Mammalian DNA polymerase δ (pol δ), a key enzyme of chromosomal DNA replication, consists of four subunits as follows: the catalytic subunit; p125, which is tightly associated with the p50 subunit; p68, a proliferating cell nuclear antigen (PCNA)-binding protein; and a fourth subunit, p12. In this study, the functional roles of the p12 subunit of pol δ were studied. The inter-subunit interactions of the p12 subunit were determined by yeast two-hybrid assays and by pulldown assays. These assays revealed that p12 interacts with p125 as well as p50. This dual interaction of p12 suggests that it may serve to stabilize the p125-p50 interaction. p12 was shown to be a novel PCNA-binding protein. This was confirmed by identification of a PCNA-binding motif at its N terminus by binding assays and by site-directed mutagenesis. The activities and reaction products of recombinant pol δ containing a p12 mutant defective in PCNA binding, as well as purified recombinant pol δ and its subassemblies, were analyzed. Our results indicate that p12 contributes to PCNA-dependent pol δ activity, i.e. the p12-PCNA interaction is functional. Our data indicate that both p12 and p68 are required for optimal pol δ activity. This supports the hypothesis that the interaction between pol δ and PCNA is a divalent one that involves p12 and p68. We propose a model in which pol δ interacts with PCNA via at least two of its subunits, and one in which p12 could play a role in stabilizing the overall pol δ-PCNA complex as well as pol δ itself.

Chromosomal DNA replication in eukaryotic cells requires the following three distinct DNA polymerases: polymerase α, polymerase δ (pol δ),3 and polymerase ε. DNA pol δ is the key enzyme that is thought to play a central role in the elongation of both the leading and the lagging strands of DNA and the maturation of Okazaki fragments (1–3). DNA pol δ was originally identified as a new type of DNA polymerase possessing an intrinsic 3′→5′-exonucleolytic activity (4). Mammalian pol δ holoenzyme consists of the p125 catalytic subunit (which harbors both 5′→3′ DNA polymerase and 3′→5′-exonuclease activities) and a tightly associated second subunit p50; this core is associated with two other subunits, p68 and p12, that are also referred to as the third and fourth subunits (5–9). The function of pol δ as a chromosomal DNA polymerase is dependent on its association with PCNA, which functions as a molecular sliding clamp (10, 11). The third subunits of pol δ in both mammalian (p68/p66) and in yeast cells (Cdc27 in Schizosaccharomyces pombe and pol 32 in Saccharomyces cerevisiae) harbor a PCNA-binding motif, and it has been shown that this provides a PCNA interaction site for pol δ (12–16). However, the exact nature of the subunit contacts of mammalian pol δ with PCNA has yet to be clarified; we (17–20) and others (8) have reported that human pol δ p125 binds to PCNA, although other reports have come to the opposite conclusion (14, 21). There is also a report that the p50 subunit of mammalian pol δ binds to PCNA (21).

The fourth subunit of mammalian pol δ, p12, is a 12-kDa protein identified by its association with highly purified pol δ from calf thymus (9). S. pombe pol δ also possesses a fourth subunit, Cdm1 (22), that is nonessential (23) but is absent in S. cerevisiae pol δ, which is a three-subunit enzyme (12). These observations suggest that the fourth subunits of pol δ play a minor role in pol δ function. However, it has been reported that small interfering RNA-mediated knockdown of p12 causes a significant decrease in the FGF2-driven proliferation rate of p12 (25) when assayed on poly(dA)/oligo(dT) templates in the presence of PCNA. These findings point to an important role for p12 in pol δ function, and the finding that the trimeric complex lacking p68 (p125/Hsp68) exhibits less than 10% of the activity of the tetrameric pol δ holoenzyme, although the trimeric complex lacking p68 (p125/Hsp50/p12) appeared to be fully active (25) when assayed on poly(dA)/oligo(dT) templates in the presence of PCNA. These findings point to an important role for p12 in pol δ function, and the finding that the trimeric complex lacking p68 exhibits PCNA-stimulated activity indicates that one or more of the other three subunits must be able to interact with PCNA. However, the trimeric complex containing p125, p50, and p12 has not been rigorously characterized, and the exact mechanisms of how the p12 subunit contributes to the PCNA-dependent activity of pol δ are largely unknown. In this study we have identified the subunit contacts of p12, and we show that it binds to both p125 and p50 and may function to stabilize the pol δ complex. In addition, we show that p12 is a PCNA-binding protein and contributes to the binding of pol δ to PCNA.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Sigma except otherwise indicated.

