The Interdomain Connector Loop of Human PCNA Is Involved in a Direct Interaction with Human Polymerase δ*

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Proliferating cell nuclear antigen (PCNA) is required for processive DNA synthesis catalyzed by DNA polymerase δ (pol δ) and polymerase ε. We have shown that the epitope of a human PCNA inhibitory monoclonal antibody (74B1), which inhibits the PCNA stimulation of DNA synthesis catalyzed by pol δ, maps to residues 121–135, which overlap the interdomain connector loop of PCNA (residues 119–133). We have mutagenized residues 122–133 of human PCNA. The mutant proteins were expressed in Escherichia coli and purified to near-homogeneity. The interactions of the mutants with antibody 74B1 were examined; mutation of Gly-127 abolished the recognition by antibody 74B1 in a Western blot analysis, confirming the epitope assignment of 74B1. Mutations of Val-123, Leu-126, Gly-127, and Ile-128 affected the ability of PCNA to stimulate DNA synthesis by pol δ in several different assays. These mutations affected the interactions between PCNA and pol δ as determined by enzyme-linked immunosorbent assays. These mutants were also affected in their abilities to form a ternary complex with a DNA template-primer, as determined by electrophoretic mobility gel shift assays. The findings show that the interdomain connector loop region is involved in binding of pol δ. This same region is involved in the binding of p21, and our findings support the view that the mechanism of inhibition of DNA synthesis by p21 is due to a competition for PCNA binding to pol δ.

DNA polymerase δ (pol δ)1 is a central enzyme involved in the replication of mammalian chromosomal DNA and is also involved in DNA repair (1, 2). Studies of in vitro SV40 replication has established the role of pol δ, as well as an understanding of the complex number of proteins that are involved in eukaryotic DNA replication (2). It is now considered that pol α is involved in the lagging-strand synthesis, while pol δ is involved in leading-strand synthesis and also for completion of lagging strand synthesis (2). A key element of the function of a replicative DNA polymerase is the ability for extended processive synthesis of DNA. This ability is conferred on replicative polymerases by accessory proteins, which function as molecular sliding clamps. These clamps form toroidal rings that encircle the DNA strand and also interact with the polymerase. This function in Escherichia coli is fulfilled by the β subunit (3), and in mammalian cells by PCNA (proliferating cell nuclear antigen), which is an essential factor for the eukaryotic DNA replication and functions as a processivity factor for pol δ (4, 5). The basis for the functions of the sliding clamps of both E. coli and PCNA has been elucidated by the determination of their crystal structures, which reveals them to be toroidal proteins that encircle the DNA strand (6, 7). PCNA is a homotrimer. Each subunit consists of two structurally equivalent domains giving the trimer a six-fold symmetry. PCNA is loaded onto double-stranded DNA by the action of RF-C (8), followed by the loading of pol δ through an interaction between PCNA and pol δ to form a ternary pol δ-PCNA-template-primer complex. However, little is known about the structural elements involved in the interaction of PCNA with pol δ.

PCNA is also implicated in DNA repair (9, 10) and cell cycle control processes (11). PCNA has been shown to interact with p21 (12, 13). The latter is a cell cycle checkpoint protein, which inhibits cyclin-dependent protein kinases, and has also been shown to inhibit DNA synthesis in vitro (12, 13). The mechanism for the latter effects has been shown to be due to the ability of p21 to bind directly to human PCNA (hPCNA); p21, however, does not inhibit the repair functions of pol δ (14, 15). A more recent study showed that a peptide derived from the C terminus of p21 when added at higher levels inhibited both DNA replication and nuclear excision repair (16). The nature of the interaction of p21 with PCNA has been determined at the atomic level by solution of the three-dimensional structure of a complex of hPCNA with a 22-residue peptide derived from the C terminus of p21 (17).

Recently, we reported that an inhibitory monoclonal hPCNA antibody (74B1), inhibits the ability of hPCNA to stimulate DNA synthesis catalyzed by pol δ in vitro using poly(dA)-oligo(dT) as a template. The epitope of this antibody has been mapped to residues 121–135 of human PCNA (18). The crystal structure shows that this region is located in the loop that connects the two conformationally conserved domains in the PCNA monomer (17). In this study, we report a mutational study of this loop region to investigate the structural basis for the interactions of hPCNA with pol δ by the use of site-directed mutagenesis in combination with electrophoretic mobility shift, processivity gel, and ELISA assays.

