Proliferating cell nuclear antigen (PCNA) is essential for eukaryotic DNA replication and functions as a processivity factor of DNA polymerase δ (pol δ). Due to the functional and structural similarity with the β-subunit of Escherichia coli DNA polymerase III, it has been proposed that PCNA would act as a molecular clamp during DNA synthesis. By site-directed mutagenesis and biochemical analyses, we have studied the functional domains of human PCNA required for stimulation of replication factor C (RF-C) ATPase and DNA synthesis by pol δ. Short deletions from either the N or C termini caused drastic changes in extraction and chromatographic behaviors, suggesting that both of these terminal regions are crucial to fold the tertiary structure of PCNA. The short C-terminal stretch from Lys254 to Glu266 is necessary for stimulation of RF-C ATPase activity, but not for stimulation of DNA synthesis by pol δ. Nine basic amino acids that are essential for activating DNA synthesis by pol δ are positioned at the internal α-helices of PCNA. This result is in good agreement with the observation that PCNA has a ring structure similar to the β-subunit and clamps a template DNA through this positively charged internal surface. Several other charged amino acids are also required to stimulate either RF-C ATPase or pol δ DNA synthesis. Some of them are positioned at loops which are exposed on one of the side surfaces of PCNA adjacent to the C-terminal loop. In addition, the β-sheets composing the intermolecular interface of the trimeric PCNA are important for interaction with pol δ. Therefore, the outer surface of PCNA has multiple functional surfaces which are responsible for the interaction with multiple factors. Furthermore, the two side surfaces seem to be functionally distinguishable, and this may determine the orientation of tracking PCNA along the DNA.

The proliferating cell nuclear antigen (PCNA) is an essential replication factor for simian virus 40 (SV40) DNA replication in vitro and is involved in the elongation stages of DNA replication (1–4). Recent studies have further demonstrated the involvement of PCNA in cellular chromosomal DNA replication in vivo (5–7). In addition to its essential role in DNA replication, PCNA is required for nucleotide excision repair of DNA (8–10) and also may participate in the cell cycle control as demonstrated by an interaction with a cyclin dependent kinase complex (11). Therefore, PCNA is multifunctional through the interaction with several specific partners, and all of the functions are crucial for cell proliferation.

Genes encoding PCNA have been isolated from various eukaryotes and are composed of highly conserved amino acid sequences of around 260 residues. The functions of PCNA during DNA replication have been elucidated by studies of SV40 DNA replication in vitro. In this reaction, three protein components, PCNA, DNA polymerase (pol δ), and replication factor C (RF-C) are required for leading strand DNA synthesis following the initiation of DNA synthesis by DNA pol α at the SV40 origin (12). PCNA and RF-C form a complex at a primer-template junction (13), and consequently, the DNA synthesizing DNA polymerase switches from pol α to pol δ (14). In this process, PCNA makes connections between the primer-end recognition reaction and the DNA synthesis reaction through physical interactions with RF-C and pol δ. These interactions have been demonstrated as the stimulation of RF-C ATPase and pol δ DNA synthesis by PCNA (15). However, little is known about the detailed mechanism of how such a complex is formed and which structures are responsible for these interactions.

The assembly of a functional replication DNA polymerase holoenzyme through protein-protein interaction has been reported for Escherichia coli DNA pol I and bacteriophage T4 DNA polymerase (for a review, see Refs. 16 and 17). The first step is a binding of an ATPase complex (E. coli γ complex and bacteriophage T4 gene 44/62 protein complex) to a primer-template junction. Following this process, a processivity factor (E. coli β-subunit and bacteriophage T4 gene 45 protein) forms a secondary complex with the ATPase. This complex tethers its specific core DNA polymerase (E. coli α, ε, δ, and bacteriophage T4 gene 43 protein) onto a DNA template mediated by hydrolysis of ATP, and a processive DNA polymerase holoenzyme is formed. Judging from the highly conserved reaction processes, there should be close structural and functional relationships between these bacterial and eukaryotic replication factors. The three-dimensional structure of pol III β-subunit has been determined by x-ray crystallography (18). The tertiary structure of β-subunit is a characteristic ring or torus-like structure composed of two homologous subunits that combine to form a DNA tracking protein (for a review, see Refs. 19–21). The central hole in the structure has a highly positively charged surface containing six α-helices which are thought to be important for interaction with the DNA template. The outer surface
has several β-sheet structures that may interact with other protein components. This model proposes three structurally related units in each monomer subunit and further more, the whole β-subunit dimer consists of six of these units. Structural similarity between PCNA and the β-subunit has been suggested, even though their primary sequences are not related (18). The prediction has been realized to be essentially correct by a recent study of the crystal structure of PCNA from Saccharomyces cerevisiae (22). Based on the crystallography results, each PCNA monomer consists of two structurally related domains and the whole molecule contains three PCNA subunits. Thus, like the β-subunit of pol III, the PCNA clamp has six structurally related units.

