The formation of a complex between DNA polymerase δ (pol δ) and its sliding clamp, proliferating cell nuclear antigen (PCNA), is responsible for the maintenance of processive DNA synthesis at the leading strand of the replication fork. In this study, the ability of the p125 catalytic subunit of DNA polymerase δ to engage in protein-protein interactions with PCNA was established by biochemical and genetic methods. p125 and PCNA were shown to co-immunoprecipitate from either calf thymus or HeLa extracts, or when they were ectopically co-expressed in Cos 7 cells. Because pol δ is a multimeric protein, this interaction could be indirect. Thus, rigorous evidence was sought for a direct interaction of the p125 catalytic subunit and PCNA. To do this, the ability of recombinant p125 to interact with PCNA was established by biochemical means. p125 co-expressed with PCNA in Sf9 cells was shown to form a physical complex that can be detected on gel filtration and that can be cross-linked with the bifunctional cross-linking agent Sulfo-EGS (ethylene glycol bis(sulfosuccinimidylsuccinamate)). An interaction between p125 and PCNA could also be demonstrated in the yeast two hybrid system. Overlay experiments using biotinylated PCNA showed that the free p125 subunit interacts with PCNA. The PCNA overlay blotting method was also used to demonstrate the binding of synthetic peptides corresponding to the N2 region of pol δ and provides evidence for a site on pol δ that is involved in the protein-protein interactions between PCNA and pol δ. This region contains a sequence that is a potential member of the PCNA binding motif found in other PCNA-binding proteins. These studies provide an unequivocal demonstration that the p125 subunit of pol δ interacts with PCNA.

Proliferating cell nuclear antigen (PCNA) was originally discovered as an antigen in autoimmune sera from patients with systemic lupus erythematosus and was reported to be found only in actively proliferating cells (1). It was later shown to be a factor that enhanced the processivity of DNA polymerase δ (pol δ) and to have key roles in both DNA replication and repair (2, 3). There have been striking recent advances in our understanding of the structure and functions of PCNA (4). Purification and expression of human recombinant PCNA and its physicochemical characterization established that it was a trimeric protein (5). The crystal structures of both yeast and human PCNA have been determined (6, 7). Like the T4 gene 45 protein and the β subunit of Escherichia coli DNA polymerase III holoenzyme, PCNA functions as a sliding DNA clamp that forms a closed ring around duplex DNA (8). The binding of pol δ to PCNA provides an elegant micromechanical solution to the biological need to maintain an extraordinarily high level of processivity during the synthesis of chromosomal DNA (8–10). Recently, it has also been found that PCNA has a number of protein partners with which it interacts (4, 9, 11). Pol δ has been shown to be involved not only in DNA replication but also in DNA repair and can be regulated by cell cycle proteins (2, 12, 13). Thus, an important area of interest is the protein-protein interaction sites of PCNA, because it may be the nexus for multiple protein-protein interactions involved in replication, repair, recombination, and cell cycle regulation.

The pol δ core enzyme consists of two subunits, p125 and p50 (14). Previous work from this laboratory has implicated the p125 subunit in an interaction with PCNA (10). A synthetic peptide conforming to the N2 region (residues 129–149) was found to inhibit PCNA stimulation of pol δ isolated from calf thymus (10). p125 and PCNA co-expressed in Sf9 cells could be co-immunoprecipitated with an antibody to PCNA, showing that the catalytic subunit of DNA polymerase δ interacted with PCNA (10). However, the recombinant p125 catalytic subunit can only be stimulated by PCNA at most 2–3-fold, and the presence of the p50 subunit is required to restore a significant level of PCNA stimulation of the p125 subunit (15–17).

Two recent studies of yeast pol δ reported contrary results. No evidence for a direct interaction between the Schizosaccharomyces pombe pol δ p125 subunit and PCNA could be found, either by co-immunoprecipitation experiments after their co-expression in insect cells or by a yeast two hybrid assay (18). It was concluded that no direct interaction occurs between S. pombe p125 and PCNA. In Saccharomyces cerevisiae, similar results were obtained using a PCNA overlay assay. In the latter studies, the interaction of S. cerevisiae p125 and p58, as well as the recently identified third subunit (p55), was studied by a PCNA overlay method. It was shown that only the third subunit (p55) of pol δ interacted with PCNA (19), indicating that the interaction of pol δ with PCNA involved the third subunit, whereas no evidence could be obtained for an interaction of PCNA with either the small second subunit p58 or the catalytic subunit.