**EXPERIMENTAL PROCEDURES**

Polymerase δ, Replication Protein A, and Replication Factor C Proteins—Calf thymus pol δ was purified by immunoaffinity chromatography as described by Ji-ang et al. (19). Human replication protein A (RPA) expressed in E. coli (a gift from Dr. M. S. Wold, University of Iowa, Iowa City, IA) was purified according to Henrickson et al. (20). RF-C was a generous gift from Dr. M. O’Donnell, Howard Hughes Medical Institute, Cornell University Medical College, New York, NY.
Construction of Site-directed Mutants of PCNA—The coding sequence for human PCNA was inserted between XbaI and SacI sites in pALTER-1 plasmid (Promega). Site-directed mutagenesis was performed as described previously (21). Primers used for generation of the mutant constructs were D122A, GTGTTGCAACAGCTAAATCCAT; V123A, CAAGTTTGCACTGACAATAC; E124Q, TTCAAGGTTGACACATCTA; Q125E, AATTCCATGTTCTCACAATC; L126S, TGGGAT- TCCAATTTGGCAA; G127A, GTCTGGAAATTCAGTGTCCT; I128A, CTGTTGAGGCTTCCATGAGT; P129A, CTCGTTGTCTGCAATTAAG; E130Q, TGATCGTCTGGTAAAGGTC; Q131E, AGCTGATCTTCTGCTTGAAAT; E132Q, CACAGGCTAAGCAGCGTTG; T133P, TACAGCTGAGTCTTTCTC. Underlined residues are those mutated from the wild type sequence. Mutations were verified by DNA sequencing.

Expression and Purification of the PCNA Mutants— Constructs containing wild type and mutant PCNA were transformed into E. coli DH5α cells for overexpression of protein. Overnight culture (5 ml) were used to inoculate 1-liter cultures (Terraform media) and grown at 37 °C until the A600 reached a value of 0.3. Isopropyl-1-thio-β-D-galactopyranoside was then added to a concentration of 0.3 mM, and the cultures were grown for another 16–18 h at 26–28 °C. The cells were harvested, and the PCNA proteins were purified as described by Zhang et al. (23).

Assays for Enzyme Activities—DNA polymerase activity was assayed with poly(dA)·oligo(dT) as described by Ji et al. (19) and with singly primed M13 template essentially as described by Burgers 1995 (24). M13mp18 single-stranded DNA was primed with a 40-mer oligonucleotide complementary to nts 7041–7080 of the M13 genome. The primer was annealed to M13 single-stranded DNA as described by Podust et al. (25). The standard 30-μl reaction mixture contained calf thymus pol δ (500 ng), RPA (850 ng), RF-C (25 ng), PCNA (250 ng), 40 mM Tris-HCl (pH 7.8), 0.2 mg/ml bovine serum albumin, 1 mM MgCl2, 100 μM each dATP, dCTP, and dGTP, 25 μM [32P]dTP, 0.5 mM ATP, 100 ng of singly primed M13mp18 DNA. The complete reaction mixtures were incubated at 37 °C for 30 min. SDS-PAGE and Western Blotting—SDS-PAGE and Western blotting were performed as described by Ji et al. (19).

Electrophoresis Mobility Shift Assays—Calf thymus pol δ was purified by immunoaffinity chromatography as described by Ji et al. (19), followed by high performance liquid chromatography gel filtration on a SEC-250 column (Bio-Rad). Wild type hPCNA and mutants (150 ng) plus calf thymus pol δ (5 ng) were incubated at room temperature for 10 min in 10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 0.1 mM dithiothreitol, 50 mM KCl, and 5% glycerol in a total volume of 20 μl. The 5′-32P-labeled 30–21-mer template-primer (0.8 ng) was added, and the mixture was incubated for an additional 10 min at room temperature after which the reaction mixtures were subjected to non-denaturing polyacrylamide gel electrophoresis (26). Acrylamide concentrations of 6% were used, and gels were run for 1 h at 80 V. Electrophoresis was performed in 2.5 x 100 V with a running buffer consisting of 45 mM Bis-Tris, pH 7.5, 45 mM boric acid, and 0.1 mM EDTA. After electrophoresis, gels were dried and subjected to autoradiography.