To study the assembly of these important eukaryotic replication factors into a functional complex, detailed information on the molecular structure and function of PCNA and the mechanism of protein-protein interactions with RF-C and pol δ is necessary. We therefore constructed mutations covering the whole PCNA gene by site-directed mutagenesis. Each mutant PCNA protein was expressed in E. coli cells and purified. Stimulation of RF-C ATPase and pol δ activity was then compared with the wild type protein.

MATERIALS AND METHODS

Oligonucleotides—Oligonucleotides for polymerase chain reaction (PCR) primers were synthesized with an Applied Biosystems model 394 DNA synthesizer (Applied Biosystems, Foster City, CA) or purchased from Bio-Oligo Co. (Nagoya, Japan) (21). Construction of a Plasmid Carrying the Wild Type PCNA—pT7PCNA which allows expression of human PCNA protein was constructed by inserting human PCNA cDNA sequence into pGEMEX-1 (Promega, Madison, WI). The 900-base pair cDNA fragment was isolated from a DNA synthesizer (Applied Biosystems model 394) oligonucleotides which allows expression of human PCNA protein was constructed by PCR-mediated, site-directed mutagenesis (24). Four deletion mutants, D2, D3, and D5, were constructed in pT7PCNA by utilizing the NdeI and XhoI sites in the pGEMEX-1 vector. The resulting plasmid, pT7PCNA, has the intact PCNA gene between the T7 RNA polymerase promoter and the terminator sequences similarly as the parental plasmid. pT7PCNA was harbored in E. coli BL21 (DE3) carrying the T7 RNA polymerase gene under the control of the lac UV 5 promoter.

Mutant Construction of the PCNA Gene—Mutations were introduced by PCR-mediated, site-directed mutagenesis (24). Four deletion mutants, Δ2–9, Δ257–261, Δ254–261, and Δ250–261 lack 8 amino acids from the second Phe and 5, 8, and 12 amino acids from the C-terminal end, respectively. 29 amino acid substitution mutants were constructed by altering the charged amino acids, Glu13, Lys14, Lys15, Lys20, Asp21, Asp22, Asp34, Lys35, Lys36, Lys37, Lys42, Lys43, Arg146, Arg149, Lys164, Lys165, Lys166, Lys170, Arg170, Arg171, Lys217, Asp232, Lys254, Glu256, and Asp259 to alanine as indicated in Fig. 1. Specific base substitutions were introduced as a mismatch between a PCR primer and a target sequence. Two mutagenic primers were prepared for each mutant construction. The first PCR was performed with a set of primers at 25 pmol each in a 50-μl reaction mixture containing 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 1 mM (NH4)2SO4, 2 mM MgSO4, 0.1% Triton X-100, 10 ng of pT7PCNA, 10 pmol of dNTP, 5 μg of bovine serum albumin, and 1-2 units of Vent DNA polymerase (New England Biolabs, Beverly, MA) by 30–35 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 2 min after heating at 94°C for 10 min. For some reactions, Pfu DNA polymerase (Stratagene, La Jolla, CA) was used instead of Vent DNA polymerase under the same condition except for the addition of 6 mM (NH4)2SO4 and 1.5 mM MgCl2. The second PCR was performed by 35–40 cycles with the first PCR products in 50 μl of reaction mixture similar to the first PCR reaction in the presence of 1 unit of Perfect Match DNA polymerase enhancer (Stratagene). The products were purified after trimming with restriction enzymes and ligated with pT7PCNA digested with the same restriction enzymes.