These findings are in conflict with our previous studies of the p125-PCNA interaction, and they suggest that the binding of PCNA in yeast and human depend on different subunit-PCNA interactions. Alternatively, it is also possible that the interaction of PCNA with pol δ involves multiple interactions with pol.
δ subunits. For this reason, we have undertaken a rigorous examination of the protein-protein interactions of the p125 subunit of pol δ and PCNA using different biochemical methods. Our studies leave little doubt of the ability of the p125 subunit to interact with PCNA.

**EXPERIMENTAL PROCEDURES**

**Ectopic Expression of p125 and PCNA in COS-7 Cells**—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal bovine serum. These cells were transfected with c-Myc-His-PCNA and pCMV-p125 using the calcium phosphate method. For each 9-cm Petri dish, the transfected DNA consisted of 5 μg of each expression vector and 10 μg of Bluescript SK DNA. The cells were washed with phosphate-buffered saline and scraped in 1 ml of radioligand precipitation buffer (10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% Nonidet P-40, 0.1% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride). After 15 min on ice, the lysed cells were spun at 5000 rpm for 15 min at 4 °C. Immunoprecipitations were performed using anti-Myc antibody (Roche Molecular Biochemicals) in 1 ml of extract. The immunoprecipitates were separated on a 5–15% gradient SDS gel and immunoblotted with anti-His antibody (Invitrogen) or pol δ monoclonal antibody. The blot was then detected by chemiluminescence (ECL, Amersham Pharmacia Biotech).

**GST Sf9 Insect Lysate Expression**—Recombinant p125 and PCNA—Sf9 cells were grown in 75-cm² flasks to 75% confluence. The cultures followed by three washes of TBST for 10 min each. The blots were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. Prestained protein standards (Sigma) were used as molecular weight markers. The nitrocellulose membranes were then blocked and blotted with biotinylated PCNA as described above.

**Pairwise Yeast Two Hybrid Interactions**—Plasmids expressing the GAL4 fusions with the coding sequences for p125, p50 and PCNA were constructed in pAS2–1 and pACT2 vectors (CLONTECH Laboratories Inc.). Sequential transformations of Y190 were performed by the lithium acetate method with dimethyl sulfoxide at a final concentration of 10%. pAS2–1-p125 and pAS2–p50 were transformed into Y190 and plated onto Trp⁻ plates. Y190 stains transformed with pAS2–1-p125 or pAS2–1-p50 were then transformed with pACT2–50 or pACT2-PCNA. After overnight recovery in Trp⁻ Leu⁻ medium, the transformants were plated on Trp⁻ Leu⁻ His⁻ 3-AT plates to select for histidine prototrophy. For the liquid assay, GAL4 and T antigen/p53 were transformed into Y190 as positive controls. pAS2–1-p50 and pAS2–1-PCNA in Y190 were used as negative controls. One ml of overnight yeast culture in liquid Trp⁻ Leu⁻ His⁻/SD selection medium was prepared. Four ml of YPD medium were added, and the reaction was incubated at 30 °C for 3–90 min. (Positive strong interactions were incubated for 3 min and negative interactions for 90 min.) The reactions were terminated by addition of 0.4 ml of 1 M Na2CO3. Cell debris was removed by centrifugation at 10,000 × g for 2 min, and the A600 was recorded. The β-galactosidase activity was calculated. Arbitrary units of activity were calculated as: β-galactosidase units = 1000 × A600/(t × V × Amax), where t = min of incubation; V = 0.1 ml.

**RESULTS**

Co-immunoprecipitation of p125 and PCNA from Crude Calf Thymus and HeLa Extracts and after Their Ectopic Expression in Cultured Cells—Previous studies have demonstrated that human PCNA could be co-immunoprecipitated with the p125 catalytic subunit of pol δ from Sf9 insect cell lysates, under conditions where both proteins were overexpressed as recombinant proteins (10). It can be argued that the interactions observed were a consequence of the supranormal concentrations of both proteins under these conditions of overexpression and may not reflect the behavior of the two proteins in a normal cellular context. Studies were therefore performed to establish whether PCNA and p125 could be co-immunoprecipitated from mammalian tissue or cell culture extracts. The results show that p125 and PCNA can be readily co-immunoprecipitated from crude calf thymus extracts that had been partially purified on phenyl agarose, as well as from HeLa cell lysates (Fig. 1, A and B). Because of the multisubunit nature of pol δ, these experiments do not show a direct interaction between p125 and PCNA, but they do confirm that an interaction between pol δ and PCNA is readily demonstrated in cell extracts.