ELISA Assays—ELISA assays were performed according to Dornreiter et al. (27) with minor modifications. ELISA plates were coated with 500 ng/well hPCNA mutant proteins in 50 μl of phosphate-buffered saline (PBS), pH 7.5 for 4 h at 4 °C. After washing twice with PBS buffer, the wells were blocked with 3% (w/v) bovine serum albumin in PBS buffer overnight at 4 °C and washed three times with PBS buffer. Monoclonal antibody 74B1 specific to PCNA (Fig. 3c) or pol δ (Fig. 6) was added at a concentration of 500 ng/well in 50 μl PBS and incubated at room temperature for 2 h. The unbound antibody or protein was removed by washing six times with PBS buffer. For detection of the binding between hPCNA mutant proteins and 74B1 monoclonal antibody, horseradish peroxidase conjugated anti-mouse IgG (1:2000, Amersham) in PBS buffer was added. For detection of pol δ, 0.5 μg of monoclonal antibody (78F5) in 50 μl of PBS buffer was incubated with the plates for 1 h at room temperature. The plates were washed six times with PBS buffer, followed by a 40-min incubation with horseradish peroxidase-conjugated mouse anti-IgG (1:2000, Amersham Corp.) in PBS buffer. The reaction was developed with tetramethylbenzidine base (TMB-ELISA, Pierce) for 2–5 min and quantitated at 450 nm with a microtiter plate reader (Bio-Rad).

CD Spectra—Near and far UV CD spectra of wild type hPCNA and hPCNA mutants were determined with a JASCO J720 spectropolarimeter. Twenty spectra were collected for each sample at a speed of 100 nm/min and subsequently averaged. A cell with a path length of 1 cm was used for near UV spectra (250–320 nm), and one with a path length of 0.1 cm was used for the far UV spectra (200–250 nm). Sample protein concentration was 0.25 mg/ml. Data were fitted re-iteratively to appropriate equations with the SigmaPlot curve fitting program.

Chemical Cross-linking—The chemical cross-linking of wild type and mutant PCNAs was performed with ethylene glycol bis(succinimidyl succinate) (Pierce) as described by Zhang et al. (23). The reactions were performed at room temperature for 2 min, and the products were analyzed by Western blotting.

Processivity Assay—The effect of hPCNA mutants on the processivity of pol δ was analyzed by polycrylamide gel electrophoresis of the reaction products as described by Prelich et al. (5). 5′-32P-end-labeled (dT)16 was annealed to poly(dA). The reaction mixtures (60 μl) contained 0.25 OD units/ml 32P-labeled poly(dA);oligo(dT) (40:1), 40 mM Tris-HCl, pH 6.5, 5 mM MgCl2, 2 mM dithiothreitol, 10% glycerol, 0.1 mg/ml bovine serum albumin, 80 μM dTTP, 150 ng of hPCNA, and 1 unit of pol δ. After incubation at 37 °C for 30 min, reactions were terminated by the addition of 10 μl of 10% glutaraldehyde in saline testis DNA in 20 mM EDTA. 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% polycrylamide, 8 μm urea gels.

RESULTS

Expression and Purification of PCNA Mutants—We have shown previously that the region between residues 121–135 of human PCNA (hPCNA) contains the epitope for an inhibitory monoclonal antibody, 74B1, by the use of overlapping peptides that were tested for their ability to inhibit the immunofluorescence flow cytometry assay of cellular PCNA (18). This region is contained in the extended loop region (residues 119–133) that extends across the surface of hPCNA and connects the two conformationally conserved regions of PCNA that constitute the basic structure of the hPCNA monomer (17). To assess the importance of this loop in the interaction of hPCNA with pol δ, we systematically mutated residues 122–133. The following mutants were constructed: D122A, V123A, E124Q, Q125E, L126S, G127A, I128A, P129A, Q131E, E132Q, and Y133F. All of the PCNA mutants were expressed as recombinant proteins in E. coli as described by Zhang et al. (23) (see “Experimental Procedures”). The hPCNA mutant proteins were characterized at levels comparable to that of the wild type hPCNA as soluble proteins. The purification of hPCNA mutant proteins was monitored by SDS-PAGE, and all were purified to near-homogeneity as judged by SDS-PAGE (Fig. 1).

