Y-family DNA polymerases are believed to facilitate the replicative bypass of damaged DNA in a process commonly referred to as translesion synthesis. With the exception of DNA polymerase η (polη), which is defective in humans with the Xeroderma pigmentosum variant (XP-V) phenotype, little is known about the cellular function(s) of the remaining Y-family DNA polymerases. We report here that an interaction between human DNA polymerase ε (polε) and the proliferating cell nuclear antigen (PCNA) stimulates the processivity of polε in a template-dependent manner in vitro. Mutations in one of the putative PCNA-binding motifs (PIP box) of polε or the interdomain connector loop of PCNA diminish the binding between polε and PCNA and concomitantly reduce PCNA-dependent stimulation of polε activity. Furthermore, although retaining its capacity to interact with polε in vivo, the pol-PIP box mutant fails to accumulate in replication foci. Thus, PCNA, acting as both a scaffold and a modulator of the different activities involved in replication, appears to recruit and coordinate replicative and translesion DNA synthesis polymerases to ensure genome integrity.

Y-family DNA polymerases are widely distributed among the three kingdoms of life. Human cells contain at least four; Rev1, polκ,1 and two RAD30 paralogs, polη and polλ (1, 2). The best characterized is polη, which has been shown to be defective in humans with the sun-sensitive, cancer-prone Xeroderma pigmentosum variant (XP-V) syndrome (3, 4). The biological functions of polκ, polλ, and Rev1 polymerases remain, however, largely unknown. Indeed, the cellular function of human polκ is particularly enigmatic given its unique enzymatic properties in vitro. Detailed biochemical analysis of the purified enzyme indicates that it exhibits up to 100,000-fold differences in the frequency of nucleotide misincorporation depending upon the template base replicated. At template T, the wobble base G is incorporated 3–11 times more often than the correct Watson-Crick base, A. However, at template A, misincorporations occur with a frequency of $10^{-7}$ (5–7).

Human polλ also exhibits a unique ability to incorporate bases opposite certain DNA lesions. Unlike the related polη, which bypasses cis-syn cyclobutane thymine dimers efficiently and relatively accurately (8, 9), polλ frequently misincorporates nucleotides opposite the 3′T of the dimer (10), and the efficiency of bypass is sequence context-dependent (11). In contrast, polλ very efficiently inserts bases opposite the more structurally distorting 6–4 pyrimidine-pyrimidone lesion (6, 10, 11), a benzo[a]pyrene adducted deoxyadenosine (12), and a noncoding abasic site (6, 13). In the latter three cases, the enzyme is unable to facilitate lesion bypass but instead requires assistance from another translesion DNA synthesis enzyme, such as polκ (6) or polλ (12).

Given the unique and generally error-prone properties of polλ in vitro, it makes teleological sense that these activities would be tightly regulated in vivo. One appealing mechanism would be to strictly control access of the polymerase to a growing primer terminus. For the replicative polymerases, such activity is coordinated by the replicative processivity factors (prokaryotic β-clamp and eukaryotic PCNA). The process is mediated by a clamp loader (which in eukaryotes is replication factor C (RFC)), which recognizes the DNA primer terminus and opens and assembles a PCNA ring around the nascent DNA (reviewed in Ref. 14). At least three human Y-family polymerases, including polλ, are believed to physically interact with the homotrimeric PCNA clamp (15–17), and such interactions have been proposed to play a central role in enabling access of the translesion DNA synthesis polymerases to a blocked replication fork (18–20).

In an attempt to understand how these interactions may specifically influence the enzymatic and biological properties of polλ, we show that the processivity of polλ is stimulated in the presence of PCNA in vitro, have identified regions in both polλ and PCNA that are important for a physical and functional interaction between the two proteins, and demonstrate that a PCNA-polλ interaction is required for the normal recruitment of polλ into cellular replication factories in vivo.

**EXPERIMENTAL PROCEDURES**

**Two-hybrid Constructs**—The cDNA for PCNA was amplified by PCR using oligonucleotides PCNA1 (5′-CGG CCT GCA TAT GTT CGA GGC GGC C-3′) and PCNA2 (5′-ATA CGG ATC CCT AAG ATC CTT C-3′) and co-transformed into yeast with the two-hybrid reporter plasmids pAct2 and pGAD22. For each of the above constructs, a polλ truncation mutant (Δ213-365) was used as a control.