The mutated DNA sequences were confirmed with an Applied Biosystems model 373A automatic DNA sequencer (Applied Biosystems) or a conventional electrophoresis method with T7 DNA polymerase, Version 2.0 Kit (U. S. Biochemical Corp., Cleveland, OH). Expression and Extraction of PCNA—BL21 (DE3) cells harboring the PCNA expression plasmid were inoculated in 1 l of L-broth with 30 μg/ml methicillin and 10 μg/ml ampicillin at 30°C until the absorbance reached 0.5 at 600 nm. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, and the culture was successively incubated at 30°C overnight. The following procedures were carried out at 4°C. The induced E. coli cells were collected at 6,000 rpm for 15 min, suspended with a 5 ml of lysis buffer (25 mM Tris-HCl (pH 7.7), 25 mM NaCl, 1 mM EDTA, 0.01% Nonidet P-40, 4 mM bezo-midine, 4 mM pepstatin A, 20 mM Na2SO4, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM EDTA) and sonicated with a microtip-horn at 0°C for 15 s, 16 times with 2-min intervals. The sample was centrifuged at 32,000 rpm for 30 min, and the cleared lysate was obtained (sonic lysate). For Δ2–9 and Δ250–261 mutants, PCNA proteins could not be extracted in the sonic lysate due to the formation of inclusion bodies. Thus, the following extraction procedure was performed. The precipitate was suspended in buffer A (25 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol, 2 μg/ml leupeptin, 1 mM PMSF, and 1 mM DTT) containing 4 μg/ml urea at 0°C for 30 min, and the lysate was obtained by ultracentrifugation as above (urea lysate).

Purification of PCNA—The sonic lysate containing about 100 mg of protein was loaded on 10 ml of Econo- pact HTP cartridge (Bio-Rad) equilibrated with buffer A containing 0.1 mM NaCl. After washing the column with 20 ml of the same buffer, proteins were eluted with a 80-ml linear gradient from 0.1 to 0.6 mM NaCl in buffer A. Presence of PCNA in fractions was monitored by SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining. A contaminated nucleic acid activity was monitored by incubating 0.5 μg of pUC118 DNA with an aliquot of each fraction containing PCNA at 37°C. PCA-containing fractions were pooled and loaded onto a Sephacyr S-300HR column (5 × 75 cm; Pharmacia Biotech, Inc.) in buffer A with 0.1 mM NaCl. PCNA fractions eluted around 120 kDa were pooled and loaded onto a Mono Q (HR 5/5; Pharmacia) equilibrated with buffer A containing 0.1 mM NaCl. The column was washed with the same buffer, and PCNA was eluted with a B-M linear gradient from 0.1 to 0.6 mM NaCl in buffer A. PCNA exhibited a discrete peak at 0.55 mM NaCl. When a contaminated nucleic acid existed, the pooled fractions were loaded onto a second Sephacryl S-300HR column (L.8 × 50 cm) and a second Mono Q (HR 5/5). Purified PCNA was aliquoted and stored at −70°C.

For mutant PCNAs, K20A, D189A, E192A, R230A, and D257A, the pooled Eco-pac HTP cartridge fractions was loaded onto a 5-ml Econo-pac HTP cartridge (Bio-Rad) equilibrated with buffer H (20 mM KPO4 (pH 6.9), 0.01% Nonidet P-40, 10% glycerol, 2 μg/ml leupeptin, 1 mM PMSF, and 1 mM DTT). Proteins were eluted with a 30-ml gradient of 0.02 to 0.6 mM KPO4. PCNA-containing fractions were pooled, mixed with an equal volume of 4 mM (NH4)2SO4, and loaded onto a 5-ml Econo-pac methyl HIC cartridge (Bio-Rad) equilibrated with buffer A containing 2 mM (NH4)2SO4. Proteins were eluted with a 30-ml linear gradient of 0.02 to 0.6 mM KPO4. The purified PCNA containing fractions were pooled, dialyzed against buffer A with 0.5 or 100 mM NaCl and loaded onto a Mono Q (HR 5/5) as above.

RF-C and Pol δ Preparation—RF-C was prepared from 293 human cells following a published method with slight modifications (13). Pol δ was isolated from calf thymus also by a slight modification from a previously published method (13).