Chemical Cross-linking—The ability of the hPCNA mutants to engage in normal subunit-subunit interactions was tested by chemical cross-linking using ethylene glycol bis(succinimidyl
Effects of Mutations in the Interdomain Connector Loop on the Immunoreactivity of PCNA—Because we had shown previously that an antibody which inhibited the PCNA stimulation of pol δ possessed an epitope that mapped to the interdomain connector loop (18), we tested the immunoreactivity of the mutants. This inhibitory antibody, 74B1, was used to Western blot the hPCNA mutants. All of the mutants could be Western blotted by the inhibitory monoclonal antibody (upper panel), and 74B1, an inhibitory monoclonal antibody (lower panel). Samples were run on a 5–15% gradient polyacrylamide gel. S refers to the prestained protein standards (maltose-binding protein-β-galactosidase, 175 kDa; maltose-binding protein-paramyosin, 83 kDa; glutamic dehydrogenase, 62 kDa; aldolase, 47.5 kDa; triosephosphate isomerase, 32.5 kDa; β-lactoglobulin A, 25 kDa; lysozyme, 16.5 kDa; aprotinin, 6.5 kDa). WT, wild type.

**FIG. 2. Western blot analysis of PCNA mutants.** Purified PCNA mutants were immunoblotted with 19F4, a non-inhibitory monoclonal antibody (upper panel), and 74B1, an inhibitory monoclonal antibody (lower panel). Samples were run on a 5–15% gradient polyacrylamide gel. S refers to the prestained protein standards (maltose-binding protein-β-galactosidase, 175 kDa; maltose-binding protein-paramyosin, 83 kDa; glutamic dehydrogenase, 62 kDa; aldolase, 47.5 kDa; triosephosphate isomerase, 32.5 kDa; β-lactoglobulin A, 25 kDa; lysozyme, 16.5 kDa; aprotinin, 6.5 kDa). WT, wild type.

Western blot analysis. Mutants D122A, E124Q, I128A, P129A, and E130Q all had weaker interactions with monoclonal antibody 74B1 (Fig. 3). The rest of the mutants had indistinguishable properties from wild type hPCNA (Fig. 3). These results independently define the epitope for antibody 74B1 as the region 122–130, in agreement with our previous studies (18).

**Functional Analysis of the PCNA Mutants**—The functional behavior of the hPCNA mutants in terms of their effects on in vitro DNA synthesis catalyzed by pol δ were examined with both poly(dA)·oligo(dT) and singly primed M13 templates (Fig. 4, A and B). The concentration dependence of the hPCNA mutants for the stimulation of pol δ activity on the poly(dA)·oligo(dT) template was examined. Eight of the 12 hPCNA mutants showed little or no difference in their ability to stimulate DNA synthesis catalyzed by pol δ, and exhibited similar concentration dependences as the wild type hPCNA (Fig. 4A). Mutant L126S was able to stimulate pol δ in this assay to a level similar to that of the wild type, but exhibited a very clear difference in concentration dependence. The mutants V123A, I128A, and G127A, in that order, showed a severe loss of ability to stimulate pol δ activity (Fig. 4A). The results obtained when the mutants were tested with the singly primed M13 assay in which DNA synthesis is dependent on RF-C and RPA are shown in Fig. 4B. The results were qualitatively similar to those observed with the poly(dA)·oligo(dT) template, in that the same mutants showed the most significant losses of activity. These results suggest that none of these mutants is specifically impaired in their abilities to interact with RF-C. In addition, we measured the RF-C-catalyzed loading of PCNA onto DNA by the ability of PCNA to stimulate the ATPase activity of RF-C. ATPase assays were performed with all the mutants as described by Fukuda et al. (28), and all of the mutants exhibited a similar ability to stimulate the ATPase activity of RF-C as wild type hPCNA (not shown).

The hallmark of the effect of PCNA on the in vitro activity of pol δ is its ability to convert the enzyme from a distributive to a processive reaction, as can be shown by examination of the reaction products on polyacrylamide gels. This was done for the PCNA mutants. As can be seen, processivity is lost with the V123A, L126S, G127A, and I128A mutants of PCNA (Fig. 5).
Fig. 4. Stimulation of pol δ activity by wild type and mutant hPCNAs. Human DNA pol δ was purified from calf thymus as described by Jiang et al. (19). Panel A shows the stimulation of pol δ activity using poly(dA)·oligo(dT) as a template as a function of PCNA concentration. Panel B shows the activity of pol δ using an M13 template primer as described by Burgers (24). WT, wild type.