Oligonucleotides were synthesized by Qiagen (Stanford, CA). Monoclonal antibodies against p125 (78F5) and p50 (13D5 and 17D2) and rabbit polyclonal antibodies against p68 and p12 were as described previously (26).

Plasmid Constructions—Wild type His-tagged p68 was constructed by insertion of the full-length p68 cDNA sequence between the NdeI and HindIII sites of the pTlac vector with eight histidine residues added at its N terminus. His-tagged p125 or p50 was constructed by insertion of the full-length p125 or p50 cDNA sequence between the
NdeI and BamHI sites of the pET33b vector, respectively. His-tagged p12 was constructed by insertion of the full-length p12 cDNA sequence between the NdeI and BamHI sites of the pET15b vector. GST full-length p21, p125, p50, p12, and p12 deletion mutants were generated by the PCR. The generated PCR fragments were digested with BamHI and EcoRI, subcloned in-frame into the BamHI-EcoRI sites of pGEX-5X-3, and sequenced. A His-p12 mutant (His-p12Mt) that lost PCNA binding activity was generated. This was done by mutation of Ile-7, Ser-10, and Tyr-11 in the PCNA-interacting protein (PIP) box to alanines using a QuickChange site-directed mutagenesis kit (Stratagene, CA). The primer pairs were as follows: p12 5’-GGCGCGGAAAGCGCCTGCACTGATGCGGACCCGGTTGTTGAAAGAGAGGGG-3’ and 5’-CCCTCTCTTTCCAAACCGGTGTCATCCTGCGAGGCGTTCGCCC-3’. A unique restriction site for NruI, which is underlined in the primer sequences given above, was created to screen the mutants. Mutations were verified by DNA sequencing after cloning into pET15b.

**Protein Purifications—**GST fusion or His-tagged proteins were expressed in *Escherichia coli* BL21DE3(plys) and purified by the use of either glutathione beads (Amersham Biosciences) or nickel-nitrilotriacetic acid-Sepharose (Sigma) or anti-p125 monoclonal antibody (78F5). His-p12 and His-p12Mt were further purified using Superdex 200 gel filtration chromatography (Amersham Biosciences). Wild-type human PCNA expressed in *E. coli* was purified using conventional chromatography as described previously (20).

**GST Pulldown Assays—**GST or GST fusion proteins (0.5 μg) in GST binding buffer (50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40 and 0.2 mM phenylmethylsulfonyl fluoride) were mixed with purified p125, His-p50, His-p12, or His-p68 (0.5 μg of protein). The reaction mixtures were incubated by gentle rocking for 2 h at 4 °C, immobilized on glutathione-Sepharose beads for another 2 h at 4 °C, and washed five times with binding buffer. The bound proteins were analyzed by SDS-PAGE and Western-blotting with either anti-His monoclonal antibody (Sigma) or anti-p125 monoclonal antibody (78F5).