ATPase Assay—The assay for RF-C ATPase activity was essentially the same as described elsewhere (15, 25). A reaction mixture (50 μl) contained 30 mM HEPES (pH 7.5), 7 mM MgCl2, 0.5 mM DTT, 0.1 mM bovine serum albumin, 40 or 100 mM NaCl, 200 pmol of poly(dA): poly(dG) (5:1), 0.1 mM [α-32P]ATP or [α-32P]ATP, RF-C (4 ng), and PCNAs (280 ng). The mixture was incubated at 37°C for the indicated periods, and the product dTMP was measured as a DE81 paper-adsorbed count after washing with 0.5 × NaHPO4.

RESULTS AND DISCUSSION

Expression and Extraction of Mutant PCNAs—Construction of a plasmid, pT7PCNA which expresses human PCNA efficiently in E. coli has been described by Fien and Stillman (23). We have constructed a similar expression plasmid, pT7PCNA, suitable for the site-directed mutagenesis in the PCNA gene using pT7PCNA as a parental plasmid. In this construct, the
PCNA gene obtained from the cDNA was placed under the control of the strong T7 RNA polymerase promoter. After the induction of the promoter with IPTG, a 36-kDa PCNA polypeptide became a major protein component in an E. coli extract and was easily purified by conventional column chromatography.

In order to investigate functional regions of PCNA, 33 mutations were introduced into the PCNA gene in the plasmid pT7PCNA, and the mutant PCNA proteins were expressed as for the wild type. Several N- or C-terminal truncations and amino acid substitution mutations at highly conserved amino acids were designed to study. Terminal deletions were designed to determine which region of the PCNA was functionally important. An N-terminal deletion mutant, Δ2–9 that lacks 8 amino acids from the second codon, and three C-terminal deletion mutants, Δ257–261, Δ254–261, and Δ250–261 lack 5, 8, and 12 amino acids from the C terminus, respectively, were constructed. The amino acids in PCNA highly conserved among several species were expected to be involved in its indispensable functions. Furthermore, since a substitution of a charged amino acid to a noncharged one will have a minimal effect on the whole structure of PCNA, any altered phenotype may simply represent an altered function rather than the denaturation of the whole protein. Twenty-nine charged amino acids as indicated in Fig. 1 were individually substituted with the noncharged amino acid, alanine.

In all cases, the mutant PCNA proteins accumulated to high amounts in the E. coli cells after the induction of the T7 promoter and became major components as observed for the wild type PCNA. Most of the mutant PCNAs, except for Δ2–9 and Δ250–261, were extracted efficiently from the E. coli cells by a sonic extraction and behaved as a single component of native molecular mass of around 120 kDa, as determined by size fractionation chromatography during the purification. This molecular mass is in good agreement with the prediction that PCNA is in a trimeric state (22).

Importance of N- or C-terminal Stretches for the Structure—In contrast to most of the altered proteins, Δ2–9 and Δ250–261 remained in an insoluble fraction after the sonic extraction, and an additional 4 M urea treatment was necessary to solubilize them. The insolubility of Δ2–9 and Δ250–261 in the sonic lysate may be due to formation of inclusion bodies in the E. coli cells. A drastic change in the mode of extraction from E. coli cells observed with two deletion mutant PCNAs probably indicates a loss of their native configuration. Indeed, they showed a heterogeneous size distribution in which the majority of the protein was eluted in fractions smaller than 100 kDa during the size fractionation step, after the removal of urea (data not shown). These results suggest that the 8 and 12 amino acid stretches from N- and C-terminal end of PCNA may be crucial to maintain a trimeric native structure. The crystal structure of yeast PCNA shows a characteristic ring structure, and its array of antiparallel β-sheets comprises the framework.
of the ring (22). These terminal deletions eliminate one of these β-sheets which disrupt the ordered alignment of β-sheets, and consequently the native molecular structure is lost.

Stimulation of RF-C ATPase and Pol δ DNA Synthesis—As mentioned above, processive DNA synthesis by pol δ requires the formation of a complex among pol δ, PCNA, and RF-C. We employed two assays, stimulation of RF-C ATPase activity and stimulation of pol δ DNA synthesis to examine the ability of each of the altered PCNA protein to function. The results with 33 mutant PCNAs are shown in Figs. 2 and 3. Five and 16 mutations out of the 33 affected the stimulatory activities for RF-C ATPase and pol δ DNA synthesis, respectively. Since ATP promotes the formation of the complex between RF-C and PCNA (13), the level of stimulated RF-C ATPase activity with PCNA may reflect directly the extent of interaction between the two components.

