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Regulation of DNA replication and repair proteins through interaction with the front side of proliferating cell nuclear antigen

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The DNA polymerase accessory factor proliferating cell nuclear antigen (PCNA) has been caught in interaction with an ever increasing number of proteins. To characterize the sites and functions of some of these interactions, we constructed four mutants of human PCNA and analysed them in a variety of assays. By targeting loops on the surface of the PCNA trimer and changing three or four residues at a time to alanine, we found that a region including part of the domain-connecting loop of PCNA and loops on one face of the trimer, close to the C-termini, is involved in binding to all of the following proteins: DNA polymerase δ, replication factor C, the flap endonuclease Fen1, the cyclin dependent kinase inhibitor p21 and DNA ligase I. An inhibition of DNA ligation caused by the interaction of PCNA with DNA ligase I was found, and we show that DNA ligase I and Fen1 can inhibit DNA synthesis by DNA polymerase δ/PCNA. We demonstrate that PCNA must be located below a 5′ flap on a forked template to stimulate Fen1 activity, and considering the interacting region on PCNA for Fen1, this suggests an orientation for PCNA during DNA replication with the C-termini facing forwards, in the direction of DNA synthesis.
Keywords: DNA repair/DNA replication/mutagenesis/PCNA/protein–protein interaction

Introduction

The proliferating cell nuclear antigen (PCNA; reviewed in Jónsson and Hübscher, 1997) was, for a long time, portrayed as a clamp to keep the replicative DNA polymerase δ (Pol δ; reviewed in Hindges and Hübscher, 1997) attached to DNA and increase the processivity of DNA synthesis during DNA replication. Recently, an abundance of papers that describe the interactions of PCNA with various proteins involved in processes as different as cell cycle control, DNA replication, nucleotide excision repair, post-replicational mismatch repair, base excision repair, apoptosis and most recently cytosine methylation (Chuang *et al.*, 1997) has changed that simple picture. The promiscuity of PCNA points towards a much wider role in coordinating the cell cycle and DNA replication with various types of DNA repair. Furthermore, the proposed involvement of PCNA in cytosine-5 methylation links it with transcriptional regulation, genetic imprinting and development. The list of proteins reported to interact with PCNA, besides the Pols δ and ε, and the clamp loader replication factor C (RF-C), includes the structure-specific flap endonuclease Fen1 (Li *et al.*, 1995), the cyclin kinase inhibitor p21 (Waga *et al.*, 1994), cyclins (Xiong *et al.*, 1992), the growth arrest and DNA damage response protein Gadd45 (Smith *et al.*, 1994) and its homologue MyD118 involved in TGF-β mediated apoptosis (Vairapandi *et al.*, 1996), the DNA mismatch repair proteins MSH2 and MLH1 (Umar *et al.*, 1996), the nucleotide excision repair endonuclease XP-G (Gary *et al.*, 1997) and most recently, human DNA-(cytosine-5) methyltransferase (MCMT; Chuang *et al.*, 1997) and human DNA ligase I (Lig I; Levin *et al.*, 1997). Many of these interactions have been characterized in some detail, and differing amounts of information about the residues involved in them are available. Since so many proteins of such diversity interact with PCNA, it would be of great advantage to have access to PCNA mutants that selectively abolish some of its interactions while leaving others intact. The roles of the various interactions could then be dissected *in vitro* and *in vivo*.

The regions on PCNA interacting with other proteins have been sought with various methods, including peptide scanning, deletion mutants, yeast two-hybrid interaction trap assays and both random and site directed mutagenesis. The publication of the crystal structure of yeast PCNA (Krishna *et al.*, 1994) made it apparent that deletion mutagenesis could only be of limited value for studying PCNA interactions and facilitated the design, and allowed a meaningful interpretation, of mutagenesis studies. Several of these studies have been conducted on *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and human PCNA. Site directed mutagenesis of human PCNA has indicated residues important for trimeric structure, stimulation of Pol δ and interaction with RF-C (Fukuda *et al.*, 1995; Jónsson *et al.*, 1995; Zhang *et al.*, 1998). Random and site directed mutagenesis studies of *S.cerevisiae* and *S.pombe* PCNA have yielded mostly complying results and pointed to binding sites for Pol ε and Fen1 (Ayyagari *et al.*, 1995; Arroyo *et al.*, 1996; Eissenberg *et al.*, 1997). Screening for complementation of a cold-sensitive (Cs^-) mutation in the large subunit of yeast RF-C (*CDC44*) has shown that mutation of buried residues in PCNA may have subtle effects on its structure, leading to altered interaction with other proteins (McAlear *et al.*, 1994), and an extensive screen for Cs^- and methyl methanesulfonate-sensitive (Mms^s) mutants of yeast PCNA further underlined the importance of the C-terminal loop of PCNA and emphasized the role of PCNA in DNA replication and repair (Amin and Holm, 1996). In summary, one of the main conclusions from mutagenic studies of PCNA is that the molecule can sustain a surprising amount of mutation without losing activity or showing obvious effects either

in vivo or *in vitro*, and that the most clear-cut effects are seen when residues in exposed loops on the PCNA surface are mutated.

In the work described here, we tried to clarify the exact roles of some of those loops, to unify some of the information gathered in other studies by making multiple alanine (Ala) mutants of human PCNA in regions where strong but specific effects could be expected and compare them side by side in several assays. Furthermore, we addressed related questions that arose during the experiments, such as on which side of the flap PCNA must be located to stimulate the flap endonuclease Fen1. The results lead to the conclusion that a hydrophobic pocket, formed between the PCNA domain-connecting loop and loops on the C-side of PCNA, is important for the interaction with Pol δ, p21, Fen1 and Lig I. We could determine that PCNA must be below a 5′ flap to stimulate the Fen1 flap endonuclease activity and in view of the binding site for Fen1, we propose that the C-side of PCNA faces forward during DNA replication. We also identified a sequence signature in Lig I, characteristic of several PCNA-binding proteins, and compared the effects of Fen1, Lig I and p21 on DNA replication with Pol δ/PCNA on linear templates, finding that Fen1 can, like p21 and Lig I, inhibit DNA synthesis by Pol δ. Additionally, PCNA can, under some conditions, inhibit ligation by Lig I.

Results

The rationale for mutating the loops of human PCNA

The PCNA monomer is composed of two domains that fold in a similar manner leading to a quasi-six-fold symmetry in the PCNA trimer (Figure 1). The two domains are connected by an exposed domain-connecting loop (aa 118–135) which is the most prominent feature on the periphery of PCNA. The domain-connecting loop emerges from domain 1 at the face where the C-termini are located (front side), and much of it lies on that side of the centre of the PCNA trimer. In addition, smaller loops protrude from the faces of the PCNA trimer. The amino acids at the C-termini form negatively charged loops on the PCNA front side, and close to them, residues 41–45 of each monomer form short connector loops that lie below the middle of the domain-connecting loop between the sheets βC_1 and βD_1. On the other side of the PCNA trimer (back side) the connection between βD_2 and βE_2 (aa 184–195) forms very prominent loops, that appear to be rather unstructured since they were not resolved in the X-ray structure of human PCNA. The structure of human PCNA was solved in complex with a peptide derived from the C-terminal PCNA-binding domain of the cyclin kinase inhibitor p21 (Gulbis *et al.*, 1996). Although the backbone structure is almost identical to that of yeast PCNA, the domain-connecting loop is somewhat displaced, probably because it forms an extensive β-sheet with the p21 peptide (Figure 1). This alteration unmasks a hydrophobic cleft on the C-side of PCNA involving the C-terminal loop, the domain-connecting loop and the βC_1–βD_1 connection (Gulbis *et al.*, 1996). This hydrophobic pocket contributes to the binding of the N-terminal part of the p21 peptide to PCNA by interacting with residues Tyr151, Phe150 and Met147, which have been identified as critical for PCNA binding (Warbrick *et al.*, 1995).

In a quest for PCNA mutants that would differ from wild-type (wt) PCNA in their affinities for interacting proteins, we selectively changed three or four residues in the above mentioned loops to Ala. Four different mutants were constructed and compared in a variety of assays. To facilitate purification, a His_6 tag was added to the C-termini of the mutants and wt PCNA, as it had been shown previously that PCNA can accommodate short sequences at both N- and C-termini without any loss of activity (Jónsson *et al.*, 1995; Mossi *et al.*, 1997). To increase the chances of obtaining mutants with strong effects, we mutated three to four residues at a time to Ala.

The first region chosen was the βC_1–βD_1 loop which forms part of the hydrophobic pocket on the C-side of the trimer, where we generated a mutant with residues Ser43, His44 and Val45 mutated to Ala (termed SHV43). This loop has been implicated in the interaction with Pol δ, Pol ε and RF-C, since a double mutant in yeast PCNA (pol30-6; DD41,42AA) shows growth defects *in vivo*, partial defects in Pol δ stimulation and is completely defective in stimulating DNA synthesis of Pol ε (Ayyagari *et al.*, 1995). In human PCNA, mutation of Asp41 to Ala leads to >50% reduction in the stimulation of Pol δ, and a lack of stimulation of RF-C ATPase activity (Fukuda *et al.*, 1995). Furthermore, Val45 forms part of the hydrophobic interacting surface with p21 as seen in the crystal structure (Gulbis *et al.*, 1996). The SHV43 mutant could thus, *a priori*, be expected to be defective in some or all of these interactions.