Next, evidence for an interaction between p125 and PCNA when they are ectopically expressed in mammalian cultured cells was sought. Human PCNA was expressed in Cos 7 cells with dual tags (a hexahistidine tag and a c-Myc tag) together with p125 expressed in the pCMV vector (see under “Experimental Procedures”). Samples of the cell extracts were immu...
Lanes 1–3 nitrocellulose membranes. The membrane was divided into two halves. PCNA. Samples were subjected to 10% SDS-PAGE and transferred to lane 1, 4, immunoprecipitate from monoclonal 38B5 against pol d 150 body for each immunoprecipitation. Immunobeads were suspended in lysate (2 mg of protein) was incubated with 30 μl of lysis buffer and protease inhibitors. The lysate was precleared with control containing purified PCNA, immunoblotted with the antibody against PCNA. Western blotting (WB) was performed using biotinylated sheep anti-mouse immunoglobulin as second antibody followed by incubation with streptavidin-biotinylated horseradish peroxidase complex. Color development was performed by incubation with 4-chloro-1-naphthol and hydrogen peroxide and terminated with sodium azide. Incubation with 4-chloro-1-naphthol and hydrogen peroxide and terminated with sodium azide.

FIG. 1. Co-immunoprecipitation of p125 and PCNA with monoclonal antibodies from calf thymus extracts or HeLa extracts. A, lane 1, a calf thymus extract was passed through phenyl agarose, and 50 μl of the extract was immunoprecipitated (IP) with 20 μg of 74B1 PCNA monoclonal antibody and blotted with monoclonal antibody 78F5 against pol δ. Lane 2, a calf thymus extract was passed through phenyl agarose, and 50 μl of the extract was immunoprecipitated with 78F5 pol δ antibody and immunoblotted with 20 μg of 74B1 monoclonal antibody against PCNA. Lane 3, control containing purified PCNA, immunoblotted with the antibody against PCNA. Western blotting (WB) was performed using biotinylated sheep anti-mouse immunoglobulin as second antibody followed by incubation with streptavidin-biotinylated horseradish peroxidase complex. Color development was performed by incubation with 4-chloro-1-naphthal and hydrogen peroxide and terminated with sodium azide.

B, HeLa cells were lysed in 1% Nonidet P-40 lysis buffer and protease inhibitors. The lysate was precleared with protein G agarose bead before incubation with antibodies (Ab). HeLa lysate (2 mg of protein) was incubated with 30 μg of monoclonal antibody for each immunoprecipitation. Immunobeads were suspended in 150 μl of lysis buffer and 150 μl of 2× SDS loading buffer. Lanes 1 and 4, 50 μg of untreated crude HeLa lysate. Lanes 2 and 6, 50 μl of immunoprecipitate from monoclonal antibody 38B5 against pol δ. Lanes 3 and 5, 50 μl of immunoprecipitate from monoclonal antibody 74B1 against PCNA. Samples were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. The membrane was divided into two halves. Lanes 1–3 were immunoblotted with monoclonal antibody 78F5 against pol δ. Lanes 4–6 were immunoblotted with monoclonal antibody 74B1 against PCNA. Western blotting was performed using horseradish peroxidase anti-mouse immunoglobulin as second antibody followed by chemiluminescence detection.

doprecipitated using a c-Myc antibody and were Western blotted with pol δ antibody. The results show that p125 is co-immunoprecipitated with c-Myc-His-tagged PCNA (not shown). Control experiments in which the immunoprecipitants were blotted with anti-His antibody confirmed that PCNA was present in the immunoprecipitate. These experiments demonstrate that pol δ p125 can be shown to co-immunoprecipitate with PCNA, either when endogenous pol δ is present or when p125 is ectopically co-expressed with PCNA.