Fig. 5. Effects of hPCNA mutants on the processivity of pol δ. Reactions were performed as described under “Experimental Procedures” and contained 1 unit of calf thymus pol δ assay plus, where added, 150 ng of hPCNA mutants. Reaction mixtures were analyzed on polyacrylamide gels. Lane 1, template alone, no additions; lane 2, pol δ alone; lane 3, wild type hPCNA alone; lane 4, pol δ + wild type PCNA; lane 5, pol δ + D122A; lane 6, pol δ + V123A; lane 7, pol δ + E124Q; lane 8, pol δ + Q125E; lane 9, pol δ + L126S; lane 10, pol δ + G127A; lane 11, pol δ + I128A; lane 12, pol δ + P129A; lane 13, pol δ + E130Q; lane 14, pol δ + Q131E; lane 15, pol δ + E132Q; lane 16, pol δ + Y133F; S refers to a 1-kilobase DNA ladder standard.

These results are consistent with those of the activity assays (Fig. 4). It may be noted that the processivity of DNA polymerase δ in response to PCNA was slightly longer and stronger in the mutant Q125E.

Interaction of the PCNA Mutants with pol δ—The interaction of the hPCNA mutants with pol δ was tested by ELISA. In this assay, hPCNA mutants were immobilized on microtiter plates, and the binding of pol δ was detected using antibody mAb78F5. The results are shown in Fig. 6. Mutants G127A and I128A exhibited the weakest interaction with pol δ. Mutants Q131E and E132Q were also weak in their interaction with pol δ in comparison to the wild type.

Electrophoretic Mobility Shift Assays (EMSA)—To gain further insight into the interactions of the mutant PCNAs with pol δ, we performed EMSA as described under “Experimental Procedures.” In these assays, the ability of PCNA to enhance the binding of pol δ to a labeled DNA template-primer by a shift of the primer in non-denaturing gel electrophoresis is examined. The results of this analysis are shown in Fig. 7. Formation of the complexes was dependent on the simultaneous presence of pol δ, PCNA, and the model template-primer (Fig. 7, lanes 1–5). The formation of pol δ-PCNA-template-primer complexes by gel shift assays was detected with wild type PCNA and with 5 of the 12 mutants (D122A, E124Q, Q125E, E130Q, and Y133F). Mutants V123A, L126S, G127A, I128A, P129A, Q131E, and E132Q did not form pol δ-PCNA-template-primer complexes in this assay (Fig. 7). Several EMSA analyses were performed with different concentrations of mutants, and the results were reproducible.

DISCUSSION

We have systematically mutated each of the residues between 122 and 133 to further define the structural elements in PCNA that are involved in its interaction with pol δ. The choice of this region was based on our previous assignment of the epitope of the inhibitory monoclonal antibody 74B1 to amino acid residues 121–135 of hPCNA by the use of peptides (18). To confirm that this was a rational basis for the functional evaluation of the mutants, we first characterized their immunological properties. Our results confirm the assignment of the epitope of antibody 74B1. One of the 12 PCNA mutants, G127A, could not be Western blotted with the 74B1 antibody (Fig. 2). This lack of interaction of the mutant G127A with the monoclonal antibody 74B1 provides solid evidence that the region between residues 122–133 of hPCNA indeed harbors the...
epitope for 74B1. The ELISA assays showed that within this connector loop region, residues Asp-122, Glu-124, and Gly-127 to Glu-130 are also involved in the interaction between antibody 74B1 and hPCNA. It is surprising that mutation of the Gly-127 exhibited such a pronounced effect on the interaction between hPCNA and pol δ. However, any mutation of glycine, because of the insertion of a large R group, may be sufficient to disrupt the local structure of the epitope that is required for interaction with the monoclonal antibody. Thus, our findings confirm the epitope assignment of monoclonal antibody 74B1 and extend our previous work, in terms of the interpretation of the inhibitory effects of this antibody as being due to its binding to a region involved in protein-protein interaction with pol δ. These findings are significant because the inhibition of PCNA stimulation of pol δ by this antibody can now be confirmed to be due to its direct interference with the binding of PCNA to pol δ.