The second region chosen for mutagenesis lies close to the middle of the domain-connecting loop (Figure 1) where we mutated Gln125, Leu126, Gly127 and Ile128 to Ala (mutant QLGI125). This region had been suggested to be part of the binding site for Pol δ since a monoclonal antibody recognizing residues 121–135 of the domain-connecting loop strongly inhibits the stimulation of Pol δ, but antibodies recognizing residues 111–125 cause no inhibition (Roos *et al.*, 1996). In agreement with this, two recently published studies have identified the yeast PCNA mutant pol30-79 (IL126,128AA) as defective in Pol δ stimulation (Eissenberg *et al.*, 1997) and residues Val123, Leu126, Gly127 and Ile128 of human PCNA as important for pol δ stimulation (Zhang *et al.*, 1998). Another question that we hoped to address with the QLGI125 mutant was where Fen1 binds to PCNA. Peptide scanning studies had identified the domain-connecting loop as the interacting site and had shown a competition in binding between p21 and Fen1 (Warbrick *et al.*, 1997). This seemingly contradicts results with yeast PCNA mutant pol30-90 where mutations in the C-terminal loop (PK252,253AA) abolish Fen1 binding and stimulation, whereas the pol30-79 mutant has wild-type activity (Eissenberg *et al.*, 1997). Finally, Gln125 and Gly127 are involved in the binding to p21 (Gulbis *et al.*, 1996).

The third region chosen was the loop between βD_2 and βE_2 on the back side of the torus (Figure 1). No proteins have been shown to interact with this side of PCNA and since this is one of the most eye-catching features in the PCNA structure, we decided to mutate some of its residues. The mutant (termed VDK188) has Val188, Asp189 and Lys190 changed to Ala. These residues are conserved in

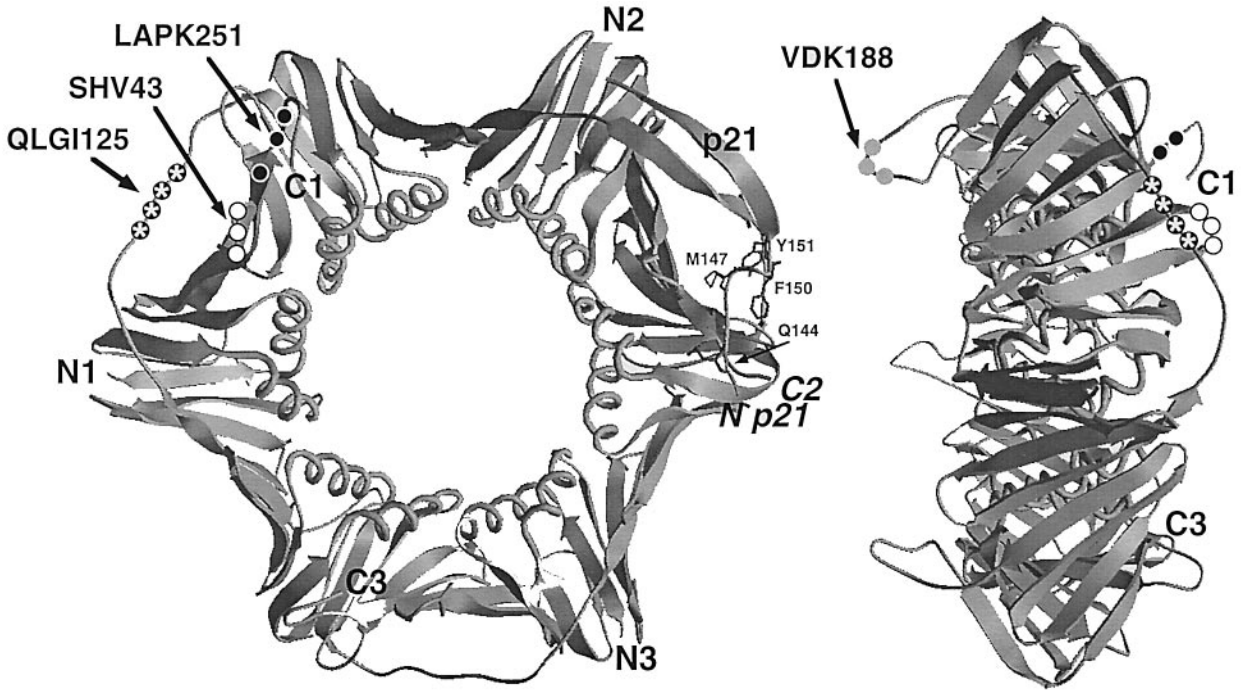

Fig. 1. Front and side views of the PCNA trimer showing the locations of mutations and the interaction with p21. A model of human PCNA was generated by homology modelling with the structure of yeast PCNA. On the left, two of the modelled monomers are shown together with the a monomer of PCNA complexed with a p21 derived peptide (monomer 2) as determined by Gulbis *et al.* (1996) viewing the front side where the C-termini are located. A side view of the modelled trimer is shown on the right. The location of mutated residues is shown: SHV43 (white dots) in a loop on the front side; QLGI125 (white stars) in the domain-connecting loop; VDK188 (grey dots) in a prominent loop on the back side and LAPK251 (black dots) immediately preceding the C-termini. On the p21 peptide, the side chains of four highly conserved amino acids (Q144, M147, F150 and Y151) that form a PCNA-binding motif (Table I) are shown. The model and the figure were generated using Swiss-Model and Swiss PDB Viewer (Guex and Peitsch, 1997).

plant and vertebrate PCNA, but not in *S.cerevisiae*, *S.pombe* or *Plasmodium falciparum*.

The fourth mutant created (LAPK251) contained a triple mutation in the C-terminal region with Leu251, Pro253 and Lys254 mutated to Ala. These three residues, as well as Ala252, are universally conserved in known PCNA sequences and can thus be expected to be of utmost importance. Six of eight cold-sensitive mutations isolated in a screen with yeast PCNA were found to be in this region (Amin and Holm, 1996). Deletion mutants of human PCNA showed that residues 254–261 were dispensable for folding and stimulation of Pol δ, but not for the stimulation of RF-C ATPase activity, whereas further deletion lead to misfolding (Fukuda *et al.*, 1995).

The three PCNA mutants SHV43, QLGI125 and VDK188, but not LAPK251, are correctly folded and trimerized

All four mutants and the wt C-His-PCNA were expressed side by side under the same conditions. Interestingly, the expression level of the SHV43 mutant was reproducibly >10-fold lower than those of the others. No obvious break-down products were visible on SDS gels before or during purification, excluding proteolysis as a cause. The LAPK251 mutant was expressed in similar amounts to wild-type and was almost exclusively found in the soluble fraction of crude extracts (data not shown). However, during purification and storage, the protein had a tendency to precipitate, indicating reduced stability of the trimer.

To verify the correct trimeric structure and folding of the PCNA mutants, we submitted them to native-PAGE

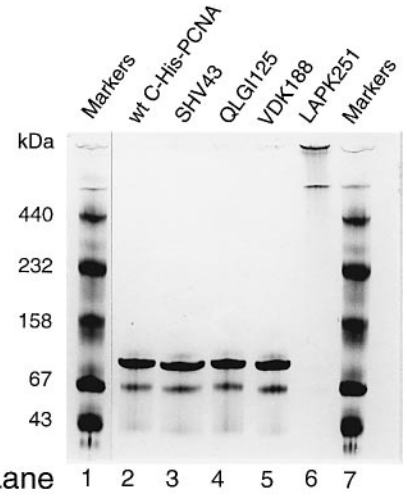

Fig. 2. The PCNA mutants SHV43, QLGI125 and VDK188, but not LAPK251, are correctly folded and trimerized. Samples containing 1 μg each of PCNA, PCNA mutants or molecular weight markers were separated on 8–25% native gradient gel using the Phast System (Pharmacia) and stained with Coomassie Blue. Markers in lanes 1 and 7 are ovalbumin (43 kDa), BSA (67 kDa), aldolase (158 kDa), catalase (232 kDa) and ferritin (440 kDa).

on 8–25% gradient gels (Figure 2). The wt C-His-PCNA, as well as SHV43, QLGI125 and VDK188 PCNAs, had identical profiles and were predominantly present as trimers with minor amounts of dimers and monomers detectable. The LAPK251 PCNA, on the other hand, was almost exclusively found in aggregates that did not enter the separating gel, although a small amount of free trimer

was visible on overloaded gels (data not shown). The observed M_r of 90 500 for the His-tagged PCNA trimers is close to the calculated mass of 92.5 kDa.