Formation of a Physical Complex between p125 and PCNA—The experimental results in which p125 co-expressed with PCNA in either baculovirus (10) or in mammalian cells (Fig. 1) indicated that p125 is able to interact with PCNA and imply that this interaction is independent of the presence of p50 or other subunits of pol δ. In these experiments, co-immunoprecipitation does not provide rigorous evidence that the p125 subunit directly interacts with PCNA, as it is possible that the p125 that is detected by co-immunoprecipitation is by virtue of an interaction of PCNA with a pol δ heterodimer, i.e., the interactions are mediated by a third polypeptide(s). In order to provide a more rigorous test, SF9 cells were co-infected with baculovirus vectors for p125 and for PCNA. The SF9 cell lysates were then subjected to gel filtration on a Sephacryl S-300 column (Fig. 2). Assays for pol δ activity showed that there were two peaks of activity, one with a relative molecular weight of 125,000 and the second with a relative molecular weight of 220,000 (Fig. 2, upper and middle panels). The fractions were analyzed for the presence of p125 and PCNA by Western blotting using specific antibodies. The results show that the 125,000 peak contained only p125, whereas PCNA co-migrated with p125 in the 220,000 molecular weight fractions. Furthermore, no free PCNA was detected in the range where either the dimer or trimer form would be expected to migrate. (In previous studies of the behavior of recombinant PCNA expressed in E. coli, we had shown that PCNA in solution is a mixture of dimers and trimers (5)). These results provide the first direct demonstration that free p125 forms a physical complex with PCNA, and moreover, the apparent molecular weight is consistent with a complex of p125 with a trimeric form of PCNA, because previous work showed that the PCNA trimer migrates with an apparent molecular weight of approximately 100,000 on gel filtration (5).

In order to establish that the co-elution of p125 and PCNA is not due to fortuitous associations with unrelated proteins, cross-linking experiments using bifunctional cross-linking...
agents were performed to establish a direct p125-PCNA protein-protein interaction in the 220,000 molecular weight complex. Sulfo-EGS was used, as we have previously shown that EGS readily cross-links PCNA (5). When the peak fractions of the complex of p125 and PCNA obtained on gel filtration were incubated with sulfo-EGS at the indicated concentrations (mM) for 15 min at room temperature and then subjected to SDS-PAGE followed by Western blotting using a monoclonal antibody against p125 (A, left panel) and a monoclonal antibody against PCNA (A, right panel). Arrows a and b show p125 immunoreactive bands of 225 and 125 kDa, respectively; arrow c corresponds to the cross-linked PCNA dimer, and arrow d corresponds to the PCNA monomer. B shows the determination of the relative molecular masses of the bands a–d (open squares); prestained protein standards (New England Biolabs, Inc.) were used as markers (solid circles). C, a similar cross-linking experiment was performed using a deletion mutant of p125 (Δ2–249) in which the N-terminal 249 residues were deleted (13). The upper panel shows the Western blot of the 97 kD immunoreactive bands of 225 and 125 kDa, respectively; the lower panel shows the immunoblot with antibody against PCNA, where 1–3 show the positions of the monomer, dimer, and trimer forms of PCNA, respectively, without evidence for formation of cross-links with p125 (Δ2–249).

In a parallel experiment, a deletion mutant of p125 (Δ2–249) in which the N-terminal 248 residues were removed (13, 20) was co-expressed with PCNA and subjected to cross-linking. In this case, only the monomer, dimer, and trimer species of PCNA were observed, as for PCNA alone, as shown in Fig. 3C. Blotting with pol δ antibodies also showed that there were no cross-links formed with the N-terminal deletion mutant of pol δ (Fig. 3C, upper panel). These results are consistent with our previous observations that the N-terminal region of p125 is required for its interaction with PCNA.

For Western Blotting with PCNA—In order to obtain additional evidence that p125 can interact with PCNA, an overlay technique for blotting of PCNA-binding proteins was used. PCNA was labeled with biotin as described under “Experimental Procedures.” Pol δ was subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were then blotted with biotinylated PCNA (see under “Experimental Procedures”) and visualized using a chemiluminescence method. A number of experiments were performed; they showed that only the p125 band provided a strong reaction with biotinylated PCNA. When immunoadfinity purified calf thymus pol δ enzyme was blotted with biotinylated PCNA, a strong reaction was observed with the p125 catalytic subunit (Fig. 4A, left panel). The p50 subunit of pol δ did not interact with PCNA in the overlay experiments (Fig. 4A, left panel). The inability of the p50 subunit to interact with PCNA was confirmed using purified recombinant p50 subunit (Fig. 4A, left panel). This experiment was repeated using recombinant pol δ heterodimer produced by overexpression of p125 and p50 in SF9 cells. The same results were obtained, namely that p125 but not p50 reacted with biotinylated PCNA (Fig. 4B). Because the overlay depends on an interaction with a polypeptide band separated by SDS-PAGE, this positive interaction demonstrates that the p125 subunit interacts with PCNA in a manner that is independent of the presence of the other subunits of pol δ. In Fig. 4A, it is noted that the overlay of immunoadfinity purified pol δ with PCNA reveals a doublet of 70 kDa. In other studies, partial protein sequence was obtained of this band, and a BLAST search identified this polypeptide as KIAA0039 (GenBank27). This was found to be a mammalian counterpart of S. pombe Cdc27.

