewer, Hex 4.2 by David Ritchie, the University of Aberdeen, Scotland, UK, and RasMol Version 2.7.2.1.
PCNA Functions as Double Trimer

RESULTS AND DISCUSSION

Mammalian PCNA Forms a Double Trimer Complex—To gain insights into the ability of PCNA to form complexes in mammalian cells, we analyzed endogenous PCNA by SDS-PAGE. As expected, only 33-kDa monomers were detected under these denaturing conditions (Fig. 1A, lane 1). However, when cells were treated with 1.5% formaldehyde for 30 min to cross-link proteins prior to SDS-PAGE, PCNA was detected at the positions of 33, ~100, and ~200 kDa (Fig. 1A, lane 2). When the cross-linking time was extended to 90 min, essentially all of the PCNA molecules in the cells formed a ~200-kDa protein complex (Fig. 1A, lane 3). Further extension of the cross-linking time to 3 h did not increase the size of complex beyond 200 kDa (Fig. 1A, lane 4), suggesting that the complex formed in a specific way and had a defined structure. The cross-linking of cell extracts, which was more efficient than that of whole cells, showed essentially the same results (see below).

To determine whether the 200-kDa complexes included other proteins, we carried out two-dimensional PAGE. As shown in Fig. 1B, 33-, 100-, and 200-kDa proteins were detected when cell extracts were cross-linked for 20 min. Essentially all of the PCNA molecules formed 200-kDa complexes when cell extracts were cross-linked for 90 min (Fig. 1C). Because the 100-, and 200-kDa complexes have exactly the same pl as the PCNA monomer (4.57), it is highly likely that they contain only PCNA molecules. Therefore, considering their molecular masses, the 100-, and 200-kDa complexes are most likely a singlet and a doublet of PCNA homotrimers, respectively. Although the double trimer may have a specific structure, it should also be noted that considerable cross-linking time was required to convert all of the single trimers into doublets that are stable enough to survive protein purification and subsequent gel electrophoresis (Fig. 1A, lanes 2 and 3). Therefore, the PCNA double trimer complex may normally exist as a dynamic, loose complex. This could be the reason why a PCNA double trimer has not been observed by crystallography (7, 8); nevertheless, this mode of dynamic and loose protein-protein interactions may be important in the regulation of many different cellular functions.

Under our cross-linking conditions, we did not observe significant cross-linking between PCNA and other proteins. This may be because essentially all of the PCNA molecules exist in the cells as homotrimer doublets (albeit as a loose complex), and none of the lysine or arginine residues in PCNA and PCNA-binding proteins present at close enough proximity to be cross-linked. It should be noted that most PCNA-binding proteins are associated with PCNA through its center loop (41DSSH44) and the interdomain-connecting loop (121LDVEQLGIPEQE132), neither of which contains an arginine nor lysine residue (1). Therefore, we do not anticipate that PCNA would be cross-linked with other known PCNA-associating proteins by formaldehyde, even when they form a complex with PCNA in vivo.

To further confirm that the ~200-kDa PCNA complex does not contain any other proteins, we repeated the cross-linking experiment using purified PCNA as substrate. High purity (~99%) PCNA was achieved using a combination of Sephacryl S200, UnoQ, and the Superdex 200 10/300 GL as described under “Experimental Procedures” (Fig. 1D). Cross-linking of purified PCNA in its native form for 15 min using 1.0% formaldehyde resulted in the formation of the trimer and double trimer complexes (Fig. 1E, lanes 2, 4, 6, and 8). In contrast to PCNA molecules purified by gel filtration, those isolated by SDS-PAGE formed double trimers extremely inefficiently (Fig. 1E, lane 10). Furthermore, they formed many nonspecific complexes when cross-linked (see the smear in Fig. 1E, lane 10). It should also be noted that, in contrast to the PCNA molecules purified by gel filtration, those from SDS-PAGE formed substantial amounts of dimers when they were renatured (Fig. 1E, lane 9). These results strongly suggest that it is difficult for PCNA to return to its native conformation once it is completely
denatured, especially when chaperones and other auxiliary factors are not available for the renaturation process. Furthermore, it appears that the native protein conformation is important for the formation of the PCNA double trimer complex.