The processivity of Pol δ in the absence of RF-C is enhanced with the SHV43 mutant and reduced with the QLGI125 mutant, compared with wt PCNA

After verifying the correct folding of the mutants, we first compared their ability to stimulate DNA synthesis by Pol δ. On a standard poly(dA)/$(dT)_{16}$ 10:1 base ratio template, SHV43 and VDK188 were indistinguishable from wild-type, whereas QLGI125 was strongly deficient in Pol δ stimulation (Figure 3A). Not surprisingly, the LAPK251 mutant was also unable to stimulate pol δ (data not shown). Comparing these results with titrations done using a more sparsely primed template (primer/template base ratio 40:1), we observed that the activity of QLGI125 was consistently lower than on the 10:1 template, consistent with reduced processivity, but the SHV43 mutant was slightly more active than wt at low concentrations suggesting an increased affinity for Pol δ (Figure 3B).

To analyse the effects of the mutants on Pol δ processivity in more detail, reactions were performed with [α-^{32}P]dTTP and the products separated on a low percentage denaturing polyacryamide gel (Figure 3C). It is apparent that the C-His tag does indeed have a slightly reducing effect on the processivity conferred upon Pol δ but the SHV43 mutation more than compensates for this, leading to significantly higher processivity with the SHV43 mutant than with wt PCNA. The products formed in the presence of VDK188 are of similar lengths to those with wt C-His-PCNA, but the QLGI125 mutant is greatly impaired in its interaction with Pol δ, leading to much shorter products. Addition of ten times more of the QLGI125 mutant (500 ng) led to increased accumulation of short products but did not shift the equilibrium towards full length products (Figure 3C, lane 12).

The SHV43 mutant reveals an important role of the βC_1–βD_1 loop for loading of PCNA onto DNA

Having shown the effects of the mutations on Pol δ stimulation, we next proceeded to see if an effect would be seen under conditions where loading of PCNA onto the DNA by RF-C is required. Using a holoezyme complex containing RF-C, Pol α and Pol δ (Maga and Hübscher, 1996) to catalyse DNA synthesis on singly primed M13 DNA, no activity with the QLGI125 mutant, and a severely reduced activity with the SHV43 mutant, was found (Figure 4A). The effect seen with QLGI125 can be explained by its inability to keep Pol δ attached to a template with extensive secondary structure. Interestingly, identical results were obtained in an RF-C-dependent assay on poly(dA)/$(dT)_{16}$ as described by Uhlmann *et al.* (1997), suggesting the possibility that QLGI125 may also be defective in loading (data not shown). The products formed on the M13 template were therefore analysed (Figure 4B). Comparing lanes 1 and 2, it is apparent that the loading of wt PCNA causes an inhibition of Pol α activity and thus fewer low molecular weight products and accumulation of full length products as had been shown previously (Maga and Hübscher, 1996). Both wt C-His PCNA and the VDK188 mutant effectively stimulate

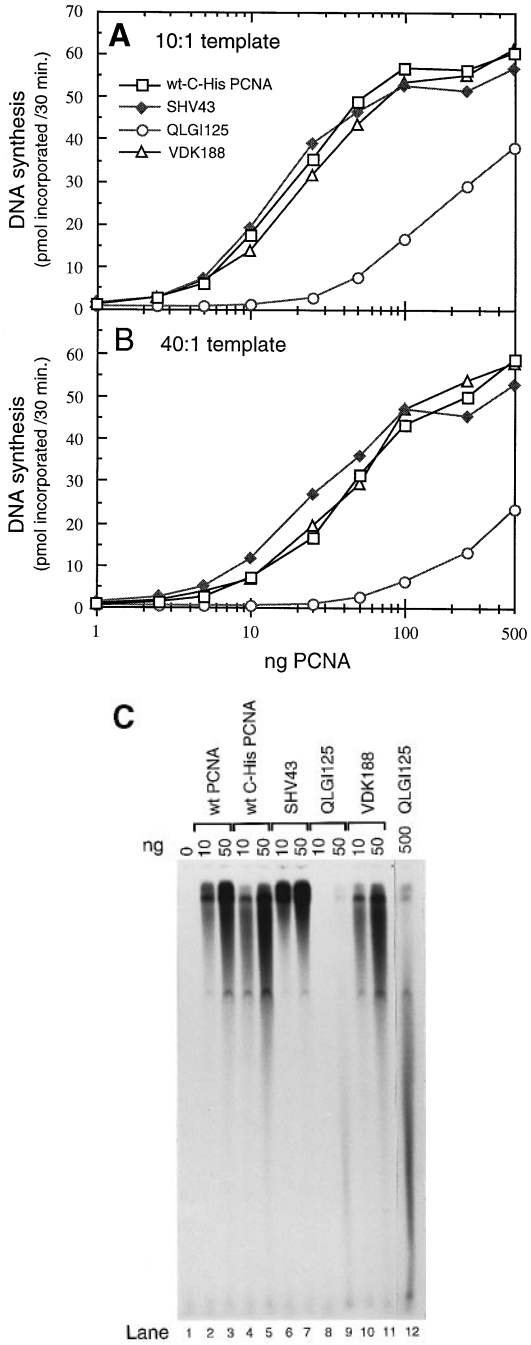

Fig. 3. Differential effects of PCNA mutants on Pol δ processivity point to the interacting site for Pol δ on PCNA. *In vitro* replication assays were performed with 0.05 U of Pol δ on poly(dA)/$(dT)_{16}$ templates as described in Materials and methods. (**A**) Normal template, template/primer base ratio 10:1. (**B**) Sparsely primed template, template/primer base ratio 40:1. The curves show pmols of dTMP incorporated into DNA in 30 min at 37°C, with the amounts of wt C-His PCNA and mutants in nanograms, as indicated in the figure legend, plotted on a log scale. (**C**) Product analysis of reactions on denaturing acrylamide gel. All reactions contain reaction mix with 0.05 U of Pol δ. Lane 1 is a control without PCNA, the other lanes have amounts (ng) of wt or mutant PCNA added as indicated above.

the synthesis of full length products, whereas the SHV43 and QLGI125 mutants are both clearly defective. Interestingly, the QLGI125 mutant produces more of intermediate products than the SHV43 mutant, in contrast with what might be expected from the lower total incorporation seen

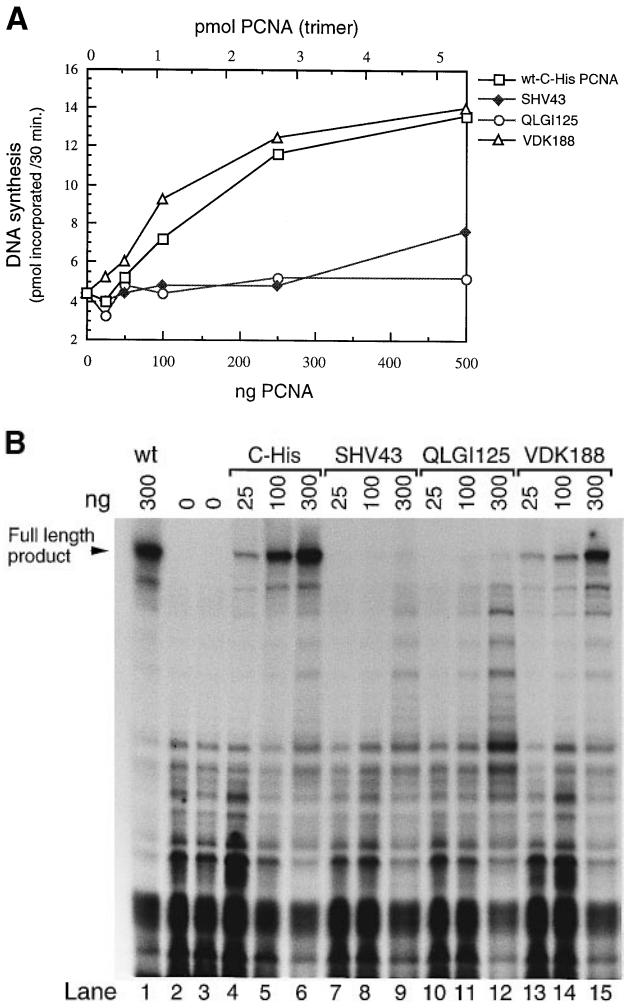

Fig. 4. The VDK188 PCNA mutant, but not the SHV43 or QLGI125 mutants, can effectively stimulate RF-C dependent replication by the pol δ holoenzyme complex on singly primed M13 template. The assays were performed as described in Materials and methods using the RC complex described by Maga and Hübscher (1996) on singly primed M13 template. (**A**) Total incorporation of dNMP plotted against amounts (ng) of wt C-His PCNA and mutants as indicated in the figure legend. *Escherichia coli* SSB (250 ng) was used in all reactions. (**B**) Product analysis of reactions on denaturing acrylamide gel. All reactions contain reaction mix with 0.01 U RC complex, 0.1 U Pol δ and 500 ng human RP-A. Lanes 2 and 3 are controls without added PCNA. Samples in other lanes contain wt or mutant PCNA in amounts (ng) as indicated above.

in Figure 4A. When 2 μg of SHV43 are added, full length products are predominant, whereas the products obtained with 2 μg of QLGI125 are only slightly longer than those seen with 300 ng and the amount of synthesis is not increased substantially (data not shown). This is in agreement with a defect in RF-C loading of SHV43, while QLGI125 seems to be loaded normally but unable to stimulate synthesis of full length products.