The analysis of the effects of mutations on residues 122–133 in the interdomain connector of PCNA on the interaction with pol δ was examined in this study at several levels. These studies provide further evidence that this region of hPCNA is directly involved in binding to pol δ. Mutation of Gly-127 and Ile-128 resulted in severe defects in the stimulation of pol δ. Two other mutants, V123A and L126S, exhibited less severe defects in the stimulation of pol δ activity. This loss of activity was established to be due to a weakened interaction between pol δ and PCNA by an ELISA assay in the case of Gly-127 and Ile-128. EMSAs that measured the ability of the PCNA and pol δ to form a ternary complex with a model substrate template proved to be a much more sensitive index of the effects of mutant on PCNA-pol δ interactions, as complex formation was lost with the same four mutants but also with P129A, L126S, and V123A. Our findings are summarized in Table I. This shows quite clearly that mutations of Gly-127 and Ile-128 had a pronounced impact on the ability of human PCNA to stimulate the human pol δ activity. A possible explanation for the pronounced impact of mutation of Gly-127 is that it causes a change in the local structure of the interdomain connector loop of hPCNA, which makes this region of hPCNA less accessible to pol δ. The interaction profile of the PCNA mutants with monoclonal antibody 74B1 is consistent with this explanation.

The loss of the ability of the PCNA mutants to stimulate in vitro DNA synthesis catalyzed by pol δ could be due to (i) the disruption of the trimer formation of hPCNA, (ii) inhibition of the RF-C-catalyzed loading of PCNA onto DNA, (iii) prevention of the binding of pol δ to the hPCNA, or (iv) interference in the translocation of the PCNA-pol δ complex along the DNA template. Chemical cross-linking assays showed that the first possibility is unlikely, and in addition, the CD spectrum of the hPCNA mutants essentially showed no secondary structural changes due to mutations. The PCNA mutants showed similar functional behavior with respect to the RF-C-catalyzed loading of PCNA onto DNA, with either the poly(dA)-oligo(dT) assays or the M13 template-primers, indicating that the inability of the four PCNA mutants to stimulate pol δ was not due to a loss in the interaction between RF-C and PCNA, because RF-C is not required in the poly(dA)-oligo(dT) assays. It is also unlikely that the loss in the ability to stimulate DNA synthesis is the result of interference in the translocation of PCNA clamp or pol δ along the DNA template because EMSA and ELISA assays showed unequivocally that the defect of these four PCNA mutants was a consequence of the loss of the binding of pol δ to PCNA.

The formation of ternary pol δ-PCNA-template-primer complexes was not detected using the hPCNA mutants, V123A, L126S, G127A, I128A, P129A, Q131E, and E132Q (Fig. 7). The stability of the pol δ-PCNA-template-primer complex is deter-

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**Table I**

Summary of mutations in the interdomain connector loop that affect PCNA function

The table summarizes the results obtained for the behavior of the PCNA mutants from the following analyses: immunoreactivity to antibody 74B1 (Western blot and ELISA assays), DNA synthesis (activities with poly(dA)-oligo(dT) or M13, PAGE analysis for processivity), and assay for PCNA-pol δ interactions (ELISA and EMSA gel shift assay). Those mutants which were negatively affected are indicated as −, +, or ++ to indicate an increasing severity of the defect. The interactions of the interdomain connector loop with p21 (17) are indicated for comparison in the last column (SHP = small hydrophobic pocket; LHP = large hydrophobic pocket; Apbs = interactions involved in hydrogen bonding/ion-pairing in the anti-parallel β-sheet interaction between p21 and PCNA).

| Mutant | Immunoreactivity | DNA synthesis | PCNA-pol δ interactions | p21 binding |
|--------|-----------------|---------------|-------------------------|-------------|
|        | Western blot    | ELISA         | Poly(dA)-oligo(dT)      | M13 PAGE    | ELISA EMSA | Apbs |
| D122A  | −               | +             | ++                      | + + + + + + | −          | ++  |
| V123A  | −               | −             | ++                      | + + + + + + | −          | ++  |
| E124Q  | −               | +             | −                       | − − − − − − | −          | Apbs |
| Q125E  | −               | −             | −                       | − − − − − − | −          | Apbs |
| L126S  | −               | −             | −                       | − − − − − − | −          | LHP  |
| G127A  | ++              | ++            | ++++                    | ++ + ++ ++  | ++        | ++  |
| I128A  | −               | −             | +                       | ++ + ++ ++  | ++        | LHP  |
| P129A  | −               | +             | −                       | − − ++ − −  | −          | −    |
| E130Q  | −               | +             | −                       | − − ++ − −  | −          | −    |
| Q131E  | −               | −             | −                       | − − − ++ +  | −          | −    |
| E132Q  | −               | −             | −                       | − − ++ + ++ | −          | −    |
| Y133F  | −               | −             | −                       | − − ++ + ++ | −          | −    |
|        |                 |               |                         |             | LHP        |      |
mained by two factors: the interaction between pol δ and PCNA, and the interaction between the pol δ-PCNA complex and the DNA template-primer. Disruption of either interaction could lead to the dissociation of the pol δ-PCNA-template-primer complex. As expected, the four PCNA mutants (V123A, L126S, G127A, and I128A) that displayed a severe loss in their ability to stimulate pol δ activity fell within this group, indicating that the loss of these four PCNA mutants in the stimulation of pol δ activity was the result of an altered interaction with pol δ. However, mutants P129A, Q131E, and E132Q, which are still functional in the stimulation of pol δ activity, showed loss of function in this assay. It is possible that even if the interactions between the pol δ-PCNA complex and the DNA template-primer were weakened such that they were not detected by EMSA, they might still be sufficient to stimulate DNA synthesis effectively.