The activity pol δ DNA synthesis was assayed with the DNA polymerase, PCNA, and a template DNA, poly(dA)-oligo(dt).

Under our assay condition, due to the presence of excess primer ends on poly(dA)-oligo(dt), pol δ is able to carry out processive DNA synthesis without RF-C, and the pol δ activity may be dependent on one or two of the following functions of PCNA, direct protein-protein interaction between PCNA and pol δ and the DNA clamping activity by PCNA. The mutant data do not discriminate between these two functions.

In the case of deletion mutants Δ2–9 and Δ250–261, which have lost the native configuration, they did not exhibit the stimulative activities for RF-C ATPase and pol δ DNA synthesis (Figs. 2A and 3A for Δ250–261, but data is not shown for Δ2–9). The loss of the stimulative activity was not simply due to the denaturation of this protein with urea during extraction, but because of an inability to fold into the native structure by these truncations, inasmuch as the wild type PCNA could be renatured and become fully active by removing urea after being extracted by the same procedure as these mutants (data not shown). Therefore, the native trimeric structure is necessary.
residues in this region, we substituted the basic residue, Lys254, with alanine. Indeed, this region is exposed as a loop in the yeast PCNA and two of the acidic residues, Glu256 and Asp257, with alanine individually, and assayed their stimulative activity for RF-C ATPase. However, all of the mutants have full activity (data not shown). Of course, they also have a full stimulation for pol δ DNA synthesis activity (Fig. 3, D257A is not shown). Thus, elimination of one charged residue from the charged amino acid stretch may be insufficient to observe an effect. This must be further tested by construction of multiple amino acids substitution mutants.

Interaction with DNA at the Inner α-Helices—The terminal deletion mutants were not very informative about the structure and function of PCNA, since even very short deletions completely eliminate the native structure. Thus, we focused attention on determining which amino acids in the internal coding region were important for function.

Among the conserved amino acids in the PCNA gene, 14 basic residues were substituted with Ala individually. Mutants K13A, K14A, K20A, K77A, R146A, R149A, and K217A lost the stimulation of pol δ activity and exhibited only a basal level DNA synthesis. The addition of K80A, K110A, or R210A to the pol δ DNA synthesis reaction caused a limited stimulation in pol δ activity at less than 50% of wild type (Fig. 3D). With mutants R64A and K168A, a partial decrease in stimulation, corresponding to 50–80% of wild type activity, was observed (Fig. 3C and E). The rest of the basic amino acid substitutions did not affect the stimulatory activity for pol δ DNA synthesis, showing that these residues were not required for the activity (data not shown except for K254A in Fig. 3).

One characteristic feature of both the pol III β-subunit and yeast PCNA is the presence of α-helices with positively charged amino acids at the inner surface of the ring (18, 22). This structural feature may be responsible for the DNA clamping activity of these DNA tracking proteins. One subunit of PCNA has 4 α-helices, and each has 2 or 3 basic residues which are highly conserved from human to yeast (Fig. 1). Lys13, Lys14, Lys70, Lys77, Lys146, Arg149, Arg210, and Lys217 are exactly positioned at each α-helix and all are necessary to stimulate pol δ activity. These contribute to the positive electrostatic potential in the center of the ring and are suggested to be necessary for an interaction with DNA. Thus, their requirement for pol δ activity may be a reflection of such an interaction with the template DNA. It is of interest that the elimination of only 1 residue out of 9 positively charged residues reduces drastically the ability of PCNA to stimulate pol δ activity. Probably, a certain positive electrostatic distribution on the inner surface is important to maintain the DNA clamping activity.

The inability to stimulate pol δ DNA synthesis with these mutant PCNAs may due to the decrease in either the initial DNA clamping activity or the sliding activity on DNA. To distinguish the mechanisms, we analyzed the products from DNA synthesis by pol δ using denatured alkaline agarose gel electrophoresis (Fig. 4). A full-length product of 200–300 nucleotides long was synthesized with wild type PCNA but DNA shorter than 20 nucleotides was produced without PCNA. If we analyzed the reaction products with mutants K13A, K14A, K20A, K77A, and K217A, all of them were significantly processive, although their incorporation of dTMP was only a few percent of the fully stimulated pol δ DNA synthesis (Figs. 3 and 4). Since the incorporation is corresponding to a DNA synthesis of less than 0.3 nucleotide from each primer end, length of the product represents the processivity of DNA synthesis. Therefore, these mutants block mainly the initiation of pol δ DNA synthesis but not the processivity of the DNA synthesis. This implies that the elimination of positive charges in the α-helices may decrease the DNA clamping activity of PCNA but not the sliding activity, leading to inefficient initiation of pol δ DNA synthesis.