The QLGI125 mutant does not bind strongly to either p21 or Fen1

Next, we turned our attention towards other proteins reported to interact with PCNA. Since three of the mutations we generated include residues involved in the binding to a p21-derived peptide, and Fen1 has been suggested to have an overlapping binding site (Warbrick *et al.*, 1997), the ability of the mutants to bind p21 and Fen1 was tested. The p21 binding is of high affinity, with a K_d of 10 nM (J.Chen *et al.*, 1996), and the K_d for the human Fen1-PCNA interaction has been estimated 60 nM (J.Chen *et al.*, 1996). We therefore examined whether PCNA/p21 or PCNA/Fen1 complexes were stable enough to survive electrophoresis on native gels. When equimolar amounts of PCNA (as trimer) and p21 or Fen1 were incubated together for 15 min at 37°C and then immediately separated by native PAGE at 15°C, clear band shifts were visible (Figure 5A). The untagged wt PCNA has an M_r of ~75 000, despite the actual mass of 86.3 kDa, reflecting the compactness of the trimer and the high overall negative charge of the protein. In the presence of p21, a band with an estimated M_r of 138 000 appears, which fits approximately to the size of a PCNA trimer with three p21 molecules bound. An alternative explanation is that the high pI of a single bound p21 molecule leads to such a large shift. His-p21 has a calculated pI of 8.4 and does not enter the gel on its own. In the presence of Fen1, a larger complex with an M_r of 263 000 appears. Fen1 has a molecular mass of 42.6 kDa, so even if three molecules are involved in the binding to PCNA, the shift is not fully accounted for. As with p21, a more likely explanation is that the high pI of Fen1 (estimated 8.78) is the cause for the extent of the shift. At pH 8.44 in the gel buffer, Fen1 has a net positive charge and does not enter the gel. When PCNA was incubated with equimolar amounts of both p21 and Fen1 followed by native PAGE, the same two band-shifts were visible in similar amounts to those seen with either p21 or Fen1 alone, and no additional bands, such as PCNA with both p21 and Fen1 bound, appeared (data not shown).

When the ability of the His-tagged PCNA mutants to bind p21 was tested, a clear band shift was seen with wt C-His-PCNA (Figure 5B) as well as the SHV43 and VDK188 mutants, whereas only faint bands were seen with the QLGI125 mutant demonstrating a marked loss of affinity for p21. Similar results were obtained for the Fen1/PCNA interaction (Figure 5C). When equimolar amounts of PCNA trimers and Fen1 were incubated for 15 min at 37°C and separated on native gel, a band-shift was evident with all PCNAs except the QLGI125 mutant. When a 2.4-fold molar excess of Fen1 over PCNA trimers was added, the amount of shifted PCNA increased but no other bands appeared (Figure 5C, compare lanes 2 and 6), and the QLGI125 mutant was not shifted either.

We also looked for native PAGE band-shifts of PCNA with Gadd45, the PCNA-binding B-fragment of the large subunit of RF-C (Fotedar *et al.*, 1996) and human DNA Lig I, but these interactions could not be detected by native PAGE (data not shown).

p21 can inhibit DNA synthesis of Pol δ, even though its binding to mutant PCNA is decreased

Since the QLGI125 mutant was seemingly unable to bind p21 but only partially defective in Pol δ binding, p21 was titrated into Pol δ assays with 0.15 U of Pol δ and 50 ng of PCNA or mutant PCNA (0.55 pmol trimer) on poly(dA)/$(dT)_{16}$ 10:1. Three different batches of untagged and His-tagged p21 gave consistent results, showing that p21 could still inhibit the Pol δ stimulation of the QLGI125 mutant, although slightly less effectively than wt or VDK188

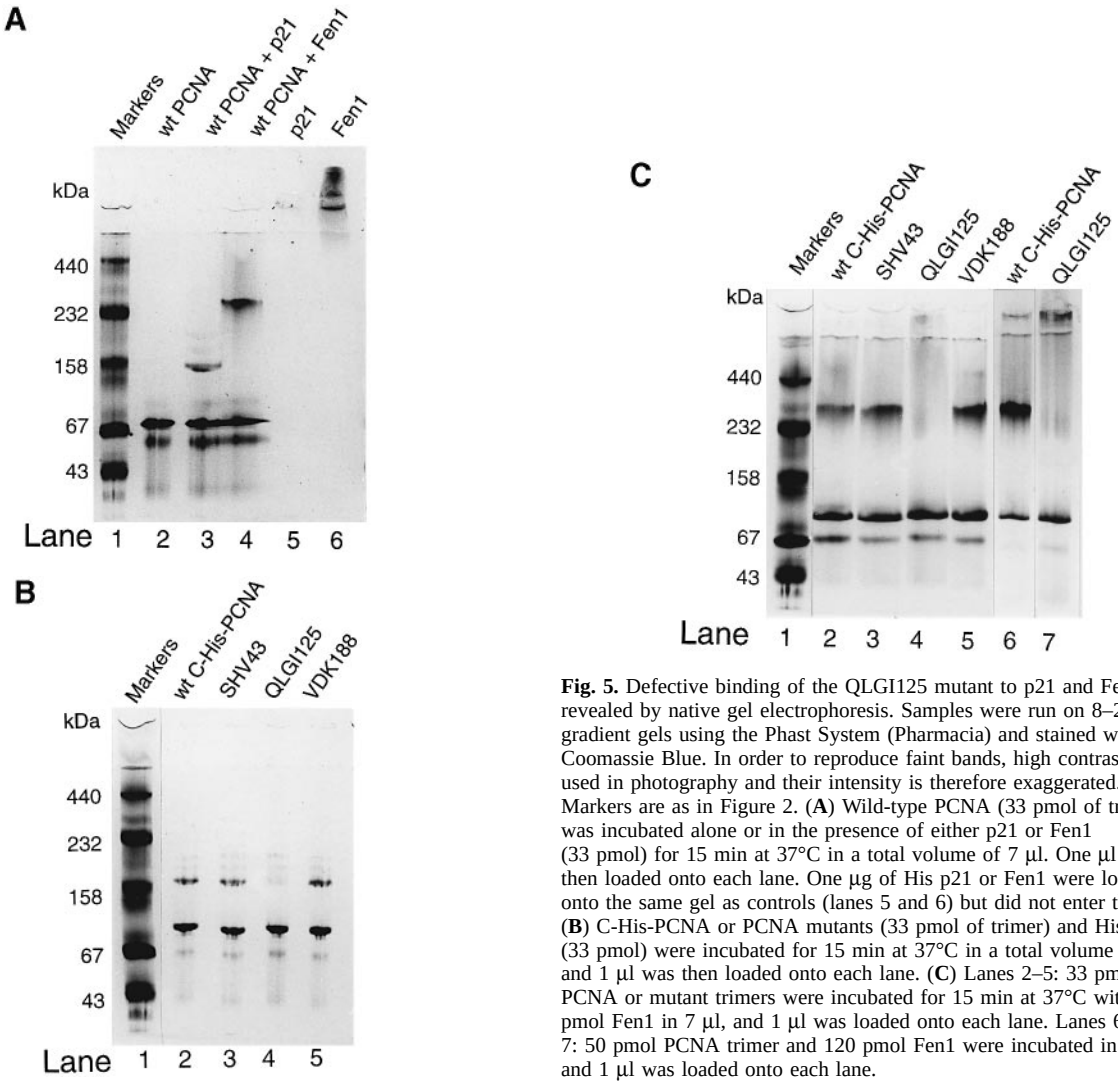

Fig. 5. Defective binding of the QLGI125 mutant to p21 and Fen1 revealed by native gel electrophoresis. Samples were run on 8–25% gradient gels using the Phast System (Pharmacia) and stained with Coomassie Blue. In order to reproduce faint bands, high contrast was used in photography and their intensity is therefore exaggerated. Markers are as in Figure 2. (**A**) Wild-type PCNA (33 pmol of trimer) was incubated alone or in the presence of either p21 or Fen1 (33 pmol) for 15 min at 37°C in a total volume of 7 μl. One μl was then loaded onto each lane. One μg of His p21 or Fen1 were loaded onto the same gel as controls (lanes 5 and 6) but did not enter the gel. (**B**) C-His-PCNA or PCNA mutants (33 pmol of trimer) and His-p21 (33 pmol) were incubated for 15 min at 37°C in a total volume of 7μl, and 1 μl was then loaded onto each lane. (**C**) Lanes 2–5: 33 pmol wt PCNA or mutant trimers were incubated for 15 min at 37°C with 33 pmol Fen1 in 7 μl, and 1 μl was loaded onto each lane. Lanes 6 and 7: 50 pmol PCNA trimer and 120 pmol Fen1 were incubated in 10 μl, and 1 μl was loaded onto each lane.