**Expression and Purification of p125, Two-subunit p125/p50, Three-subunit p125/p50/p12, and Four-subunit p125/p50/p68, and p125/p50/p68/p12Mt Pol δ Enzymes—**Baculoviruses expressing p125, p50, p68, and p12 are as described previously (26). pFastBac-p12Mt was generated by PCR using pET15b-12Mt as template. p12Mt baculovirus was generated using the Bac-to-Bac system (Invitrogen). For expression and purification of the recombinant enzymes, 50 ml of 2 × 10⁶/ml insect Hi Five cells in suspension culture were co-infected with the different combinations of p125, p50, p68, and p12 baculovirus at a multiplicity of infection of 5 for 48 h. Cell pellets were lysed in 20 ml of TGEE (40 mM Tris-HCl, pH 7.8, 10% glycerol, 0.5 mM MgCl₂, 25 mM HEPS, pH 6.0, 100 cpm/pmole [³²P]dATP, and ~0.2 unit of pol δ in the absence or presence of 100 ng of PCNA in a total volume of 30 μl. Reaction mixtures were incubated for 30 min at 37 °C and were terminated by spotting onto DE81 papers that were then washed four times with 0.3 mM ammonium formate, pH 7.8, once with 95% ethanol, and counted. One unit of pol δ activity catalyzes the incorporation of 1 nmol of dTMP per h at 37 °C.

**Reaction products were analyzed by PAGE of the products as described (28). The reaction mixtures (30 μl) contained 0.25 OD units/ml of poly(dA)/oligo(dT) (40:1), 200 μg/ml BSA, 5% glycerol, 10 mM MgCl₂, 25 mM HEPS, pH 6.0, 100 cpm/pmole [³²P]dATP, the indicated amounts of PCNA, and 10–40 ng of pol δ. After incubation at 37 °C for 30 min, reactions were terminated by the addition of 10 μl of salmon testis DNA (10 mg/ml) 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 12% polyacrylamide, 8 μm urea gels.

**Assays using M13 DNA as the template were performed as follows.** M13mp18 DNA (New England Biolabs) was primed with 20-mer oligonucleotide (5’-CTAGAGGATCCCCGGGTAC-3’) complementary to nucleotides 6262–6243. An M13 DNA/primer mixture (1:2 molar ratio) in buffer containing 10 mM Tris-HCl, pH 7.8, 2.5 mM MgCl₂, 125 mM NaCl was heated at 70 °C for 15 min and annealed by slow cooling overnight to room temperature. The standard reaction contained 10 ng of pol δ, 40 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 0.2 mg/ml BSA, 10 mM MgCl₂, 0.5 mM ATP, 50 mM NaCl, 250 μM each of dTTP, dCTP, dGTP, and 25 μM dATP, 3 μCi of [α-³²P]dATP, 100 ng of primed M13 template, 80 ng of RFI, 200 ng of RPA, and variable amounts of PCNA in a 30-μl reaction volume. The reaction mixtures were incubated at 37 °C for 30 min and were terminated by the addition of 20 mM EDTA. The reaction products were run on 1.5% alkaline agarose gels at 50 V for 2.5 h. The gels were visualized with a PhosphorImager.

**PCNA Overlay Assay—**PCNA gel overlay for the detection of PCNA-binding proteins was performed as described previously by the use of digoxigenin (DIG)-labeled PCNA as a probe (20).

**Yeast Pairwise Assay—**Yeast pairwise assays for protein-protein interactions were performed as described previously (19).
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RESULTS

p12 Binds to the p125 and p50 Subunits and Can Thereby Function as a Bridge That Provides Additional Stability to the p125–p50 Subassembly—In order to approach the question of whether p12 may have some structural role in the assembly of the pol δ complex, we determined its ability to interact with the other subunits of pol δ. By using the yeast two-hybrid assay, we tested the ability of p12 to interact with p125, p50, or p68 (Fig. 1A). Human p125, p50, and p68 coding sequences were inserted into the pAS2-1 vector and tested for their pairwise interactions with p12 inserted in the pACT-2 vector using the yeast two-hybrid system. For these experiments, the yeast transformants were grown and the lysates tested for β-galactosidase activity. The results were compared on the basis of the relative specific activities (arbitrary units of β-galactosidase activity per unit of cell culture density). The results show that p12 interacts with both p125 and p50 but not with p68 (Fig. 1A).