The Amino Acid Residues Arg-5 and Lys-110 Are Crucial for Double Trimer Formation—To further characterize the double trimer complex in mammalian cells, we analyzed GFP-PCNA fusion proteins produced in CHO cells transfected with pEGFP-PCNA (24). It was previously shown that the GFP portion of this fusion protein did not interfere with the formation of a functional PCNA trimer (24). Consistent with this previous data, the GFP-human PCNA fusion protein produced in CHO cells behaved similarly to endogenous PCNA following cross-linking (Fig. 2, A and B). The relative amount of endogenous PCNA is much lower than that of the PCNA fusion protein in this particular experiment (Fig. 2B, arrow). As reported previously (24), the GFP-PCNA fusion proteins appear to be localized in the replication foci (Fig. 2C). To further characterize the GFP-PCNA fusion protein, extracts prepared from CHO cells transfected with pEGFP-PCNA were subjected to two-dimensional PAGE using a pH 4–7, 13-cm gel strip and subsequent immunostaining using PC10. As shown previously (25, 27), three distinct isoforms of endogenous PCNA were detected (Fig. 2D, M, A, and B are for the main (pI 4.57), acidic (pI 4.52), and basic (pI 4.62) isoforms, respectively). The GFP-PCNA fusion protein also contained three distinct isoforms (Fig. 2D, denoted as $M^*$, $A^*$, and $B^*$) that are nearly identical with the three endogenous isoforms, except their molecular mass (−66 kDa) and pI (5.0, 4.95, and 5.05, respectively) were shifted. The differences in size and pI were predicted because of the addition of GFP. As expected, a 30-min cross-linking of the cell extracts resulted in the formation of trimer and double trimer complexes by endogenous PCNA (Fig. 2E, T and DT, respectively). Similarly, the GFP-PCNA fusion protein also formed trimer and double trimer complexes (Fig. 2E, $T^*$ and $DT^*$, respectively). Consistent with the data in Fig. 1A, the monomer and large complexes have exactly the same pI (Fig. 2E), again suggesting that no other proteins are included in the trimer or double trimer complexes formed by the GFP-PCNA fusion proteins.

To map the precise contact points between the two trimer subunits of the doublet, we analyzed complex formation by several pEGFP-PCNA point and deletion mutants on the surface of the PCNA structure. Both anti-GFP and PC10 antibodies were used for immunostaining to distinguish the (mutant) GFP-PCNA fusion protein and endogenous PCNA. The following mutants did not alter the formation of either trimer or double trimer: K13A, K14A, K254A (Fig. 3A), K20A, K80A, K117A, K191A, K240A, and K248A (data not shown). It should be noted that Lys-13 and Lys-20 are located inside the PCNA trimer ring, whereas Lys-117 and Lys-254 are on the surface of the front side. The rest of the mutation sites are located on the back side of the PCNA trimer ring. Further mutation analysis identified Arg-5 and Lys-110 as two critical amino acid residues for stable double trimer formation by formaldehyde-mediated cross-linking (Fig. 3A, lanes 8 and 10).

We also examined the ability of synthetic peptides to inhibit PCNA double trimer formation. As shown in Fig. 3B, synthetic peptides encompassing Arg-5 (140EFARLVQGSIL152) and Lys-110 (160EAPNQEK111) inhibited formation of the double trimer complex, whereas a peptide (251LAPKIEMDEG260) corresponding to the amino acid sequence at the C terminus region (i.e. located on the front side) did not. None of these peptides inhibited the formation of the PCNA homotrimer complex (Fig. 3B). Consistent with the data obtained by mutation analysis (Fig. 3A), these results strongly suggest that the Arg-5 and Lys-110 residues are essential contact points between the two homotrimers of a PCNA doublet. It is interesting to note that the inhibition of double trimer formation by the aforementioned two peptides was only effective with cell extracts using hypotonic lysis buffer containing 15 mM NaCl (Fig. 3B) and was not effective in the cells extracted with radioimmune precipitation assay buffer containing 150 mM NaCl (data not shown). This result suggests that PCNA normally exists as a double trimer complex in physiologic salt concentrations. Although the complex may be loose and dynamic, it cannot be easily disrupted by short synthetic peptides under physiological salt conditions.