(Figure 6A). A virtually complete inhibition was seen at a 50-fold molar excess of QLGI125, versus an ~20-fold excess of wt C-His PCNA and VDK188. Interestingly, the SHV43 mutant was even less influenced by p21, requiring a 90-fold molar excess over the trimer for complete inhibition. We thus conclude that QLGI125 can still bind p21, although not strongly enough to retain it during native PAGE, and that the reduced interaction with Pol δ allows p21 to compete effectively for an overlapping binding site. The SHV43 mutant does not seem to be defective in p21 binding, and the increased affinity for Pol δ could lead to less inhibition by p21.

Both the domain-connecting loop and the C-side of PCNA are important for Fen1 stimulation

It has been shown that both yeast and human Fen1 (Lieber, 1997) and PCNA interact (Li *et al.*, 1995; J.Chen *et al*., 1996; Wu *et al.*, 1996). Residues at the C-terminus of Fen1 have been identified as important for the interaction and show some limited sequence similarity to the p21 PCNA-binding peptide (Table I) with which they compete for interaction (J.Chen *et al*., 1996; Warbrick *et al.*, 1997). Using PCNA deletion mutants in the yeast two-hybrid system, a region including the domain-connecting loop was found to bind Fen1. These results, and comparison with the co-crystal structure of PCNA and the p21 peptide, lead the authors to conclude that Fen1 binds to the domain-connecting loop (Warbrick *et al.*, 1997). As mentioned above, this is seemingly in contradiction with mutagenesis studies with yeast PCNA, which located the interaction at the C-terminus (Eissenberg *et al.*, 1997), but is in agreement with what can be concluded from our results with band-shifts on native gels. PCNA has been shown to stimulate the yeast Fen1 activity by at least 50-fold, and the same has been claimed for human PCNA and human Fen1 (Li *et al.*, 1995) although the extent and significance of the stimulation has been called into question (J.Chen *et al*., 1996). It has also been shown that human PCNA can stimulate yeast Fen1 but not *vice versa* (Wu *et al.*, 1996). We consequently set out to investigate the extent of stimulation seen with human PCNA and Fen1 in more detail.

The claimed stimulation of human Fen1 by PCNA could easily be reproduced using assay conditions and template described by Harrington and Lieber (1994). However, as already noted with yeast Fen1 and PCNA, the stimulating effect is dependent on the presence of salt (Li *et al.*, 1995). In the absence of NaCl or KCl, Fen1 has high activity on flap substrates and does not need PCNA stimulation (data not shown). In the presence of

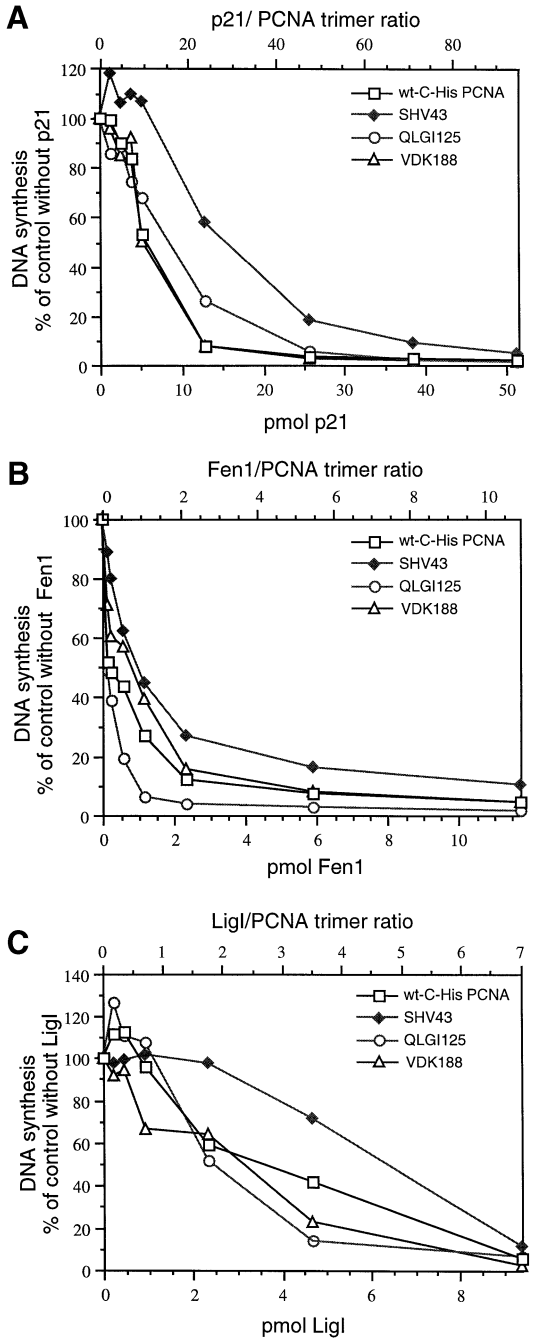

Fig. 6. p21, Fen1 and Lig I differentially inhibit RF-C independent DNA synthesis by pol δ with PCNA and PCNA mutants. Pol δ assays were performed on poly(dA)/$(dT)_{16}$ base ratio 10:1 and the inhibiting proteins titrated into them as indicated. The lower X-axis of each graph indicates the amount of protein added in picomoles and the upper X-axis the molar ratio between the inhibitor protein and PCNA trimers. The Y-axis shows relative DNA synthesis compared with samples without added inhibitor. (**A**) His-p21 was titrated into assays containing 0.15 U of Pol δ and 50 ng of wt or mutant PCNA (0.55 pmol trimer). (**B**) Human Fen1 titrated into assays with 0.15 U of Pol δ and 100 ng of each PCNA (1.1 pmol trimer). (**C**) Human Lig I titrated into assays with 0.025 U of Pol δ and 125 ng of each PCNA (1.3 pmol trimer).

50–100 mM salt, the activity is severely reduced but can be rescued completely by addition of an excess of PCNA. In the absence of PCNA, Fen1 activity is optimal at pH 8.0 (Harrington and Lieber, 1994), but from experience with Pol δ assays we knew that the spontaneous loading of PCNA onto linear DNA is ineffective at pH 8.0 (Weiser *et al.*, 1991). We therefore compared the PCNA stimulation at pH 8.0 with stimulation in our standard Pol δ assay buffer (pH 6.5), complemented with 60 mM NaCl. As expected, lower amounts of PCNA were required to stimulate Fen1 at pH 6.5 (data not shown).

After finding conditions under which the PCNA stimulation of human Fen1 was evident, we compared the ability of the PCNA mutants to stimulate Fen1. When 250 ng mutant were added, both untagged and His tagged wt PCNA as well as VDK188 stimulated the cleavage 7-fold, SHV43 3.5-fold, QLGI125 1.7-fold and LAPK251 not at all (Figure 7). We thus conclude that both the C-side and the domain-connecting loop are important for Fen1 binding, at least to human PCNA, and that Fen1 probably utilizes the same surfaces as p21, including the hydrophobic pocket on the C-side. Since yeast PCNA does not stimulate human Fen1, the binding of Fen1 to human and yeast PCNA probably differs slightly. The LAPK251 folding mutant provides a control that excludes that the stimulation seen is an artefact caused by buffer effects, since all the PCNA mutants were in the same buffer, and that proper trimerization is required for the effect to be seen.

Human Fen1 can interfere with DNA synthesis by Pol δ

It has been reported that Fen1 does not interfere with DNA synthesis by Pol δ or ε (Lieber, 1997). This may depend on the template used, since in our hands a clear inhibition of RF-C-independent Pol δ assays was seen (Figure 6B). This inhibition might be an artefact caused by the endo- and exonucleolytic activities of Fen1 when the Pol δ/PCNA complex collides into the 5′ end of a downstream product on the template strand or engages in strand displacement synthesis. On the other hand, the inhibition may well be caused by a competition with Pol δ for PCNA, since p21 has been shown to inhibit Pol δ by competition (Podust *et al.*, 1995) and Fen1 binds to the same site on PCNA with high affinity. Furthermore, the inhibition is less pronounced when more PCNA is present (data not shown) and since PCNA stimulates the nucleolytic activities of Fen1, the opposite would be expected if they are the main cause for the apparent inhibition. The inhibition seen with Fen1 is apparently stronger than with p21 (compare Figure 6A and B), although p21 reportedly binds PCNA with higher affinity. The SHV43 mutant, which shows reduced Fen1 stimulation but normal binding, seems to be less affected than wt. This could reflect less Fen1 activity or the fact that this mutant is more processive with Pol δ.