In order to confirm the yeast two-hybrid data, interactions between p12 and the other three subunits were examined by systematic GST pulldown assays using purified pol δ subunits. GST-p12 and GST-p50 were tested for their ability to pull down purified recombinant p125 or p68. GST-p50 could pull down purified p125 and His-p68, although GST-p12 interacted with p125 but not with His-tagged p68 (Fig. 1B,a and b). The interaction between p12 and p50 was confirmed by a pulldown assay using GST-p12 and p50 (Fig. 1B,c) and by the reciprocal pulldown assay using GST-p50 and p12 (Fig. 1B,d). GST-p125 could pull down p50 and p12 but not p68 (Fig. 1B,e–g). A diagram of the interactions between the subunits of human pol δ based on these experiments is shown in Fig. 1B,h. The p125, p50, and p68 subunits form a linear arrangement. p12 bridges p125 and p50. This ability of p12 to bind to both p125 and p50 is consistent with the view that it serves to strengthen the interaction between them, effectively functioning as a brace.

p12 Interacts with PCNA Directly and Is Part of a Network of pol δ-PCNA Interactions—We have tested the ability of p12 to bind to PCNA. A PCNA overlay assay was performed using DIG-labeled PCNA (20) on His-p125, His-p50, His-p68, and His-p12. GST-p21 (29,30) was used as a positive control. The overlay assay (Fig. 2A) showed that p12 interacted strongly with DIG-PCNA, although His-p50 and GST did not. In the same experiment, His-p68 and His-p125 also interacted with PCNA. The band for p12 and GST is weaker than those for p68 and p12, but this may be due in part to lower protein loading as shown by the protein stain for the membrane in Fig. 2B.

These results show that p12 is a PCNA-binding protein and that p68 and p125 are also detected as PCNA-binding proteins in the same overlay blot. This clarifies previous issues as to which subunits of pol δ have the capacity to interact with PCNA and raises some important questions regarding the network or architectural arrangement of the overall protein-protein interactions of the pol δ-PCNA complex that is required for DNA replication (see “Discussion”).
Identification of the Interaction Site of p12 That Is Involved in PCNA Binding—A panel of GST-p12 deletions was constructed and purified. These purified proteins and control proteins, GST-p12 (29, 30) and GST, were loaded at equal amounts onto SDS-PAGE and subjected to the PCNA overlay assay. Only the GST-p12-(1–31) fusion protein bound to PCNA (Fig. 3A). GST, GST-p12-(32–72), GST-p12-(73–107), GST-p12-(20–107), and GST-p12-(30–107) fusion proteins did not show any interaction with PCNA (Fig. 3A). This indicates that the binding region for PCNA lies in the N-terminal 31 residues of p12. The failure of the GST-p12-(20–107) fusion protein to interact with PCNA (Fig. 3A) narrows the region to residues 1–19.

PCNA, besides being a critical partner of pol δ in the process of chromosomal DNA replication, also binds to a number of other proteins involved in DNA transactions (31, 32). The sequences responsible for PCNA binding have been found to conform to a loose consensus sequence, the PIP box (Table 1). Examination of the first 19 N-terminal amino acid residues of p12 indicates that there is a putative PCNA-binding motif (KRL

\begin{align*}
\text{K} & \, \text{R} \\
\text{L} & \, \text{I} \\
\text{T} & \, \text{D} \\
\text{S} & \, \text{Y}
\end{align*}

between amino acid residues 4 and 11 cant role in the pol δ-PCNA interaction. p12, together with p68 and p125, could form a triad of interactions that mediate pol δ-PCNA interaction, assuming that all three subunits are capable of interacting with PCNA when in the pol δ-PCNA complex. In order to gain further insights into the roles of the p12 and p68 subunits in PCNA-pol δ interaction, we characterized the PCNA-dependent enzyme activities of pol δ and its subassemblies. For this study the recombinant proteins were expressed in S9 cells and purified to near-homogeneity by immunofinity chromatography and Mono-Q fast protein liquid chromatography (see “Experimental Procedures”). The subunits were not tagged in order to avoid potential steric effects on pol δ assembly that could compromise the data. The first parameter we examined was the specific activities of the purified preparations when determined by the commonly used assay for pol δ activity. This assay determines activity using a poly(dA)/oligo(dt) template in the presence of PCNA (see “Experimental Procedures”). We compared the p125 subunit alone, p125/p50, p125/p50/p68, p125/p50/p12, and p125/p50/p68/p12. The data are