The Formation of a PCNA Double Trimer Complex May Be Essential for Proliferation and Survival in Mammalian Cells—To gain insights into the effects of double trimer disruption in mammalian cells, we transfected CHO cells with wild type or the R5A/K110A double mutant construct. (Note that the amino acid sequences of hamster and human PCNA proteins are identical except for three residues.) We found that −5 and 35% of cells transfected by wild type and the mutant construct, respectively, exhibited a “rounded-up” phenotype 24 h post-transfection (Fig. 4, A and B). By 48 h post-transfection, the rates of rounded cells were 10 and 70% for cells transfected by wild type and R5A/K110A mutant, respectively. Flow cytometry showed that the rounded cells by R5A/K110A transfection were arrested in G0/G1 and died within a few days. However, most of the rounded cells in the wild-type PCNA-transfected population were normal mitotic cells and did not die. We also found that a R5A or K110A single mutant showed similar
effects to the R5A/K110A double mutant (data not shown). These data, together with those shown in Fig. 3, suggest that Arg-5 and Lys-110 are essential for a double trimer formation and mammalian cell growth and survival.

A Back-to-Back PCNA Double Trimer Complex, but Not a Homotrimer Alone, Can Simultaneously Accommodate Both DNA Polymerase δ and CAF-1—The front side and outer surface of the PCNA trimer can bind to many different proteins, including pol δ and CAF-1 (1, 4, 5, 10–13, 19). It was previously suggested that the back side might be involved in the regulation of various PCNA functions by interacting with other regulatory proteins (6). However, there is no direct evidence that any protein binds to the back side of the PCNA homotrimer. A back-to-back double trimer model can explain why no proteins normally bind to the back side of the PCNA trimer ring. In addition, the availability of two front sides in the trimer doublet to allow protein binding makes it possible for one PCNA complex to simultaneously “coordinate” multiple functions such as DNA replication, repair, and cell-cycle control (5). To test this possibility, we used a chromatin immunoprecipitation assay to analyze the potential of PCNA on the chromatin to interact simultaneously with pol δ and CAF-1. We found that PCNA, CAF-1, and pol δ were present on chromatin in close proximity (data not shown). To determine whether PCNA can physically bind to pol δ and CAF-1 simultaneously, extracts prepared from cells transfected by wild-type PCNA were subjected to DNase I digestion and immunoprecipitated with PC10. The PCNA complexes in the precipitate were dissociated from the antibody beads and then were subjected to a second immunoprecipitation with an anti-pol δ antibody. The final precipitate, which was expected to be a PCNA-pol δ complex, was subjected to SDS-PAGE separation. The proteins on the membrane were then analyzed by sequential immunostaining without stripping off the previous antibodies with anti-CAF-1 (Fig. 4C, lane 1), -pol δ (lane 2), and PC10 (lane 3) antibodies. These data showed that the PCNA-pol δ complex also contained CAF-1 (Fig. 4C, lane 3), strongly suggesting that pol δ and CAF-1 are simultaneously associated with PCNA.