The crystal structure of hPCNA complexed with a 22-residue peptide derived from the C terminus of cell-cycle checkpoint protein p21 (residues 139–160) has been solved recently (17). There are three major structural elements of PCNA that are involved in the interaction with pol δ. First, the formation of an anti-parallel β sheet structure between residues 152–160 of p21 and residues 119–127 of PCNA. Following this region of interaction, there is a second major interaction involving residues Leu-126, Ile-128, and Tyr-133 of the PCNA loop. These three residues, together with Met-40, Val-45, Leu-47, Pro-234, Tyr-250, Ala-252, and Pro-253, form a large hydrophobic pocket that interacts with residues 147–151 (MTDFY) of p21. Gly-127, Ile-128, and Pro-129 form one edge of a binding cleft in the large hydrophobic pocket into which the side chains of Met-147, Phe-150, and Tyr-151 of p21 are inserted. There is a small hydrophobic pocket, formed by residues Leu-121 and Val-123 of the PCNA loop, which interacts with Ile-158 of the p21 peptide (17). When the interaction sites of p21 are listed against the mutants of PCNA that affect its interaction with pol δ (Table 1), it is seen that there is a very close correlation between the residues whose mutation are strongly affected and those involved in the PCNA/p21 peptide interaction.

It is strong evidence that the effects of p21 on the inhibition of DNA pol δ activity is mediated by a competition with PCNA (12, 13). The current information of the p21-PCNA structure is thus highly relevant, as it leads to a reasonable proposition that the binding of pol δ to PCNA may involve similar interactions as with p21 to PCNA, namely interaction of an extended region of peptide sequence with the interdomain loop via formation of an anti-parallel β sheet structure and possibly interaction with the two hydrophobic pockets. The proposal that a short peptide sequence of pol δ is involved in the interaction with PCNA is supported by experiments showing that a synthetic peptide corresponding to residues 129–149 of pol δ is able to inhibit PCNA activation of pol δ (32). This sequence is part of a conserved region in the N terminus between yeast, mammalian, Epstein-Barr, and herpes viral DNA polymerases (33).

There have been several other mutational studies of PCNA, which, however, have not provided information on the role of the interdomain connector loop in the interaction with pol δ. A systematic analysis based on the mutation of 29 charged residues of hPCNA to alanine on the basis that alteration of charged residues might have the least effect on the structure of PCNA has been reported (28). Eight of the mutants (Lys-13, Lys-14, Lys-20, Lys-77, Lys-110, Arg-146, Arg-149, and Lys-217) showed defects in the stimulation of pol δ; these residues are located in the helices on the inner surface of the PCNA toroid. Of the residues mutated that lie on the outer surface of PCNA, effects on pol δ activity were observed with Asp-41, Arg-64, and Asp-122. The activities reported for the stimulation of pol δ were <50% for the Asp-41 and between 50 and 80% for the Arg-64 and Asp-122 mutants. The latter is one of the residues we mutated; however, we did not observe significant effects in any of the functional assays. A mutational analysis of Saccharomyces cerevisiae PCNA in which 21 pairs of proximal charged residues were mutated has also been reported (34). In this study, the D41A/D42A mutation exhibited a reduced activity with pol δ. Two double mutants in the loop region, K127A/E129A and E129A/E130A, displayed no phenotypic defects. Arroyo et al. (35) have mutated Schizosaccharomyces pombe PCNA, choosing 7 residues in the loop regions of PCNA that are conserved in S. pombe, S. cerevisiae, rice, and human PCNA. Their results showed that mutation of S. pombe Leu-2 and Arg-64 resulted in reduced abilities to stimulate pol δ, and they concluded that these residues may play a role in interaction with pol δ. The agreement between the fission yeast and the study of hPCNA that Arg-64 is involved is of interest, as mutation of Asp-63 of hPCNA had no effect (28). These studies show some agreement regarding the effects of Asp-41 and Arg-64 on PCNA stimulation of pol δ. Asp-41 is located in the loop between β sheets βC1 and βD1 of hPCNA, which forms one edge of the large hydrophobic pocket of hPCNA. Arg-64 is located in the loop between β sheets βE1 and βF1 of hPCNA, which provides residues that form one edge of the small hydrophobic pocket of hPCNA. Mutation of D41A could cause local structure perturbations of the large hydrophobic pocket, whereas mutation of R64A could affect the local structure of the small hydrophobic pocket, which includes Ala-67 and Gly-69. While a direct interaction between residues Asp-41 and Arg-64 with pol δ is not excluded, these findings provide provocative evidence that the hydrophobic pockets that are involved in p21 binding may also be important in the binding of pol δ. Leu-2 in S. pombe and S. cerevisiae PCNA is not conserved in hPCNA where it is a phenylalanine residue. In both S. cerevisiae and human PCNA, this residue is buried but is adjacent to residues that form the small hydrophobic pocket (7, 17).