| Protein | Residues | PIP box |
|---------|----------|---------|
| Hs p12  | 144–151  | QTSTMDFY |
| Hs p68  | 456–663  | QVSITGFF |
| Hs FEN1 | 337–344  | QGRDLDFF |
| Hs DNA ligase 1 | 2–9 | QRISMSSF |
| Hs MCMT | 164–171  | QTITTSHF |
| Sp Cdm1 | 14–21    | NTNIRDVF |
| Hs p12Mt | 4–11     | KRLITDSY |
| PIP box | X/XX/XXF | QXXXXVF |

* The PIP box in p12 is conserved in S. pombe Cdm1, although it is as yet unknown if the latter is a PCNA-binding protein.
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FIGURE 5. ELISA analysis of the binding of p12 and p12Mt to PCNA. A, 96-well ELISA plates were pre-coated with 100 ng of His-p12, His-p12Mt, or BSA and incubated with increasing amounts of PCNA as indicated. The bound PCNA was detected using a monoclonal antibody against PCNA. B, 96-well plates were coated with 100 ng of His-p12, His-p12Mt, or BSA were then performed using a monoclonal antibody against His to detect the interaction between PCNA and p12. Absorbance readings were taken at 450 nm, and the values were plotted after subtraction of the control values with BSA (His-p12, circles; His-p12Mt, squares). Each assay was performed three times and the standard deviations are shown by the error bars.

FIGURE 6. Comparison of the specific activities of pol δ and its subcomplexes. Recombinant p125, p125/p50, p125/p50/p68, p125/p50/p12, and p125/p50/p68/p12 pol δ complexes were expressed in insect cells and purified to near-homogeneity (see "Experimental Procedures"). Assays were performed using poly(dA)/oligo(dT) as the template in the presence of PCNA (see "Experimental Procedures"). Each assay contained the same amount of protein (40 ng). One unit of pol δ activity catalyzes the incorporation of 1 nmol of dTMP per h at 37 °C. The specific activities as units/mg protein were determined and are shown relative to the specific activity of the holoenzyme (solid bars). The relative specific activities were also normalized to reflect the activities based on the molar content of the p125 subunit (shaded bars).

The data, interpreted in the simplest context that there are no conformational changes that affect the p125 catalytic subunit when it is assembled in larger complexes, would reflect the strength of the PCNA-pol δ interaction and thereby the combined contributions of the individual subunits to PCNA binding. The p125 catalytic subunit and the p125/p50 assembly were not significantly stimulated by PCNA, as we have shown previously (26, 33), nor was the p125/p50/p68 trimer (Fig. 6). However, the trimer containing p12 subunit and the p125/p50 core exhibited comparable activity to the holoenzyme on a per mg basis or, more correctly, to a 73% relative activity based on the molar content of the p125 subunit (Fig. 6). Similar findings using His-tagged subunits have been observed previously in recombinant pol δ assemblies (25). Until now, these data have posed a conundrum, because the p68 subunit possesses a PCNA-binding site and was thought to be a primary site for PCNA interaction. Our experiments indicate that p12 plays a role in PCNA binding when it is assembled into the holoenzyme. The lack of response to PCNA of the p125/p50/p68 trimer could be explained by assigning p68 a smaller role in PCNA binding compared with p12 or by assigning p68 a smaller role in PCNA binding compared with p12 or by the likelihood that p12, in addition to its PCNA-binding function, plays a structural role in pol δ or the combined pol δ-PCNA assemblies.