PCNA must be below the flap to stimulate Fen1

Considerable effort has been devoted to analysing how Fen1 threads onto the flap strand of its template (Murante *et al.*, 1995), but the question of where PCNA acts to stimulate Fen1 remains unanswered. Indeed, PCNA is drawn arbitrarily on either side of the flap in the published literature (Li *et al.*, 1995; Lieber, 1997). To address this issue, we constructed templates that allowed us to determine on which side of a flap PCNA must be located to stimulate Fen1. By adding biotin labels to either or both sides of the template, we could block the entry of

Table I. A conserved amino acid signature for PCNA binding is found at the N-terminus of Lig I

Protein[1]	aa	PCNA binding sequence	Ref.
p21 human	141	------KRRQTSMTDFYHSKRRLIFS---	(Warbrick *et al.*, 1995)
p21 mouse	136	------KRRQTSLTDFYHSKRRLIFS---	(I.T.Chen *et al.*, 1996)
Fen1 human	331	---SRQGSTQGRLDDFFKVTGSL------	(Warbrick *et al.*, 1997)
Rad2 pombe	332	---ASKTIPQGRLDSFFKPVPSS------	(Warbrick *et al.*, 1997)
Rad27 yeast	334	---GLKSGIQGRLDGFFQVVPKT------	(Warbrick *et al.*, 1997)
XP-G human	981	LKQLDAQQTQLRIDSFFRLAQQEKEDAKR	(Gary *et al.*, 1997)
MCMT human	162	-------TRQTTITSHFAKG---------	(Chuang *et al.*, 1997)
MCMT mouse	159	-------TRQTTITAHFTKG---------	(Chuang *et al.*, 1997)
MCMT chicken	182	-------GRQPTILSVFSKG---------	(Chuang *et al.*, 1997)
LigI human	1	MQRSIMSFFHPKKEGKAKKPE	
LigI mouse	1	MQRSIMSFFQPTKEGKAKKPE	
LigI frog	1	MQRTIKSFFQPKLGAEVKTKE	

[1]The published interacting sequences of p21, Fen1 and homologues, XP-G and DNA-(cytosine-5) methyltransferase (MCMT) from different species are shown aligned with the N-termini of human, mouse and *Xenopus laevis* DNA LigI.

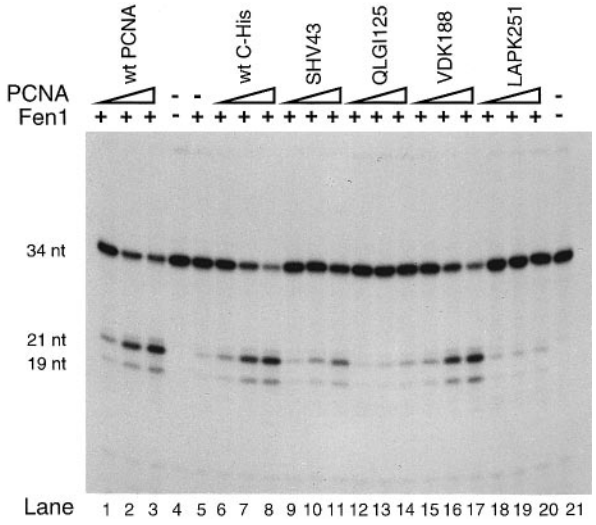

Fig. 7. Fen1 binding to PCNA is necessary but not sufficient to stimulate Fen1 activity. The QLGI125 mutant neither binds nor stimulates Fen1 but although the SHV43 mutant can bind Fen1 stimulation is reduced. Fen1 assays were performed as described in Materials and methods in BDB buffer at pH 6.5. Lanes 4 and 21 contain control reactions without added Fen1. All other reactions contained 5 ng of Fen1 (2 U) and different amounts of PCNA or mutants as indicated above, amounts of 10, 50 or 250 ng each. Lane 5 is a control containing Fen1 without added PCNA. Fen1 cuts the [^{32}P]-labelled 5′-flap at two different places 19 or 21 nt away from the 5′ end.

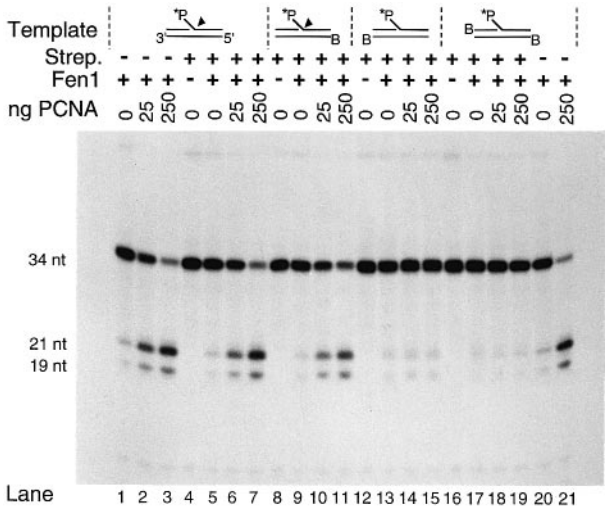

Fig. 8. PCNA must be below the 5′-flap to stimulate Fen1. Fen1 assays were performed as described in Materials and methods using four different versions of the template as indicated above the figure. Lanes 1–7, normal template; lanes 8–11, template with biotin at the 5′ terminus of the bridge strand; lanes 12–15, template with biotin at the 3′ terminus of the bridge strand; lanes 16–21, template with biotin at the 5′ termini of the flap-adjacent and the bridge strand. The labelled templates were incubated with or without 18 ng of streptavidin (0.25 U) as indicated, for 15 min at room temperature before adding Fen1 (5 ng, 2U) and two different amounts of wt PCNA followed by incubation for 30 min at 30°C. Lanes 4, 8, 12 and 16 show controls without added Fen1 that demonstrate the equal labelling of the templates. Comparison of lanes 1–3 and 5–7 shows that streptavidin does not affect the reaction when not bound to the template. Lanes 20 and 21 show that the biotin label alone does not inhibit PCNA loading or cleavage by Fen1. In the absence of NaCl all four templates are cleaved effectively by Fen1 without the need for PCNA stimulation, and the cleavage is not inhibited by streptavidin (data not shown).

PCNA from each end with streptavidin, and since PCNA cannot slide past the flap in either direction, we could identify where PCNA must act to stimulate Fen1. The results in Figure 8 clearly demonstrate that PCNA must be on the 5′ side of the flap (i.e. below it) to stimulate Fen1. This would be the logical position for PCNA after strand displacement synthesis and would fit a role for PCNA in coordinating polymerization and 5′-end processing of Okazaki fragments. Furthermore, these results suggest that the PCNA would be loaded onto DNA with the C-termini facing in the direction of DNA synthesis.

PCNA mutants and sequence similarities indicate that DNA ligase I interacts with a hydrophobic pocket on the C-side of PCNA

The recent description of an interaction between human Lig I and PCNA (Levin *et al.*, 1997) led us to investigate whether we could find where Lig I interacts with PCNA. Levin *et al.* (1997) located the PCNA-binding region

within the N-terminal 118 residues of Lig I, and another recent publication describes a bipartite sequence (residues 1–28 and 111–179) that targets Lig I to replication foci in the nucleus (Cardoso *et al.*, 1997). We therefore looked for similarities between these regions and other known PCNA-binding regions, and identified a hitherto overlooked potential PCNA-binding motif at the very N-terminus (Table I). The most important features are a conserved Glu residue (Glu2), a conserved hydrophobic residue (Ile5) and two conserved aromatic residues, Phe8 and Phe9, which coincide with identical or similar residues shown to be critical for the PCNA binding of p21, Fen1, XP-G and MCMT. These residues would be expected to interact with the hydrophobic pocket on the PCNA C-side (Figure 1).

Experiments performed in our laboratory also showed that human Lig I could interfere with DNA synthesis by Pol δ/PCNA on both linear and circular templates (R.Mossi, E.Ferrari and U.Hübscher, submitted). To verify that the effects seen were because of an interaction between PCNA and Lig I and, if possible, to identify where Lig I binds to PCNA, we titrated purified recombinant Lig I into DNA replication assays on poly(dA)/$(dT)_{16}$ template (Figure 6C). From the titration, it is clear that SHV43 is less inhibited than wt or the other mutants, which would be consistent with the hypothesis that the inhibition is caused by a competition of Lig I and Pol δ for PCNA, and a binding of Lig I to the hydrophobic pocket on the PCNA C-side. An alternative explanation for the inhibition would be that Lig I and Pol δ compete for the 3′ termini of the template. The hyper-processive SHV43 mutant could then presumably be less inhibited. However, the inhibition occurs although the template is in excess over Lig I and an excess of T4 ligase does not have any effect on the assay (data not shown).

PCNA can inhibit ligation by DNA ligase I

Since inhibiting DNA synthesis is unlikely to be the purpose for the interaction between Lig I and PCNA, we set out to see if PCNA could stimulate Lig I as it stimulates Fen1. Experiments with the template described in Materials and methods under standard ligase assay conditions at pH 8.0 showed no effect of PCNA on ligation by Lig I, but a strong inhibition by NaCl (Figure 9A and B). However, when assays were performed at pH 6.5 to favour PCNA loading, the effects were reversed. One hundred mM salt caused only minimal inhibition but PCNA effectively inhibited ligation in the absence of NaCl. The inhibition was >6-fold with 25 ng Lig I and 20 ng PCNA (Figure 9A and B). The effect was less pronounced at 50 mM NaCl, and no inhibition was visible at 100 mM NaCl. As before, there are at least two plausible explanations. A specific interaction between PCNA and Lig I could be responsible, or PCNA could simply be covering the nick and hindering the ligase sterically. The latter possibility is unlikely since the inhibition is evident when the template is in 20-fold molar excess over PCNA trimers and furthermore, PCNA caused no inhibition of ligation by T4 ligase.