**Assay of Interaction between PCNA and Polλ**—The above constructs were transformed into yeast strains BY4741 (MATa his3Δ leu2Δ ura3Δ ade2Δ) and YPH499 (MATa his3Δ leu2Δ ura3Δ ade2Δ). Yeast were grown on YPD (1% dextrose, 2% Bacto-yeast extract, and 4% bactopeptone) at 30 °C, and then grown in synthetic medium lacking amino acids. Cells were then induced with 100 mM 3-aminotriazole (3-AT). The yeast strains were grown in liquid YPD (1% dextrose, 2% Bacto-yeast extract, and 4% bactopeptone) at 30 °C, and then grown in synthetic medium lacking amino acids. Cells were then induced with 100 mM 3-aminotriazole (3-AT).
Functional Interactions between PCNA and pol

C-3’), from pET39hPCNA (21) digested with NdeI-BamHI and cloned into the NdeI-BamHI sites of vectors pGBK7 and pGADT7 (Clontech) producing plasmids pGBK7-PCNA and pGADT7-PCNA. To generate NdeI and BamHI mutations in the IDCL of PCNA, the last 432 nucleotides of hPCNA cDNA were amplified with mutagenic primer PCNA3 (5’-GGA ATT CGA CGT CGA AGC TGG AGC GCC AGA GTA CAT CCG TG-3’) and PCNA2, digested with EcoRI-BamHI and cloned into the EcoRI-BamHI sites of vector pGBK7, generating plasmid pGBK7-PCNA[586–786]. Next, the first 363 nucleotides of hPCNA were amplified by PCR with PCNA1 and PCNA4 (5’-GCA ATT CGA GCT TTA AAT CCA TCA ACT TCA TTG C-3’) digested with NdeI-AatII and cloned into the NdeI-AatII-digested pGBK7-PCNA[364–786] vector to produce pGBK7-PCNA-IDCL. pACT2-poli (1–278), pACT2-poli (492–715), pGBK7-pol2, and pGBK7-pol7 were constructed as previously described (22). To produce pACT2-poli, PIP1 (Y426A,Y427A), pACT2-poli; PIP2 (F546A,F547A), and pGBK7-pol1, pGBK7-PCNA[674-nucleotide fragment from peYFP-pol; pol1 or peYFP-pol; pol2 was subcloned into the similarly digested pACT2-poli, pGBK7-pol7 plasmids, respectively. The pol1 PIP3 (F710A,H711A) mutant was constructed by PCR amplification of the POL1 gene with primers: 5’-GTC GGG TCA TGT ATA CAA TAA TCA G-3’ and 5’-GCG GAT CCT TAG TAT TTA TGG CCA ATG GCA GCA GGA GCA GTC GAT CATT GCT G-3’, and ligation of the AstII-BamHI digested pGBK7-pol1 (5) to the similarly digested pGBK7-pol7 and pGBK7-pol2 vectors. The PCR product was then digested with EcoRI-Sall and cloned into the EcoRI-Sall sites of pGBK7.

**Protein Purification Constructs**—To generate His-tagged PCNA and an IDCL-PCNA mutant, NdeI-BamHI-digested protein expression constructs containing full-length hPCNA and IDCL mutant were inserted into the NdeI-BamHI sites of pET16b (Novagen). The GST-pol[484–713] expression vector was produced by subcloning a 690-bp EcoRI-Sall pol7 fragment from pGBK7-pol[484–713] into the EcoRI-Sall sites of pGEX-4T1 (Amersham Biosciences). **Proteins**—Glutathione S-transferase-tagged human pol was purified from infected insect cells as previously described (5). Histidine-tagged human PCNA and IDCL-PCNA proteins were purified on Ni2+-charged nickel-nitritiotriacetic acid His-Bind Resin (Novagen) as recommended by the manufacturer. The His-PCNA containing eluates were then dialyzed against H Buffer (20 mM potassium phosphate, pH 7.5, 10 mM 2-mercaptoethanol, 0.01% Nonidet P-40, 10% glycerol) and applied to a hydroxylapatite (Bio-Rad) column previously equilibrated with H Buffer. The column was subsequently washed with H Buffer. His-PCNA containing fractions were aliquoted and stored at 80 °C.