The processivity of the pol δ p125/p50/p12 enzyme was analyzed by examination of the reaction products by gel electrophoresis in the presence of increasing amounts of PCNA. When poly(dA)/oligo(dT) template primer was used, the reaction products were comparable in terms of the maximum size of the DNA (~1000 bp) that are made by the

also normalized to reflect the activities based on the molar content of the p125 subunit (Fig. 6, shaded bars). (The relative specific activities of all five pol δ assemblies in the absence of PCNA were very similar, at ~2,000 units/mg.)
holoenzyme, although it is noted that the three subunit enzyme is less sensitive to PCNA (Fig. 7A).

The p125/p50/p12 enzyme was then tested for its ability to carry out highly processive synthesis using singly primed M13 DNA as the template. In this assay, pol δ activity is dependent on the presence of replication protein A (RPA) and replication factor C (RFC) for the loading of PCNA. The reaction products were examined by agarose gel electrophoresis for the appearance of the completed M13 DNA (~7 kb). When challenged with the M13 template, p125/p50/p12 is poorly active, in contrast to its behavior on poly(dA)/oligo(dT). The amount of completed full-length M13 is small compared to that for the holoenzyme (Fig. 7B), and there appear to be more reaction products at ~1 kb than for the four subunit pol δ, suggesting that there is also significantly greater pausing by p125/p50/p12. Thus, despite the fact that the enzyme activities are very similar on poly(dA)/oligo(dT), pol δ/p125/p50/p12 is deficient in the M13 assay. From this it can be inferred that p68 (as well as p12) is essential for optimal activity on the M13 template.

The Effects of Mutation of the PCNA-binding Motif of p12 on the Activity of the pol δ Holoenzyme—The recombinant pol δ holoenzyme containing the p12 mutant (p12Mt) was prepared and purified to near-homogeneity. This holoenzyme contains a p12 subunit that does not bind to PCNA (Figs. 4 and 5). In order to confirm that mutation of p12 did not affect its reconstitution into the holoenzyme, equal amounts of the purified enzymes were subjected to Western blotting with antibodies to all four subunits. The amounts of each of the four subunits in the two preparations are indistinguishable by this comparison (Fig. 8A).

The specific activities of the pol δ[p12Mt] preparations were found to vary from 60 to 85% of the wild type pol δ based on the data obtained for three preparations.

The effects of PCNA on the activities of pol δ and pol δ[p12Mt] with poly(dA)/oligo(dT) as the template were compared (Fig. 8B). The PCNA concentration that is required to elicit half-maximal activation of the two enzymes was determined by nonlinear regression analysis. Because the activity is dependent on PCNA binding, these values represent the apparent \( K_d \) values for PCNA binding to pol δ. In the experiment shown in Fig. 8B, the \( K_d \) values for PCNA binding to pol δ and pol δ[p12Mt] were found to be 17 and 35 nM, respectively. We have repeated the analyses with a separate set of enzyme preparations and obtained values that reflect a 3-fold increase in \( K_d \) (Fig. 8B, see legend). The relative specific activities of the pol δ[p12Mt] preparations used above were 61 and 67% that of the wild type pol δ based on the analysis of the \( V_{max} \). This difference is significant and is consistent with the view that loss of the PCNA-binding site of p12 affects the interaction of pol δ with PCNA.

The reaction products of pol δ[p12Mt] were compared with those of pol δ by gel electrophoresis as a function of PCNA concentration (Fig. 9A). The processivity of the two enzymes is similar within the range of products examined (up to 1000 bp). However, the response to PCNA of pol δ[p12Mt] is lower than for pol δ, as can be seen by comparison of the products formed with 25, 50, and 100 ng of PCNA, respectively (Fig. 9A, lanes 2–4). The data confirm the kinetic analyses of pol δ[p12Mt] (Fig. 8B), which showed a 2–3-fold increase in \( K_d \).