Finally, we compared the Lig I inhibiting effects of the different PCNA mutants. The reactions were carried out without addition of salt. C-His-PCNA and the VDK188 mutant were indistinguishable from wt PCNA in their

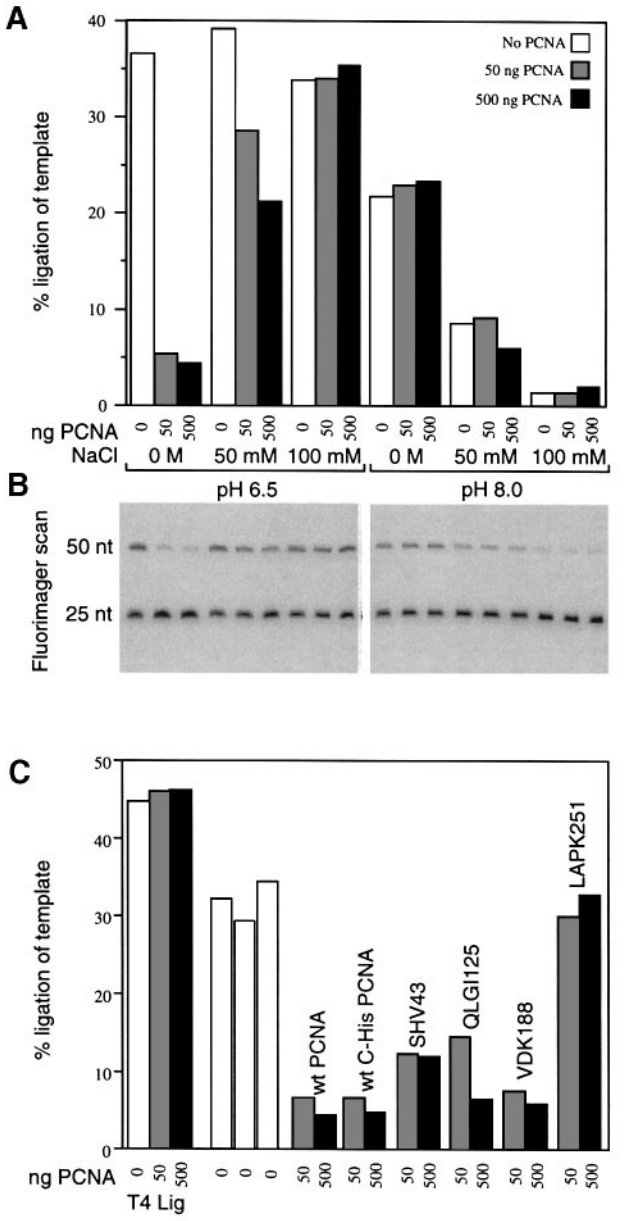

Fig. 9. PCNA can inhibit ligation by DNA ligase I. Ligase assays were performed as described in Materials and methods. (**A**) Effects of NaCl and PCNA on human Lig I. Twenty-five ng Lig I were incubated with 10 pmol of template for 15 min in two different buffers and with salt and wt PCNA as indicated. (**B**) The fluorimager scans used for quantitation, lanes coincide with the respective columns above. (**C**) The effects of PCNA mutations of Lig I inhibition. Twenty-five ng Lig I were incubated at pH 6.5 with 50 or 500 ng of wt or mutant PCNA. The rightmost three columns show a control experiment with 0.01 U T4 ligase instead of Lig I, and the next three columns show three different controls with 25 ng Lig I without added PCNA.

behaviour, but the SHV43 and QLGI125 mutants showed slight differences (Figure 9C). Even when 500 ng of the SHV43 mutant were added, the inhibition was less than that with 50 ng of wt PCNA. The QLGI125 mutant inhibited less at lower concentrations, but increasing the concentration led to similar inhibition as wt. The LAPK251 mutant had no inhibiting effects even at high concentrations, which rules out the possibility that the inhibition seen is an artefact caused by buffer effects and that proper

Table II. Summary of the effects caused by three PCNA mutations[a]

Effect on	PCNA-mutation		
	SHV43AAA	QLGI125AAAA	VDK188AAA
Trimerization	normal	normal	normal
Pol δ stimulation	normal	reduced	normal
Pol δ processivity	increased	reduced	normal
Loading by RF-C	reduced	apparently normal	normal
p21 binding	normal	reduced	normal
Inhibition by p21	reduced	slightly reduced	normal
Fen1 binding	normal	reduced	normal
Fen1 stimulation	reduced	defective	normal
Inhibition by Fen1	slightly reduced	increased	normal
Inhibition of Lig I	reduced	slightly reduced	normal
Inhibition by Lig I	reduced	normal	normal

[a]The summary is based on data the presented in Figures 2–9. The LAPK251 mutant is defective in folding and direct comparison is therefore not appropriate.

folding and trimerization of PCNA is needed. It will be of interest to characterize the inhibiton using different substrates and analyse which step of the ligation reaction is inhibited.

Discussion

Taken together, our results show that many proteins involved in DNA replication and repair bind to the same, or at least partially overlapping sites on the outer front side of PCNA (summarized in Table II). It seems clear that the PCNA back side, with its protuberant loops, is not a player in any of these interactions. Since p21 and Fen1 share a sequence motif in their PCNA-binding regions, it is not surprising that they target the same site on PCNA. Other proteins sharing the same motif, such as XP-G and MCMT, can be expected to bind to the same region as well, and it has indeed been shown that p21 competes with MCMT for PCNA (Chuang *et al.*, 1997). We could identify a region in vertebrate Lig I proteins that contains this sequence signature, consistent with the interaction between Lig I and PCNA discovered recently, and the observation that p21 can dissociate Lig I/PCNA complexes (Levin *et al.*, 1997). Importantly, this signature coincides with a region demonstrated to be required for the association of human Lig I with sites of DNA replication (Cardoso *et al.*, 1997). We demonstrated an inhibitory effect of PCNA on Lig I activity and we could show an inhibiting effect by Lig I on the PCNA stimulation of Pol δ. It remains to be seen what the implications of these effects are during DNA synthesis *in vivo*. The PCNA-binding signature is not found in all PCNA-binding proteins. For example, we did not find the motif in the subunits of human RF-C or Pol δ, even though they seem to interact with similar regions on the PCNA surface. It must also be stressed that the PCNA-binding signature described is both short and general, and has therefore limited predictive power. However, a pattern search based on the alignment in Table I revealed that a similar pattern is present close to the N-terminus of *S.pombe* Lig (*cdc17*) and, interestingly, the signature shows up at the very C-terminus of the *Methanococcus jannaschii* homologue of the large subunit of RF-C.

Experiments with the Fen1 endonuclease allowed us to consolidate two different views about the site of Fen1 binding, and showed that Fen1 binding to PCNA is not enough to stimulate its activity, since the SHV43 mutant can bind Fen1 with high affinity, but is impaired in its stimulation. We could also show that PCNA must be below a 5′ flap to stimulate the Fen1 flap endonuclease activity and, in view of the binding site for Fen1, we propose that the C-side of PCNA faces forward during DNA synthesis and that Pol δ and RF-C sit in front of the trimer. This is in agreement with models of the orientations of the β subunit of *Escherichia coli* Pol III holoenzyme, which is a homolog of PCNA (Stukenberg and O'Donnell, 1995).

The previously undescribed inhibition of Pol δ by Fen1 and the similar inhibition caused by Lig I are remarkably strong compared with the inhibition we see with p21. Care must be taken, however, when comparing the three graphs in Figure 6 directly, since the amounts of PCNA and Pol δ used for each experiment differ. The replication-inhibiting effects of p21 have been analysed quite extensively (Waga *et al.*, 1994; Podust *et al.*, 1995), but the significance of the direct inhibition in preventing DNA replication after extensive DNA damage is still unclear.

Although each PCNA trimer contains three of the potential front side interaction sites, it is obvious that all the PCNA interacting proteins described cannot be bound to a single PCNA trimer at the same time, and this leads us to contemplate how the interactions are ordered spatially and temporally. The replication of the lagging strand is of particular interest in this respect, since many cycles of priming by Pol α, PCNA loading, switching to a processive polymerase (Pol δ or ε), primer processing by Fen1 and/or RNaseH1 and ligation, presumably by Lig I (reviewed in Tomkinson and Levin, 1997), are required. We know that PCNA is required for the first steps on this path, the switch from Pol α to Pol δ (Stillman, 1988) and processive DNA synthesis. In view of the interactions of PCNA with Fen1, Lig I, MCMT and mismatch repair proteins, it seems likely that PCNA plays a larger role in coordinating these activities. A possible scenario would be that a Pol α–RF-C–Pol δ complex stays attached to PCNA until the 5′ end of a down-stream Okazaki fragment is encountered. Pol δ could then initiate strand displacement synthesis and continue until it is displaced by Fen1, releasing PCNA

from the complex. PCNA would then stimulate the removal of the flap as has been proposed (Murante *et al.*, 1996) and stay attached to the DNA to coordinate DNA methylation by MCMT and mismatch repair by its interaction with the MSH2–MSH3 complex and MLH1. PCNA may, by its orientation on the DNA, provide the signal necessary for discrimination of the parent and newly synthesized strand (Jónsson and Hübscher, 1997). Another possible strand discrimination signal is the presence of nicks in the newly synthesized strand (Holmes *et al.*, 1990; Thomas *et al.*, 1991) which PCNA may preserve by inhibiting Lig I. The unloading of PCNA could then allow the ligase to finish ligation and, thus, Okazaki fragment processing. Similar models may be envisioned for the other DNA repair pathways involving PCNA, where a single PCNA trimer may bind and coordinate the repair process.