GST and GST-pol-pool[484–713] proteins were purified by glutathione-Sepharose affinity chromatography following the manufacturer’s instructions (Amersham Biosciences). Human RFC (23) and RPA (24) were purified as previously described.

**In Vitro Transcription/Translation of Proteins**—In vitro transcription/translation of full-length pol, pol1, pol1, and PCNA was performed using a TNT-coupled reticulocyte lysate system (Promega) according to the manufacturer’s instructions. The expression vectors encoding the pol genes were subcloned into pGEX-4T1 and expressed in E. coli DH5α for overexpression. The His-PCNA and IDCL-PCNA proteins were purified as described above. The interaction between human pol and PCNA was analyzed in vivo using the Saccharomyces cerevisiae two-hybrid Matchmaker III system (Clontech). Strain AH109 was co-transformed with the GAL4 binding domain (pGBK7-X) and GAL4 activation domain (pGADT7) expressing constructs of PCNA[484–713] and pol1, pol2, pol3, pol4, pol5, pol6, pol7, pol8, pol9, and pol10, respectively. Co-transformants were selected on DOBA-Trp-Leu (Bio 101) plates. Colonies were subsequently replated on DOBA-Trp-Leu-His-Ade (Bio 101) plates. An interaction between the two fusion proteins resulted in the ability of the transformed yeast cells to grow in the selective medium.

**Pull-down Assays**—Equal amounts of nickel-nitritiotriacetic acid beads alone or coupled to His-tagged PCNA or His-IDCL-PCNA proteins (20 μg) were mixed with 10 μl of a standard TNT-lysate reaction containing [35S]-labeled pol, pol1, pol1, or PCNA in 500 μl of binding buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 50 mM imidazole, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM diethiothreitol) in the presence of a “Complete EDTA-free” mixture of protease inhibitors (Roche Applied Science). After incubation for 16 h at 4 °C, the beads were washed four times with binding buffer and the bound proteins were separated on a 4–20% SDS-PAGE. The dried gels were scanned using a FujiFilm FLA-3000 Phosphor Imager.

**GST Pull-down Assay**—Equal amounts of GST or GST-pol-pool[484–713] (20 μg) coupled to glutathione-agarose beads were mixed with [35S]-labeled pol or pol1, pol1, pol1, or PCNA in 500 μl of binding buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 50 mM imidazole, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM diethiothreitol), with or without PCNA, RFC, and RPA were preincubated with an excess of competitor DNA (1 mg/ml of nonradialed herring sperm DNA) to capture pol molecules that dissociated from the radiolabeled template and incubated at 37 °C for 10 min. As a control, a reaction mixture containing pol, PCNA, RFC, and RPA was preincubated with an excess of DNA competitor and the DNA template prior to addition of the DNTPs.

**Two-hybrid Assay**—The interaction between human pol and PCNA was analyzed in vivo using the Saccharomyces cerevisiae two-hybrid Matchmaker III system (Clontech). Strain AH109 was co-transformed with the GAL4 binding domain (pGBK7-X) and GAL4 activation domain (pGADT7) expressing constructs of PCNA[484–713] and pol1, pol2, pol3, pol4, pol5, pol6, pol7, pol8, pol9, and pol10, respectively. Co-transformants were selected on DOBA-Trp-Leu (Bio 101) plates. An interaction between the two fusion proteins resulted in the ability of the transformed yeast cells to grow in the selective medium.

**Plasmids**—peYFP-pol and peYFP-pol were produced in a similar way to peYFP-pol and peYFP-pol described in Ref. 22. The pol1, pol2, pol3, pol 4, pol5, pol6, pol7, pol8, pol9, and pol10. A minimum of 10 μl of a standard Trx-lysate system reaction in 500 μl of binding buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 50 mM imidazole, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM diethiothreitol) was used to generate the “Complete EDTA-free” mixture of protease inhibitors (Roche Applied Science).

**Model Building Procedure**—The pol peptide suspected to bind PCNA was aligned with the p21 peptide co-crystallized with PCNA (Protein Data Bank code 1AXC). To make the model complex of pol and PCNA, the graphic program ONO (25) was used to substitute residues of p21 in place of the pol peptide.
1AXC with those of pol based on the sequence alignment. Interestingly, residues 423–427 of pol are predicted to form a short helix corresponding to the $\alpha_3$ helix formed by residues 147–151 of $\gamma 2$. Most of the rotamers of mutated side chains were kept the same as in the original crystal structure. Side chains of Lys$^{420}$ and Lys$^{421}$, which replaced Gln$^{144}$ and Thr$^{145}$, were adjusted choosing one of the favored rotamers and manually fitted to avoid clashes.