The activities of pol δ and pol δ[p12Mt] were then compared in the presence of increasing amounts of PCNA using singly primed M13 DNA as the template. Pol δ[p12Mt] was less active than the wild type pol δ in the presence of similar concentrations of PCNA (Fig. 9B). Even at the highest concentration of PCNA used, it is apparent that the amount of DNA synthesis is only a fraction of that of pol δ (Fig. 9B, cf. lanes 6). Thus the efficiency of pol δ[p12Mt] in this assay is reduced, unlike its behavior with the poly(dA)/oligo(dT) template. Nevertheless, pol δ[p12Mt] is able to synthesize products of similar length to pol δ. These experiments provide further supportive evidence that p12 contributes to the function of pol δ through its PCNA binding ability. In addition, the fact that pol δ[p12Mt] is stimulated by PCNA, whereas the p125/p50/p68/p12 trimer is not (25),4 indicates that the role of p12 is not limited to its PCNA binding function. The ability of p12 to act as a bridge between p125 and p50 may be as important as a stabilizing factor as is its contribution to PCNA interaction.

**DISCUSSION**

In this study we have examined the functions of the p12 subunit and have made two major experimental findings. The first is that p12 binds to both the p125 and the p50 subunits. p12 would act as a stabilizing factor for the structure because the free energy of the binding interactions for p125/p50/p12 would be greater than that of the same structure where p12 binds to either p125 or p50 only. However, these observations on their own do not reveal whether this expected contribution of p12...

4. H. Li, B. Xie, Y. Zhou, A. Rahmeh, S. Trusa, S. Zhang, Y. Gao, E. Y. C. Lee, and M. Y. W. T. Lee, unpublished observations.
p12 to the structural stability is required or contributes to the overall function of pol δ. Examination of the properties of the pol δ subassemblies and the pol δ[p12Mt] enzymes provides additional support for the view that p12 has a role in the stabilization of the pol δ enzyme, as well as for the pol δ-PCNA complex.

Our second major finding is that p12 is a PCNA-binding protein. This is based on PCNA overlay and ELISA analysis. The PCNA-binding site was shown to be located at residues 4–11 by site-directed mutagenesis and was identified as a member of the PIP box family. That p12 is a PCNA-binding protein adds to the complexity of the issue as to which subunits of pol δ are involved in PCNA binding. The p68 subunit has a well defined PCNA-binding motif (12–16), but it appears to be dispensable for pol δ activity assayed using poly(dA)/oligo(dT) as the template because the p125/p50/p12 trimer is fully active. On the other hand, the PCNA-dependent activity of pol p125/p50/p12 is strongly affected on the singly primed M13 DNA templates (Fig. 7B). These experiments also indicate that the p68 subunit is essential in this assay. Thus, both p12 and p68 are required for optimal pol δ-PCNA function.

We observed that the p12 homolog in S. pombe, Cdm1 (22, 23), also contains a putative PIP box (Table 1), which leads us to suggest that it too might be a PCNA-binding protein. Although S. cerevisiae pol δ lacks a p12 subunit, it is notable that the heterodimer (pol δ*) that is equivalent to mammalian p125/p50 is capable of interacting with PCNA, although much higher concentrations are required to solicit activity than with the heterotrimer (12), i.e. the S. cerevisiae pol δ catalytic subunit and/or its p50 homolog must be able to interact with PCNA.

Recent analyses of the thermodynamic and structural interactions of PCNA with peptides derived from p21, p68, and FEN-1 that contain the PIP box have shed some insights into the issue of whether a single interaction between PCNA and a PIP box of a given protein is sufficient in vivo. Analyses of the binding constants for PIP box peptides derived from p21, the p68 subunit, and FEN-1 were determined by isothermal titration calorimetry. The p68 and FEN-1 peptide bound to human PCNA 189–725-fold less tightly than those derived with p21, with $K_d$ values of 16 and 60 μM respectively (34). The authors suggested that the weaker binding of the p68 and FEN1 to PCNA would be enhanced by additional binding interactions, for which they favored potential interactions with DNA.

The properties of pol δ[p12Mt], in which the PCNA-binding motif of p12 was mutated, were examined in order to assess whether p12 contributes to the PCNA binding of pol δ to PCNA. The apparent $K_d$ value for PCNA was reproducibly found to be 2–3-fold higher than that for pol δ. Although this difference is small, other evidence that pol δ[p12Mt] has a decreased affinity for PCNA was obtained by gel electrophoretic analysis of the reaction products with both poly(dA)/oligo(dT) and M13 template-primers. pol δ[p12Mt] was able to synthesize full-length M13 DNA, although with a great deal less efficiency.