None of the basic amino acids in the internal α-helices affected stimulation of RF-C ATPase (Fig. 2 and data not shown). Taking the complex formation among RF-C, PCNA, and a DNA template into consideration, the DNA clamping activity might potentially affect the RF-C/PCNA interaction. However, the stimulation of RF-C ATPase was independent of the DNA clamping of PCNA, implying that the initial contact between RF-C and PCNA seems to occur in the absence of DNA, or the DNA binding of RF-C alone may have primary significance for the complex formation.

Interaction with Pol δ—Other positively charged mutants, K64A, K110A, and K168A, which were severely affected the stimulation of pol δ activity, are separated from the internal α-helices and are located at the outer surface of the PCNA ring (22). This suggests that their outer surface is probably involved in interacting with pol δ. There are also several highly conserved acidic amino acids on the protein outer surface. We have constructed mutants, E3A, D21A, D41A, E55A, D63A, D86A, D97A, D122A, E143A, E174A, D189A, E192A, and D232A...
Fig. 5. The mutation sites and their activities to stimulate RF-C ATPase or pol δ DNA synthesis. Filled circles indicate locations of mutations. Arrowheads indicate the end points of deletions. Symbols in each stimulatory activity represent extents of the activity by mutant PCNAs. +++, more than 80% activity of the wild type; ++, between 80 and 50% of the activity; +, between 50 and 20% of the activity; and --, less than 20% of the activity, respectively.

(Fig. 5). Among them, less than 50% of the pol δ activity found in the presence of wild type PCNA was observed with the D41A mutant and between 50–80% of that with the D122A mutant (Fig. 3C). The other acidic amino acid mutations allowed a full stimulatory activity at wild type level (data not shown except for D232A in Fig. 3).

If these affected mutation sites are placed on the crystal structure of PCNA, they are positioned in several specific structures. Lys110 and Lys168 are in two β-sheets (β1 and β2, respectively; see Fig. 6) corresponding to the intermolecular interface of the PCNA trimer (22). It has been suggested that the interface may be necessary to hold the trimeric structure. However, since these mutant proteins behave as a native trimer during gel filtration chromatography (data not shown), this does not appear to be the explanation of why these mutants are defective. Thus, the mutation effects may be due to some local structural changes which influence the DNA clamping activity or the contact with pol δ. Asp41, Lys64, and Asp122 are in the loop between sets of β-sheets. These loops are exposed on the outside of the protein and we suggest that they provide a structure to contact directly with pol δ.

Interaction with RF-C—Using the same set of mutants, we studied their effect on RF-C ATPase stimulation. Mutant D41A is severely affected for the stimulatory activity on RF-C ATPase and another mutant, D97A, showed only a small but reproducible decrease in the activity (Fig. 2, B and C). The other mutations had no effect on this stimulation (Fig. 2 shows D63A, D86A, and D122A, but the others are not shown). Therefore, only two out of 29 mutated amino acids exhibited an apparent effect on the RF-C ATPase stimulation (Fig. 5). This may indicate that a very limited surface of the protein is used to contact with RF-C. As mentioned above, the C-terminal loop of PCNA is required for the specific interaction with RF-C. The crystal structure of yeast PCNA shows that both Asp97 and Asp122 are exposed to a surface on the same side of PCNA as the C-terminal loop. This implies that the three clustered loops projecting to one side of PCNA compose a pocket to contact RF-C.

Interestingly, D41A affects both RF-C ATPase stimulation and pol δ DNA synthesis. To exclude the possibility that the D41A mutation would change the protein structure, we analyzed the circular dichroism profile of D41A. The signals of short wave range (200–250 nm) which represent peptide secondary structure were completely same between wild PCNA and D41A. This indicates that D41A has the same structure as wild PCNA. Thus, Asp41 is required to interact with both RF-C and pol δ, and this loop has two targets for interaction. But it is unclear whether or not this amino acid is able to interact with pol δ and RF-C simultaneously.