We have also examined the Δ2–249 N-terminal deletion mutant of p125, in which the N2 region is absent (13), by PCNA overlay. The results (Fig. 5A) show that this deletion mutant is not recognized by overlay with biotinylated PCNA. In parallel, experiments were performed using deletion mutants Δ186–321, Δ336–715 (core region deleted), Δ675–1107, and Δ778–1047 (C-terminal regions deleted) expressed in SF9 cells (Fig. 5B). All of these deletion mutants, covering essentially the entire p125 sequence from residue 186 (Fig. 5C), interacted with PCNA. These results, taken with the inability of the Δ2–249 deletion mutant to bind to biotinylated PCNA, restricts the binding region on p125 to within the first 186 residues.

**Yeast Two Hybrid Assay—**Human p125, p50 and PCNA coding sequences were inserted into the pAS2–1 and pACT2 vectors and tested for pairwise interactions using the yeast two hybrid system. For these experiments, the yeast co-transformants were grown and the lysates tested for β-galactosidase activity. The results were compared on the basis of relative specific activities (arbitrary units of β-galactosidase activity per unit of cell culture density). The results are shown in Fig. 6. This analysis confirms the biochemical data that p125 and PCNA interact and that p50 and PCNA do not interact.

**The N2 Peptide Binds to PCNA—**Previous work had identi-
fied a region of the N terminus of pol \(\delta\), the N2 region (GVT-DEGFSVCCHIHGFAAPFY, residues 129–149) as being involved in the interaction of pol \(\delta\) with PCNA. This was based on the ability of a synthetic peptide with this sequence to inhibit the PCNA stimulation of pol \(\delta\) (10). However, these experiments were performed with purified pol \(\delta\), and in the context of evidence that p125 does not interact with PCNA in other systems, it could be argued that these findings were due to an interference of the peptide with an interaction between p125 and an intermediary protein that leads to loss of PCNA response. Experiments were performed to test for a direct interaction of PCNA with the N2 peptide as well as with several variants with selected alanine replacements by dot blot analysis using biotinylated PCNA (Fig. 7). The peptides were bound to nitrocellulose and then blotted with biotinylated PCNA. The results (Fig. 7) show that immobilized N2 peptides, but not the mutant N2 peptide in which the three terminal YFY residues were replaced with alanine (GVTDEGFSVCCHIHGFAAPAAA), are blotted by biotinylated PCNA. p21 peptide and p125 were also blotted in this dot blot assay (Fig. 7). The loss of interaction of the mutant in which the three terminal aromatic residues were changed to alanine (GVTDEGFSVCCHIHGFAAPFY, residues 129–149) as being involved in the interaction of pol \(\delta\) with PCNA. This was based on the ability of a synthetic peptide with this sequence to inhibit the PCNA stimulation of pol \(\delta\) (10). However, these experiments were performed with purified pol \(\delta\), and in the context of evidence that p125 does not interact with PCNA in other systems, it could be argued that these findings were due to an interference of the peptide with an interaction between p125 and an intermediary protein that leads to loss of PCNA response. Experiments were performed to test for a direct interaction of PCNA with the N2 peptide as well as with several variants with selected alanine replacements by dot blot analysis using biotinylated PCNA (Fig. 7). The peptides were bound to nitrocellulose and then blotted with biotinylated PCNA. The results (Fig. 7) show that immobilized N2 peptides, but not the mutant N2 peptide in which the three terminal YFY residues were replaced with alanine (GVTDEGFSVCCHIHGFAAPAAA), are blotted by biotinylated PCNA. p21 peptide and p125 were also blotted in this dot blot assay (Fig. 7). The loss of interaction of the mutant in which the three terminal aromatic residues were changed to alanine is highly significant because it provides supportive evidence that the N2 region contains a variant of the PCNA binding motif (see under “Discussion”).