RESULTS

Stimulation of pol-dependent Polymerase Activity in the Presence of PCNA, RFC, and RPA—Previous studies using steady-state kinetic analysis revealed that the catalytic activity of pol is stimulated up to 150-fold in in vitro reactions containing RFC, PCNA, and RPA, yet processivity was unaltered, and pol continued to behave in a highly distributive manner (15).

Studies on pol suggest, however, that the properties of the enzyme are especially sensitive to both the template base replicated and the sequence context in which it is located. At enzyme are especially sensitive to both the template base replicated and the sequence context in which it is located. At

FIG. 1. In vitro stimulation of pol activity by PCNA, RFC, and RPA in the absence or presence of a molecular DNA “trap.” A, pol (2.5 nM) was incubated in a standard 10-μl reaction at 37 °C for 5 min with 5 nM of the primed M13 circular template and all four dNTPs (100 μM). Where indicated, PCNA (50 nM), RFC (2 nM), and RPA (475 nM) were included in the reactions. The immediate template sequence context is given on the right-hand side of the figure. B, reactions were performed as in A and were initiated by the addition of dNTPs (100 μM) (lanes 1 and 2), or dNTPs and an excess of competitor DNA (1 mg/ml), to capture pol molecules after they dissociated from the radiolabeled primer-template (lanes 4 and 5). The reaction mixtures were then incubated at 37 °C for 10 min. In a control reaction pol, PCNA, RFC, and RPA were preincubated with an excess of DNA competitor and the radiolabeled primer-template prior to the addition of the dNTPs (lane 3). The locations of pol-dependent pause sites corresponding to misincorporation at template T are indicated by arrows.
however, the interaction with PCNA was clearly not as robust as that observed between full-length pol and PCNA. The two-hybrid results, although not definitive, tentatively mapped the PCNA-binding site between amino acids 279 and 492 (Fig. 2C).

In this region, we identified a potential PIP box, PIP1 (KKGLIDYY), between pol/H9259 amino acids 420 and 427. However, because the interaction between the truncated pol/H9259-[1–492] and PCNA was suboptimal, we felt compelled to further investigate the possibility that the two other potential PIP boxes of pol/H9259 may nevertheless facilitate an interaction between pol/H9259 and PCNA, especially because PIP2 (SRGVLSFF) had previously been proposed as a potential PCNA-binding site (15), and PIP3 (KRTGSDFH), which although far from the consensus PIP sequence, aligns with the pol/H9257 PIP box.

We therefore generated base substitutions in full-length POLI that resulted in amino acid substitutions in each potential PIP box and assayed the mutants via the two-hybrid assay. As shown in Fig. 2D, both the PIP2 and PIP3 mutants retained the capacity to interact with PCNA, whereas amino acid substitutions of Y426A and Y427A in PIP1 completely eliminated the interaction with PCNA (Fig. 2D). The diminished ability of the PIP1 mutant to interact with PCNA is specific, because like wild-type pol/H9259 (22), the mutant (along with the PIP2 and PIP3 mutants) retains an ability to interact with both full-length pol and a C-terminal fragment of pol/H9257.

To confirm that the PIP1 mutant disrupts pol-PCNA interactions, we used purified His-tagged PCNA protein in a pull-down assay (Fig. 3). pol and the pol/PIPI mutant under the control of the T7 promoter on the two-hybrid vector pGBKT7 were used as a template for in vitro transcription and translation in the presence of 35S-labeled methionine. As shown in Fig. 3A (lanes 5 and 6), SDS gel electrophoresis of the labeled
proteins showed bands of the expected size for human pol. In vitro translated 35S-labeled pol or pol-PIP1 were incubated with either Ni2+-charged beads alone or with beads coupled with His-PCNA. After extensive washing, the bound proteins were resolved by SDS-PAGE. Consistent with the reported binding of wild-type pol with His-PCNA, but not to the Ni2+-charged beads alone (Fig. 3A, cf. lanes 1 and 2). In contrast, the amount of the pol-PIP1 mutant retained on either the Ni2+-charged beads or His-PCNA was dramatically reduced and similar to the nonspecific binding of wild-type pol to the Ni2+-charged beads (Fig. 3A, cf. lanes 1, 3, and 4). Our in vitro findings are therefore in good agreement with the two-hybrid assay identifying PIP1, but not PIP2 or PIP3, as a bona fide pol-PCNA-binding site. As a control, we carried out a parallel pull-down experiment using GST protein and a GST-pol-PIP1-binding site. As a control, we carried out a parallel pull-down experiment using GST protein and a GST-pol-PIP1 construct. Both, pol and the pol-PIP1 mutant protein retained an ability to interact with pol and were bound to the beads coupled with GST-pol[484–713] but not to GST alone (Fig. 3B and Ref. 22).