Recently, a sequence motif has been identified in p21 (36), Fen1 (37), Fen1 homologues in yeast (RTH1, RAD2, rad2, and rad13), and human XP-G. All these proteins bind to the same site in PCNA and have the consensus sequence QGRDXXFF (37). Cdc27, a putative third subunit of DNA polymerase δ in the fission yeast (38, 39), also contains a similar sequence motif. Deletion or mutation of this motif in Cdc27 abolishes PCNA binding in vitro and Cdc27 also binds to the interdomain connector loop region of PCNA. In p21, the cognate sequence QTSMTDFY (residues 144–151) is contained within the binding site with PCNA (17), and as discussed above, the Met, Phe, and Tyr residues interact with the large hydrophobic pocket. The question raised in relation to our studies is whether the N2 region of pol δ also contains a sequence that can be recognized as a member of the consensus. There is no obvious strong relationship; however, the N2 region (33) contains the highly conserved feature of 3 adjacent aromatic residues, IHGFAPYFY (residues 141–149), which could potentially serve the same role of providing a hydrophobic interaction with PCNA.

Current research now provides a structural basis for the findings that PCNA interacts with a number of other proteins in the form of the identification of a putative peptide motif for PCNA binding (36–39), and the description of the binding of p21 to PCNA at the atomic level (17). Interestingly, the exist-

2 L. S. Cox, personal communication.
3 S. A. MacNeill, personal communication.
ence of a putative third subunit (Cdc27) of yeast pol δ that binds to PCNA^{3} raises the issue of redundancy in the binding of the pol δ holoenzyme to PCNA, since our studies indicate that the catalytic subunit of pol δ (p125) interacts directly with PCNA. An additional issue is whether it is p125 or the third subunit (or both) that is responsible for the functional interaction of pol δ with PCNA. The enzyme we used in this study was purified by the immunoaffinity chromatography and gel filtration, and may contain other components besides the two subunit form that is isolated after stringent conventional chromatography (19). In our hands, the pol δ p125 subunit overexpressed in SF9 cells could be stimulated only 5-fold with PCNA in the presence of recombinant p50 expressed in E. coli (40). Polymerase δ obtained by co-expression of the p125 and p50 subunits (pol δ core) in the baculovirus system could be stimulated by PCNA at most 8-fold, far less than the stimulation obtained by the holoenzyme prepared from the immunoaffinity column. Thus, it is possible that the full functional effects of PCNA on pol δ may involve multi-point interactions. That these interactions may involve the same site on PCNA is feasible, since its trimeric structure should contain three binding sites.

In summary, we have obtained mutational evidence for the localization of the region of PCNA that is involved in binding to pol δ to the interdomain connector loop of PCNA. This evidence is consistent with, and strengthened by, a previous analysis of the epitope of an inhibitory antibody. In addition, the recent molecular analysis of the p21-PCNA interaction site, which is also localized to the interdomain connector loop, provides a basis for the prediction that pol δ may interact in a similar way, and a rational mechanism for the ability of p21 to inhibit DNA synthesis.

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