**DISCUSSION**

Biochemical evidence for a direct interaction of the p125 catalytic subunit with PCNA was obtained. The demonstration of a physical complex between recombinant p125 and PCNA by gel filtration and chemical cross-linking with sulfo-EGS provides rigorous evidence for this interaction. In addition, the use of biotinylated PCNA in overlay experiments also shows that
this interaction is not dependent on the presence of other pol δ subunits, and the use of deletion mutants of p125 restricts the location of the interaction site to the N-terminal 186 residues of p125. Further evidence for a region on p125 that is involved in the interaction with PCNA was obtained by the use of synthetic peptides to the N2 region (residues 129–149). These findings confirm and extend previous studies from this laboratory that show that p125 directly interacts with pol δ and that the N2 region in p125 can be implicated in the protein-protein interaction with PCNA. The need for a more detailed investigation of whether there is a direct interaction between p125 and PCNA was raised by studies of pol δ of whether there is a direct interaction between p125 and PCNA was obtained. Tratner et al. (18) reported that recombinant S. pombe p125 did not interact with hemagglutinin epitope-tagged PCNA when they were co-expressed in insect cells and tested for co-immunoprecipitation of 35S-la-

beled proteins or when tested for pairwise interactions by the yeast two hybrid system. In S. cerevisiae, a third subunit of pol δ encoded by the POL32 gene has been identified (22, 23). A 32P-tagged PCNA containing a fused PKA site at the N terminus was used in overlay experiments against p125 (POL3), p58 (POL31), and p55 (POL32) subunits. The results showed that only the p55 subunit bound to the tagged PCNA and no evidence of interaction of either p58 (the p50 homologue) or p125 with PCNA was found (19). The different results that were obtained in the yeast system could be due to differences in the experimental conditions used, as noted by Eisenberg et al. (19). The present studies confirm that, unlike the yeast proteins, human PCNA and p125 co-immunoprecipitate with either p125 or PCNA antibodies when they are co-expressed in Sf9 cells, COS 7 cells, and are also co-immunoprecipitated from calf thymus or HeLa extracts. As already noted, co-immunoprecipitation methods do not eliminate the possibility that positive results are due to the intervention of an intermediary polypeptide(s).

An important result obtained in these studies is the first biochemical demonstration of a complex between free recombinant p125 and PCNA by gel filtration. All the gel filtration studies were performed in the presence of 150 mM NaCl, a standard biochemical practice to avoid nonspecific associations. Thus the association between p125 and PCNA takes place at ionic strengths that are near physiological. The cross-linking of the complex with sulfo-EGS showed that there was a rapid cross-linking to a high molecular weight species of a size that was consistent with a p125-PCNA trimer. These results must be taken in the context that under the conditions used we have reproducibly found that PCNA itself is only slowly cross-linked to the trimer and that the bulk of the reaction products are the monomer and dimer species (5). Previous observations from this laboratory have shown that PCNA in solution is an equilibrium mixture of the dimer and trimer species (5). The findings that the only complex present was a PCNA trimer-p125 complex, with an absence of dimeric or monomeric PCNA complexes with p125, suggests that p125 either selectively binds to the PCNA trimeric form or stabilizes the PCNA trimer. This preference for trimeric PCNA is consistent with the physiologically expected interaction of pol δ with PCNA, the function of which is associated with a trimeric state. It is also consistent with current models of the assembly of the replication complex.

### Table I

| PCNA Binding Motif | Gen Bank no. |
|--------------------|-------------|
| Pol δ              | 12345678    |
| S. cerevisiae      | 901234567   |
| UDG                | 78912345    |
| Human              | 12345678    |
| PCNA               | 12345678    |
| Tigger Cds2        | 12345678    |
| Human              | 12345678    |
| Rad2               | 12345678    |
| S. pombe           | 12345678    |
| Human              | 12345678    |
| p57                | 12345678    |
| Human              | 12345678    |
| p21                | 12345678    |
| S. pombe           | 12345678    |
| Drosophila         | 12345678    |
| S. cerevisiae      | 12345678    |
| Cdc27              | 12345678    |
| S. pombe           | 12345678    |
| POGO               | 12345678    |
| Drosophila         | 12345678    |
| FEN1               | 12345678    |
| Human              | 12345678    |
| XPG                | 12345678    |

UDG, uridine DNA glycosylase; MCMT, methyl 5′-cytosine DNA methyl transferase. The eight residue PCNA binding motif is numbered 1–8 at the top. Conserved residues are in bold at positions 1, 4, 7, and 8. In the C-terminal regions (numbered 9–17), proline residues are underlined and basic residues (arginine and lysine) are in bold.
in which replication factor C first loads PCNA onto DNA, following which pol δ is recruited (8). Recent findings (24) have shown that replication factor C disengages from PCNA upon sliding clamp formation and that the loaded PCNA clamp was able to recruit and bind polymerase δ and stimulate DNA replication.