### Experimental Procedures

#### Fig. 3. **In vitro** Ni2+- and GST pull-down assays demonstrating that the pol-PIP1 mutant [Y426A,Y427A] does not interact with His-PCNA, but still binds to the C-terminal region of pol.** In vitro translated 35S-labeled pol and pol-PIP1 proteins were incubated with Ni2+-charged beads alone (A) or coupled to His-tagged PCNA (20 μg) or glutathione-Sepharose beads (B) and equal amounts of GST- or GST-pol[484–713] as indicated under “Experimental Procedures.” Bound proteins were eluted and resolved by 4–20% SDS-PAGE. A portion of the in vitro translated 35S-labeled pol and pol-PIP1 proteins corresponding to ~10% of the labeled protein in the binding reaction was loaded as input (lanes 5 and 6 in A).

|        | 1 | 2 | 3 | 4 | 5 | 6 |
|--------|---|---|---|---|---|---|
| Pol    | + | + | - | - | - | - |
| Pol-PIP1 | - | - | + | + | + | + |
| His:PCNA | - | - | + | + | - | - |
| Ni2+ Beads | + | + | + | + | - | - |

**A**

| 1 | 2 | 3 | 4 |
|---|---|---|---|
| Pol | + | + | - |
| Pol-PIP1 | - | - | + |
| GST:Pol1 | - | + | - |
| GST | + | - | + |

**B**

| 1 | 2 | 3 |
|---|---|---|
| PCNA | - | + |
| RFC | - | + |
| RPA | + | + |

**Control**

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|
| Pol | - | + | + | + | + | + |
| Pol-PIP1 | - | + | + | + | + | + |

**Fig. 4. Effect of PCNA, RFC, and RPA on the DNA polymerase activity of the pol-PIP1 mutant [Y426A,Y427A].** 1:40 dilutions of TnT-coupled reticulocyte lysate reactions containing either template pGBK7T (lanes 1–3), pGBK7T-pol (lanes 4–6), or pGBK7T-pol-PIP1 (lanes 7–9) were incubated for 20 min at 37 °C with the circular M13 primer-template substrate (5 nM) and dNTPs (100 μM) in the absence or presence of PCNA (0 nM), RFC (2 nM), and RPA (475 nM) as indicated. **In vitro** synthesized pol-PIP1 mutant exhibited activity comparable with the wild-type enzyme in the absence of PCNA, RFC, and RPA. In the presence of these accessory factors, there was a modest stimulation of polymerase activity (Fig. 4, cf. lanes 7–9), but this appears to be largely independent of RFC (and presumably PCNA) because the level of stimulation was similar in the presence and absence of RFC (Fig. 4, cf. lanes 8 and 9).
The pol-PIP1 mutant does not localize into UV-induced foci after damage. MRC5 human cells were transfected with plasmids encoding eCFP-pol1, and wild-type eYFP-pol (top row), eYFP-pol-PIP2 [F546A,F547A] (middle row), or eYFP-pol-PIP1 [Y426A, Y427A] (bottom row). 20 h post-transfection, the cells were irradiated with 7 J/m². After 12 h, the distribution of the eCFP and eYFP fusion proteins was examined following paraformaldehyde fixation. The autofluorescent signal of eCFP-amined following paraformaldehyde fixation. The autofluorescent signal of eCFP and eYFP fusion proteins was examined following paraformaldehyde fixation. The autofluorescent signal of eCFP-pol and eYFP-pol (red) in the same cell are shown. Co-localization of eCFP-pol with wild-type eYFP-pol and eYFP-pol-PIP2 is indicated by a yellow pattern in the merged top and middle panels in the right-hand column. Since eYFP-pol-PIP1 does not form foci, there is no co-localization with eCFP-pol1 and the foci are therefore green (bottom panel, right-hand column).