To further confirm this result, we compared the binding patterns of CAF-1 and pol δ between wild type and mutant PCNA. The CAF-1 complexes were immunoprecipitated with a monoclonal anti-CAF-1 (p150 subunit) antibody from cells transfected with either the wild type or the R5A/K110A mutant construct. The CAF-1 complexes were then analyzed by SDS-Western blot analysis using antibodies against pol δ (p125 subunit), CAF-1 (p150 subunit), or PCNA (i.e. PC10). The authenticity of the bands representing these proteins in the immunoprecipitates was verified by comparing with the immunostaining patterns of samples prepared from total protein extracts (Fig. 4, D–F, lanes 1, 2, 5, 6, 9, and 10) and immunoprecipitates (lanes 3, 4, 7, 8, 11, and 12). As expected, the p150 subunit of CAF-1 was present in the total extracts and the immunoprecipitate prepared from the cells transfected by the wild type or the mutant construct (Fig. 4D, lanes 1–4). Significantly, the CAF-1 complex immunoprecipitated from the cells transfected with wild-type PCNA also contained pol δ (Fig. 4E, lane 7). In contrast, the CAF-1 complex immunoprecipitated from cells transfected by the R5A/K110A mutant construct did not contain pol δ (Fig. 4E, lane 8), although wild type and the R5A/K110A mutant apparently have similar binding affinity for CAF-1 (Fig. 4F, lanes 11 and 12). It is therefore clear that PCNA homotrimers can bind either pol δ or CAF-1 (complex) but not both. Because the wild-type PCNA can form a back-to-back double trimer complex, it can accommodate both pol δ and CAF-1. However, the R5A/K110A mutant cannot form a double trimer complex and, therefore, cannot simultaneously accommodate both pol δ and CAF-1. This set of data, along with Fig. 4C, confirms that wild-type PCNA, but not the R5A/K110A
back-to-back arrangement using PhotoEditor and PowerPoint to show the contact points from the side view (the RasMol and Swiss-PDB Viewer programs, and the file 1AXC.pdb from the Protein Data Bank. Two PCNA trimers were then laid out in a back-to-back arrangement using PhotoEditor and PowerPoint to show the contact points from the side view (lower part of A). For simplicity, only two of the six contact points are shown. B, a possible lock-in position is shown using the slab mode of RasMol Version 2.7.2.1. L and * are, respectively, the protuberant $\beta_D$/$\beta_E$ loop and the groove around the $\alpha_A$, $\alpha_B$, $\beta_H$, and $\beta_I$ motifs (6, 8).

Although a PCNA trimer contains three identical surfaces to accommodate proteins with PCNA-binding motifs, the binding to a given protein (complex) appears to occur in a competitive manner at the expense of other PCNA-binding proteins (6, 7, 19, 28, 29). This is probably because one PCNA-associating protein (complex) can occupy most of the PCNA homotrimer surface as in the case of the interaction between a PCNA homotrimer and DNA ligase 1 (30). It should be noted that both pol $\delta$ and CAF-1 are quite large and comprise at least four (p125, p60, p50, and p12) and three subunits (p150, p60, and p19, 28, 29). This is probably because one PCNA-associating protein (complex) can occupy most of the PCNA homotrimer back side (8). This could make it difficult for Arg-5 to physically interact with both the pol $\delta$ and CAF-1 complexes.

A Functional Model of Mammalian PCNA—The $\beta_D$/$\beta_E$ loop forms a handle-like structure that protrudes from the PCNA homotrimer back side (8). This could make it difficult for Arg-5 on one trimer to closely interact with Lys-110 on the other trimer. Furthermore, formaldehyde is a tight homobifunctional amine cross-linker that requires less than a 2-Å distance between the two amino acid residues (31). However, it may be possible that sufficient proximity between Arg-5 and Lys-110 is achieved by a “locking-in” of the two PCNA trimers through loops and grooves. Consistent with this idea, topological analysis using the Hex program predicts that the lock-in can occur if the two trimers rotate slightly so that the $\beta_D$/$\beta_E$ loop (Fig. 5 B, L) of one trimer fits into a nearby groove around $\alpha_A$ (amino acids 9–20, GSILKKVLEALK), $\alpha_B$ (amino acids 209–211, LRYLNFKATPL), $\beta_H$ (amino acids 235–241, LV-VYEKLY), and $\beta_I$ (GHIKLKYYL) of the second trimer (Fig. 5B, *). By this lock-in, Arg-5 and Lys-110 could be in sufficiently close proximity to be cross-linked by formaldehyde.

Henderson et al. (32) argued that their data, which was obtained from Drosophila PCNA genetic studies, could make sense only if two PCNA trimer rings formed a back-to-back complex. Overall, our data provides direct evidence to support their speculation. This back-to-back double trimer model predicts that many cellular functions such as epigenetic control, DNA methylation, and mismatch repair processes are tightly coupled with DNA replication via the PCNA doublet complex.

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