The CDC44 gene in S. cerevisiae encodes the large subunit of RF-C, and five independent suppressors of its cold sensitive mutations were isolated. All of them were mapped to the PCNA gene (pol 30), suggesting that the 140-kDa subunit interacts directly with PCNA (26). Two of these mutations occurred at positions completely or partially buried in the structure and are likely to cause changes in the structure of PCNA (22). Although these suppressor mutation sites are involved in interacting with RF-C, they are physically close to sites which have been shown to be involved in interacting with pol δ, but not with...
RF-C. This discrepancy may due to a difference of the assay system. In our system, interaction of RF-C and PCNA is studied by a biochemical assay and only strongly affected mutations may have been appeared. In the case of suppressor mutation, such strong mutations might not be isolated, but rather, mutations that only moderately affect the protein could be isolated, for example, those which alter the partial molecular structure will be selected. It is therefore likely that the amino acid residues involved in maintaining the tertiary structure may contribute to interaction with RF-C as well as pol δ, but their effect may not be detected in our biochemical assay.

Asymmetrical Sides of PCNA—Our studies indicate the importance of exposed loop structures for interacting with RF-C and pol δ. Some of them containing Arg and Asp that may be involved in interaction with pol δ are located at the peripheral surface of the ring and do not contribute much to its orientation. But other loops containing Asp, and the C-terminal loop that may interact with both RF-C and pol δ or RF-C alone are of interest. These are projected to a common side of the PCNA trimer, implying that PCNA has an orientation for the function where one side is used to interact with RF-C and pol δ. This side should face to a primer/template junction and the other should face to a double stranded DNA region (Fig. 7). Are there any functions on the other free side? PCNA has multiple target proteins to interact with in addition to replication proteins, for example the interactions of PCNA with cell cycle proteins, cyclin-dependent kinase, cyclins, and p21 and with a DNA damage-induced protein, Gadd45, have been reported (11, 27, 28). It is therefore possible that the free side may provide a structure to contact with such proteins. Mapping of PCNA regions interact with cyclin D has been reported using deletion constructs of PCNA, but it is difficult to localize finely the regions for the interaction on the crystal structure, since relatively large deletions were used (29). This will be tested in future studies using our mutant PCNA proteins.

CONCLUSION

We have introduced 33 mutations into the human PCNA gene and studied their effects on the functions of PCNA. Stimulatory effects on RF-C ATPase activity and pol δ DNA synthesis activity were examined and the results are summarized in Figs. 5 and 6. Amino acid substitution at residues Lys, Lys, Lys, Lys, Lys, Arg, Arg, Arg, and Lys in PCNA, which are located at the α-helices, exhibited a limited stimulation of pol activity. The α-helices provide an internal surface to clamp a DNA template through their highly positive charges facing to the same side of each α-helix (Fig. 7). In addition to the internal surface, the β-sheets at the intermolecular interface and the exposed loops have a significant role in interaction with pol δ. The assay to stimulate RF-C ATPase with mutant PCNAs demonstrates a strict requirement for specific residues that are located at the loops projecting to one side of the PCNA trimer. PCNA has 6 redundant units in the trimeric structure, each of which may have surfaces to interact with either pol δ or RF-C, or both.

How do pol δ and RF-C share the redundant structures of PCNA? RF-C consists of five subunits, and their cDNA sequences have demonstrated a significant structural redundancy among them (30, 31). Thus, we propose a model to assemble the processive DNA polymerase complex as described in Fig. 7. At least three pockets of the side loops may interact with three subunits of RF-C specifically. Other structures, such as the intermolecular interface, may also contribute to interactions with RF-C, as suggested by the yeast genetics. This means that the five subunits of RF-C may occupy five out of six units of PCNA trimer. Interaction between RF-C and PCNA in the presence of ATP may induce a conformational change in PCNA, which could then open one of the interfaces and pass a DNA helix through the PCNA ring. Then pol δ may be recruited onto the template DNA using one free unit of PCNA as a contact site.

Our study with single amino acid mutations and deletion mutations in PCNA demonstrates the presence of multiple functional surfaces in the characteristic ring structure of PCNA. In addition, several loops projected from one specific side provides the orientation which is important for the function of PCNA as a tracking molecule.

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