Recently, a number of additional proteins that bind to PCNA have been identified (4, 9, 11, 25). These findings have major implications for understanding the roles of PCNA. These PCNA-binding proteins fall into three major groups: DNA replication proteins (pol δ, replication factor C and pol ε, flap endonuclease 1, and DNA ligase), DNA repair proteins (xeroderma pigmentosum G and methyl-5-cytosine/methyl transferase), and cell cycle regulatory proteins (p21 and p57). These proteins contain a short PCNA binding motif in which there is a conserved glutamine and two conserved aromatic residues. The motif in question lies at the N terminus of the p21 peptide that forms an α-helical region in which the two aromatic residues interact with the large hydrophobic pocket of PCNA, whereas the C-terminal region forms an anti-parallel β sheet with the interdomain connector loop of PCNA (7).

An obvious question that arises is whether the N2 region of the p125 subunit of pol δ harbors a sequence that corresponds to the PCNA binding motif that has been identified in these PCNA-binding proteins. The alignment of the N2 sequence of pol δ from human and yeast with the PCNA binding motifs of a number of proteins is shown in Table I, which shows the eight-residue motif bounded by the conserved glutamine and the aromatic residues. The main features of the motif are the conserved glutamine at position 1, the presence of an aliphatic residue (leucine or isoleucine) at position 4, and a pair of aromatic residues at positions 7 and 8. Comparison of the N2 sequence shows that it possesses the aromatic residues and the aliphatic residue at position 4, but does not have the conserved glutamine. Thus, the region of the N2 sequence does not carry a complete consensus with the known PCNA binding motif. However, this glutamine residue is not conserved in p57, which also binds PCNA (Table I). Evidence obtained in this study using peptides to the N2 region of pol δ provides strong evidence that it contains a variant of the PCNA binding motif (Table I). The loss of binding when the aromatic residues are mutated in the N2 peptide does provide significant evidence that this region of the N2 sequence may be a member of the PCNA binding motif family of sequences.

Table I, the regions C-terminal to the PCNA binding motif are also aligned. In the case of p21, this region is involved in the second major protein-protein contact of the peptide with PCNA and forms an anti-parallel β-sheet with the interdomain connector loop of PCNA (7). In previous studies, it had been shown that the interdomain connector loop of PCNA is important for the interaction of pol δ, and it was proposed that the N2 region of pol δ may interact with PCNA in a manner that may be similar to the interaction of p21 with PCNA (26, 27). There is a group of PCNA-binding proteins in which there is a preponderance of basic residues in this region (Table I), as previously noted by Warbrick et al. (28). However, it is also seen that there is a second group of sequences, in which there is a high percentage of proline residues. This includes pol δ from human and yeasts, human uracil DNA glycosylase, methyl 5′-cytosine methyl transferase, the human Tigger sequences (28), and S. pombe RAD2. The positions of the prolines is well conserved in these eight examples (Table I). The presence of this “proline-rich” motif provides additional support for the view that the N2 sequence belongs to the family of PCNA binding sequences. Furthermore, it should not be forgotten that from the example of p21 that the QXXLXXFF motif reflects an interaction only with the large hydrophobic pocket of PCNA. Although examples also exist that indicate that this interaction alone (Drosophila POGO and S. pombe Cdc27) is sufficient for PCNA binding, the presence of the two features of the C-terminal region in the form of the proline rich motif and the basic motif suggests that these may represent sequences that are suited to the formation of a β-sheet with the interdomain connector loop of PCNA.

In summary, a detailed approach was undertaken to investigate the issue of whether the p125 polypeptide directly interacts with PCNA. Our findings provide strong biochemical confirmation that there is a direct interaction between the p125 subunit and PCNA, although they do not eliminate the possibility that the pol δ holoenzyme has multiple sites of interaction with PCNA through one or more of its subunits. The latter possibility is one that could be facilitated by the trimeric nature of PCNA, because this provides for extended interactions of individual PCNA subunits with different subunits of pol δ.