Taken together, our results indicate that the pol-PIP1 mutant has a greatly reduced ability to participate in RFC-PCNA-dependent replication reactions (Fig. 4, cf. lanes 5 versus 8).

PCNA Targets pol to the Replication Machinery in Human Cells—The precise mechanisms by which Y-family polymerases are recruited to the replication machinery and how the cell chooses a specific polymerase to bypass any particular lesion remain to be determined. In an earlier study, we demonstrated that wild-type pol localizes in replication factories during S phase and that these replication foci accumulate after UV irradiation. Furthermore, pol foci formation was coordinated with the appearance of pol1 foci (22). To investigate the role that PCNA might play in targeting pol into replication factories, we analyzed the nuclear localization pattern of the pol-PIP1 mutant (Y426A,Y427A) fused to enhanced yellow fluorescent protein (eYFP) and compared it with the pattern of foci observed for similar constructs expressing wild-type pol. Since pol-PIP2 does not form foci, there is no co-localization with eYFP-pol and the foci are therefore green (bottom panel, right-hand column).
Functional Interactions between PCNA and pol

**Fig. 6.** PCNA interacts physically and functionally with pol through its IDCL domain. A, two-hybrid assay demonstrating that the IDCL mutant PCNA (I126A,L128A) does not interact with pol. Yeast strain AH109 was transformed separately with the GAL4-AD expression vectors pACT2, pACT2-pol, pGADT7, and pGADT7-PCNA, in combination with each one of the following GAL4-BD expression vectors pGBK7 and pGBK7-[IDCL-PCNA]. Colonies from each transformation were grown overnight at 30°C in selective medium, and a sample was spotted on to a DOBA-Trp-Leu-His-Ade plate and incubated at 30°C for 3 days. B, *in vitro* pull-down assay. *In vitro* translated 35S-labeled pol (upper panel) and PCNA proteins (lower panel) were incubated with Ni²⁺-charge beads alone or coupled to His-tagged PCNA or the His-tagged IDCL-PCNA mutant (20 µg) as indicated under “Experimental Procedures.” Bound proteins were eluted and resolved by 4–20% SDS-PAGE. A portion of the *in vitro* translated 35S-labeled pol and PCNA proteins corresponding to ~10% of the labeled protein in the binding reaction was loaded as input (lane 1). C, primer extension assay comparing stimulation of pols by wild-type PCNA, or the IDCL-PCNA mutant. A limiting amount of pol (2.5 nM) was incubated under standard assay conditions with the circular M13 primer-template (5 nM) and all dNTPs (100 µM). PCNA (50 nM; lane 5), IDCL-PCNA mutant (50, 100, and 200 nM; lanes 6–8, respectively), RFC (2 nM), and RPA (475 nM) were included as indicated.

In summary, our results provide the first direct evidence for functional interactions between PCNA and pol, and suggest that the IDCL region of PCNA is likely the primary docking site for pol. These interactions therefore most likely explain why our ICDL mutants exhibited a greatly reduced ability to interact with pol, but the PCNA-pol interactions were not entirely eliminated.

**Modeling of the pol PIP1 Residues onto PCNA**—Having identified the contacting residues between pol and PCNA necessary for binding and stimulation of the processivity of pol, we were interested in gaining structural insights into these protein-protein interactions. Although the homology of the PIP box of pol to the PIP consensus is low outside the (I/L/M)XX(F/Y)(F/Y) five-residue region (Fig. 2), secondary structure prediction of pol by PsiPred (bioinf.cs.ucl.ac.uk/psipred/) indicated that the residues immediately flanking Tyr426 and Tyr427 of pol fold into a short helix (residues 423–427) preceded and followed by extended regions just like the residues of p21 that intimately interact with PCNA (Fig. 7A). Moreover, the most prominently conserved aromatic side chains, Tyr426 and Tyr427, are readily modeled onto the crystal structure of p21 peptide and PCNA complex replacing the Phe150 and Tyr151 of p21 (30).
Fig. 7. Model of the pol β and PCNA complex. A, the pol β peptide complexed with one of the three equivalent PCNA subunits is shown in a ribbon diagram (44). Like residues 147–150 of p21, pol β is predicted to have a $3^1_0$ helix between residues 423 and 427. The backbone of the pol β peptide is shown in purple and that of the PCNA subunit is shown in pink. Tyr$^{426}$ and Tyr$^{427}$ of pol β and Leu$^{126}$ and Ile$^{128}$ of PCNA, which anchor the interactions between the two proteins, are highlighted in ball-and-stick presentations. B, close-up of the hydrophobic pocket in PCNA that holds Tyr$^{427}$ of pol β. PCNA is presented in molecular surface format using graphic program PyMol (www.pymol.org). The subsurface backbone trace and side chains of PCNA are shown with Leu$^{126}$ and Ile$^{128}$ highlighted in orange. The pol β peptide trace is shown as a worm in purple, and the side chains of Tyr$^{426}$ and Tyr$^{427}$ are highlighted as green sticks.