Our data support a model in which both the p68 and p12 subunits interact with a single PCNA homotrimer. Mammalian pol δ has been shown to bind to PCNA (8, 17–20); however, the question of whether the p125 interaction with PCNA is also engaged in the pol δ-PCNA complex remains to be experimentally resolved by site-directed mutagenesis. The fact that PCNA is trivalent makes it possible that pol δ might be able to interact with a single molecule of PCNA homotrimer through its three subunits, p125, p68, and p12.

Examination of the network of interactions in a model of the PCNA-
pol δ as an integrated structure is shown in Fig. 10. In this model the interactions between the pol δ and PCNA subunits are shown with the assumption that these contacts take place with a single PCNA homotrimer. This map highlights the potential importance of p12 as a brace for the p125-p50 subunit pair, as a keystone for the pol δ-PCNA interaction. The latter can be deduced by examining the number of protein–protein interactions in the network (considering pol δ and PCNA as a unitary assembly without regard for the origin of the subunit). The potential role of p12 in this overall structure is supported by comparison of the complete network (Fig. 10A) with that for the trimer where p12 is removed (Fig. 10B). In the complete structure, six of the seven participants are each involved in three protein–protein interactions. Removal of p12 converts the interaction network to one in which only two of the seven proteins (two PCNA monomers) retain trivalent interactions. From this perspective, p12 is a keystone for the pol δ-PCNA complex because it creates four of the six trivalent interactions. By contrast, loss of the p68 subunit (Fig. 10C) has a lesser effect, in that four of the trivalently bonded proteins still remain. Thus, based on our model we would argue that p12 not only stabilizes pol δ but may have an equally important role in converting pol δ and PCNA into an integrated assembly. This model may be incomplete or only partially correct but does provide a novel perspective of pol δ-PCNA interactions.

In summary, we have shown that p12 binds to p125 and p50 and may play a stabilizing role in the pol δ complex. P12 was shown to be a novel PCNA-binding protein, with a PIP box at its N terminus. Analyses of pol δ complexes provided evidence that the PCNA binding of p12 contributes to the pol δ-PCNA interaction. These findings suggest that p12 plays a significant role in pol δ function.

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Note Added in Proof—It is noted that if only two of the three PCNA-binding pol δ subunits interact with a single PCNA homotrimer at a given time, then there will be three potential arrangements of the pol δ-PCNA complex, i.e. pol δ bound to PCNA via p125 and p12, p125 and p68, or p68 and p12. This may permit pol δ to adopt flexible configurations with PCNA in which its spatial orientation can be shifted between several modes. A paradigm for a dynamic arrangement involving a PCNA-protein complex has emerged from the crystal structure of the human FEN1-PCNA complex (35). FEN1 is able to adopt conformations in which it can swing out from PCNA via a hinge region, and it has been proposed that this could allow for a tracking mode for the complex, as well as for a “locked down” mode that is catalytically functional. In addition, it was noted that there would be sufficient spatial access for a molecule of DNA ligase I, pol δ, or pol ε to bind to the same PCNA molecule in the “swung out” mode of the FEN1 core (35). Such flexibility would provide a functional economy during lagging strand DNA replication, where DNA transactions involving several PCNA-bound enzymes take place, since it would reduce or eliminate multiple exchanges of different enzyme-PCNA complexes on the DNA template-primer. A similar spatial flexibility of pol δ-PCNA conformational states would support such a concept. An alternative possibility is that the three interaction sites of pol δ could allow a single pol δ molecule to interact with two PCNA molecules. It has been reported that PCNA may function as a “back to back” dimer of two PCNA homotrimers (36). If this were the case, then pol δ might bind to one PCNA molecule via p125 and p12, whereas p68 may have sufficient extension to bind to the second PCNA molecule.

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