REFERENCES
1. Miyachi, K., Fritzler, M. J., and Tan, E. M. (1978) J. Immunol. 121, 2228–2234
2. Zhang, X.-R., Jiang, Y., Zhang, S.-J., Hao, H., and Lee, M. Y. W. T. (1994) J. Biol. Chem. 269, 13748–13755
3. Burgers, P. M. J. (1998) Chromosoma 107, 218–227
4. Warbrick, E. (1998) BioEssays 20, 195–199
5. Zhang, P., Zhang, S.-J., Zhang, Z., Weensner, J. F., and Lee, M. Y. W. (1995) Biochemistry 34, 10703–10712
6. Krishna, T. S., Kong, X. P., Gary, S., Burgers, P. M., and Kuriyan, J. (1994) Cell 79, 1233–1243
7. Gullits, J., Melman, Z., Hurwitz, J. O’Donnell, M., and Kuriyan, J. (1996) Cell 87, 297–306
8. Kelman, Z. (1997) Oncogene 14, 629–649
9. Cox, L. R. (1997) Trends Cell Biol. 7, 493–498
10. Zhang, S.-J., Zeng, X.-R., Zhang, P., Toomey, N. L., Chuang, R. Y., Chang, L. S., and Lee, M. Y. W. T. (1995) J. Biol. Chem. 270, 7988–7992
11. Lou, G., Zhang, S. J., Zhang, P., Toomey, N. L., and Lee, M. Y. W. T. (1997) Nucleic Acids Res. 25, 5041–5046
12. Zeng, X.-R., Hao, H., Jiang, Y., and Lee, M. Y. W. T. (1994) J. Biol. Chem. 269, 24027–24033
13. Wu, S.-M., Zhang, P., Zeng, X.-R., Zhang, S.-J., Mo, J., Li, B. Q., and Lee, M. Y. W. T. (1998) J. Biol. Chem. 273, 9561–9569
14. Lee, M. Y. W. T., Tan, C. K., Downey, K. M., and So, A. G. (1984) Biochemistry 23, 1906–1913
15. Zhang, P., Frugulietti, I., Jiang, Y., Holt, G. L., Condit, R. C., and Lee, M. Y. W. T. (1995) J. Biol. Chem. 270, 7993–7998
16. Sun, Y., Jiang, Y., Zhang, P., Zhang, S.-J., Zhou, Y., Li, B. Q., Toomey, L. N., and Lee, M. Y. W. T. (1997) J. Biol. Chem. 272, 13013–13018
17. Zhou, J. Q., He H., Tan, C. K., Downey, K. M., and So, A. G. (1997) Nucleic Acids Res. 25, 1094–1099
18. Tsurumi, I., Piard, K., Grenon, M., Perederiset, M., and Baldacci, G. (1997) Biochem. Biophys. Res. Commun. 231, 321–328
19. Eisenberg, J. C., Ayyagari, R., Gomes, X. V., and Burgers, P. M. J. (1997) Mol. Cell. Biol. 17, 6367–6378
20. Yang, C. L., Chang, L. S., Zhang, P., Hao, H., Zhu, L., Toomey, N. L., and Lee, M. Y. W. T. (1992) Nucleic Acids Res. 20, 735–745
21. Jiang, Y., Zhang, S. J., Wu, S. M., and Lee, M. Y. W. T. (1995) Arch. Biochem. Biophys. 329, 297–304
22. Gerik, K. J., Li, X., Pautz, A., and Burgers, P. M. J. (1998) J. Biol. Chem. 273, 19747–19753
23. Burgers, P. M. J., and Gerik, K. J. (1998) J. Biol. Chem. 273, 19756–19762
24. Podust, V. N., Tiwari, N., Stephen, S., and Fanning, E. (1998) J. Biol. Chem. 273, 31929–31999
25. Watanabe, H., Pan, Z. Q., Schreiber-Agus, N., Depinho, R. A., Hurwitz, J., and Xiong, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1392–1397
26. Roos, G., Jiang, Y., Landberg, G., Nielsen, N. H., Zhang, P., and Lee, M. Y. W. T. (1996) Exp. Cell Res. 226, 208–213
27. Zhang, P., Sun, Y., Hou, H., Zhang, L., Zhang, Y., and Lee, M. Y. W. T. (1996) J. Biol. Chem. 271, 713–719
28. Warbrick, E., Heatherington, W., Lane, D. P., and Glover, D. M. (1998) Nucleic Acids Res. 26, 3925–3932