(Fig. 7B). The additional hydroxyl group of Tyr$^{426}$ in pol β is easily accommodated by slight adjustment of torsion angles between Cβ and Cγ. Like Tyr$^{151}$ of p21, Tyr$^{427}$ of pol β fits snugly into a hydrophobic pocket on the surface of PCNA encompassed by Leu$^{126}$ and Ile$^{128}$ in the IDCL (Fig. 7). The less conserved residues, such as the replacement of Gln$^{144}$ of p21 by Lys$^{420}$ of pol β, is also readily achieved because the extended Lys side chain is flexible and can replace solvent molecules surrounding Gln$^{144}$.

This model of the pol β-PCNA complex provides a clear explanation for the reduced interaction between the two proteins carrying the mutations in pol β and IDCL. Substitution of the two aromatic residues in pol β with alanines obviously removes the most extended hydrophobic interactions between the two proteins (Fig. 7B). Vice versa, replacement of the bulky hydrophobic Leu$^{126}$ and Ile$^{128}$ with alanines enlarge the hydrophobic pocket that snugly accommodates Tyr$^{427}$ of pol β. We therefore predict that pol β and PCNA interactions are similar to those occurring between p21 and PCNA, particularly surrounding the two consecutive aromatic residues.

**DISCUSSION**

PCNA plays a pivotal role in coordinating the ability of the cell to duplicate its genome accurately and efficiently. This critical function occurs largely through protein-protein interactions between PCNA and key proteins, such as the main replicases of the cell, pols and pols. The accumulation of pols into replication factories but that an interaction between pols and PCNA is the key determinant for foci formation. Indeed, in XPV cells lacking polη, the number of UV-induced pol foci dropped 3–6-fold (from $–60\%$ in a wild-type cell to 10–20% in an XP-V cell), indicating that pol foci formation is at least partially dependent upon polη (22). In our present study, we show that foci formation is essentially abolished in a full-length pol PIP mutant ($<3\%$ cells with foci formation), whereas polη foci formation remains unaltered. Our data suggest, therefore, that a functional interaction with PCNA is the key determinant for the accumulation of pol into replication factories but that an interaction between pols and PCNA also contributes to stabilizing these structures.

Because of the homotrimeric structure of PCNA, up to three DNA polymerases could potentially bind to the clamp simultaneously, in a manner similar to the 'tool belt' model proposed by Pages and Fuchs (19). Support for such an idea comes from the recent structural analysis of Bunting et al. (20), who co-crystallized the little finger domain of *Escherichia coli* pol IV with the β-clamp and demonstrated that in principle, the clamp could accommodate multiple polymerases without any steric hindrance of the polymerase engaged at the primer terminus. Because different regions of pols are involved in the interactions with PCNA (Fig. 4) and polη (22), it is conceivable to a possible scenario in which pols δ, η, and ε could potentially form...
a constitutive translesion-synthesis complex, whose stability would depend ultimately on pairwise interactions between its components. Further regulation may occur through post-translational modifications of PCNA by mono- and polyubiquination and sumoylation (reviewed in Ref. 41) or acetylation (42), which change the respective affinities of the various polymerases for PCNA and help facilitate switching between replicative and translesion DNA synthesis polymerases. Indeed, in support for such an idea, human pol\(\text{\textalpha}\) has recently been shown to bind much more tightly to monoubiquitinated PCNA than to unmodified PCNA (43).

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