Although our goal of generating mutations that would selectively knock out the interaction of PCNA with individual interacting proteins seems elusive in view of the similar binding sites, the effects of the mutations are different enough to be useful for furthering our understanding of the pathways involving PCNA. It will be of interest to see what the effects of the mutations are *in vivo* and if any effects can be seen in some of the established *in vitro* DNA repair systems where a requirement for PCNA has been demonstrated [nucleotide excision repair (Shivji *et al.*, 1992), base excision repair (Matsumoto *et al.*, 1994) and mismatch repair (Umar *et al.*, 1996)]. In particular, it will be interesting to see if the back-side loops on PCNA have a specific function and if effects on cell-cycle regulation can be observed.

Materials and methods

Nucleic acids

Poly(dA)$_{1000–1500}$ was from Sigma and Oligo(dT)$_{16}$ from Microsynth, Balgach, Switzerland. Poly(dA)/(dT)$_{16}$ (base ratio 10:1 and 40:1) was prepared as described (Weiser *et al.*, 1991). Singly primed M13 DNA (spM13) was prepared according to Podust and Hübscher (1993). Oligonucleotides for mutagenesis and sequencing of the PCNA gene and Fen1 assays were from Microsynth. The plasmid pT7/hPCNA carrying the cDNA of human PCNA (Fien and Stillman, 1992) was kindly provided by B.Stillman, the construction of pET23/hPCNA was described by Jónsson *et al.* (1995), the plasmid pET11/HligI(2–919) (Mackenney *et al.*, 1997) encoding full length human DNA Lig I with a N-terminal His$_6$ tag was a gift of T.Lindahl, the pET14b-Gadd45 expression plasmid (Carrier *et al.*, 1994) was a gift of, A.J.Fornace Jr, and the plasmid pET-Fen1 (J.Chen *et al.*, 1996) encoding full length human Fen1 was a gift of A.Dutta.

Proteins

Calf thymus Pol δ (Weiser *et al.*, 1991) and the replicative complex (RC) holoenzyme (Maga and Hübscher, 1996) were isolated as described. Human wt PCNA was produced in *E.coli* using the plasmid pT7/hPCNA and purified to homogeneity as described (Schurtenberger *et al.*, 1998). *Escherichia coli* SSB was purified according to Lohman *et al.* (1986). Recombinant human RP-A was purified according to Henricksen *et al.* (1994). His$_6$-Gadd45 was expressed in *E.coli* BL21(DE3)pLysS and purified from the soluble fraction on a Ni-NTA spin column (Qiagen) according to the manufacturers protocol. Restriction enzymes, T4 polynucleotide kinase (PNK), T4 ligase and T4 Pol were from New England Biolabs.

Buffers

The following buffers were used: buffer A [50 mM $NaPO_4$ pH 8.0, 300 mM NaCl, 20 mM imidazole-HCl, 1mM phenylmethylsulfonyl fluoride (PMSF)]; buffer B (50 mM HEPES–NaOH pH 7.5, 300 mM NaCl, 20 mM imidazole–HCl); buffer C (20 mM Bis-Tris–HCl pH 6.7); buffer D (8M urea, 100 mM $NaPO_4$ pH 8.0, 10 mM Tris–HCl, 2 mM β-mercaptoethanol); buffer E [50 mM $NaPO_4$ pH 8.0, 30 mM Tris–HCl, 150 mM NaCl, 1 mM PMSF, 10% (v/v) glycerol]; buffer F [50 mM $NaPO_4$ pH 6.0, 300 mM NaCl, 10% (v/v) glycerol]; buffer G [20 mM Tris–$NaPO_4$ pH 7.7, 10 mM KCl, 1 mM EDTA, 1 mM dithiotreitol (DTT), 1 mM PMSF, 0.1% (v/v) NP-40, 10% (v/v) glycerol]; buffer H [20 mM ethanolamine–HCl pH 9.2, 1mM DTT, 0.05 % (v/v) Nonidet P-40, 10% (v/v) glycerol]; buffer I [50 mM HEPES–NaOH pH 7.4, 0.02% (v/v) Nonidet P-40, 1 mM DTT, 0.5 mM EDTA, 10 % (v/v) glycerol]; buffer J [50 mM HEPES–NaOH pH 8.0, 500 mM NaCl, 10 mM imidazole HCl, 10% (v/v) glycerol, 1 mM β-mercaptoethanol, 1 mM PMSF]; buffer K [40 mM Bis–Tris–HCl pH 6.6, 0.5 mM EDTA, 10% (v/v) glycerol, 1 mM β-mercaptoethanol, 1 mM PMSF; BDB buffer, 50 mM Bis–Tris pH 6.5, 1 mM DTT, 0.25 mg/ml bovine serum albumin (BSA)]; buffer L (10 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithioerythrol); gel loading buffer [95% (v/v) formamide, 10 mM EDTA, 0.25 mg/ml bromophenol blue, 0.25 mg/ml xylene cyanol]; Lig I buffer (60 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 50 μg/ml BSA); TDB buffer (40 mM Tris–HCl pH 7.5, 1 mM DTT, 0.2 mg/ml BSA); TBE (90 mM Tris–borate pH 8.0, 2 mM EDTA).

Construction, expression and purification of C-His-PCNA and PCNA mutants

A clone expressing the full length human PCNA protein with the sequence RIRAPSTSLHHHHHH fused to the C-terminus was generated by removing the stop codon from pET23/hPCNA (Jónsson *et al.*, 1995) by site-directed mutagenesis with the oligonucleotide pGGATCCTAGATCCTTCTTC. Site-directed mutagenesis was performed using T4 Pol as described by Jónsson *et al.* (1995). The pET23/C-His-PCNA clone was used as a template for mutagenesis to generate PCNA mutations using the following oligonucleotides: SHV43AAA, pACCAAAGAGGCGGCGGCCGAGTCCATG; QLGI125AAAA, pTGTTCTGGAGCTGCAGCGGCTTCAACATCTA; VDK188AAA, pTTCCTCCTCTGCAGCGGCATTACTTGT; LAPK 251AAAA: pCCTCGATCGCGGCAGCCGCGTAGTATTTTA

Wild-type and mutant C-His PCNA were expressed in *E.coli* BL21(DE3)pLysS. For purification 750 ml cultures were grown to an OD_{600} of ~0.6 in LB [with 200 μg/ml ampicillin (Amp) and 34 mg/ml chloramphenicol] at 37°C, and then expression was induced by adding IPTG to a final concentration of 0.5 mM and growing for 5 h. The bacterial pellets were resuspended in 20 ml buffer A and sonicated to disrupt cells and reduce viscosity. Insoluble material was removed by centrifugation for 30 min at 40 000 *g* and the supernatants loaded onto 1 ml Hi-Trap chelating columns (Pharmacia) charged with Ni^{2+} and equilibrated in buffer A. After loading, the columns were washed with 5 ml buffer A followed by 5 ml buffer B and wt C-His-PCNA, and mutants were then eluted with a 5 ml gradient to 500 mM imidazole in buffer B, collecting 0.4 ml fractions. The purity of the proteins was evaluated by SDS–PAGE and peak fractions were diluted with 2 vol buffer C and loaded onto a Mono Q HR5/5 column (Pharmacia) equilibrated in the same buffer. The LAPK251 protein partially precipitated upon storage over night at 4°C at high concentration (5 mg/ml) and the precipitate had to be removed by centrifugation and filtration through a 0.22 μm filter before proceeding. After washing with 5 ml buffer C, the proteins were eluted with a 15 ml gradient to 650 mM NaCl in buffer C, collecting 0.5 ml fractions. Peak fractions, containing practically homogenous PCNA, were identified by SDS–PAGE and pooled. After measuring protein concentration using the Bio-Rad protein assay, the proteins were brought to 1 mg/ml in buffer C containing 30% (v/v) glycerol, either by dilution or by buffer exchange and concentration on centricon 10 (Amicon) columns (SHV43 and LAPK251). The exact protein concentrations were then measured again, side by side, in the Bio-Rad protein assay, and the absence of nuclease contamination was verified by incubating 1 μg of each PCNA mutant with 1 μg of linear and circular pUC19 in buffer L for 1 h at 37°C, followed by separation on an agarose gel.

Expression and purification of p21

Expression and purification of untagged p21 was according to Podust *et al.* (1995). His-p21 expression and washing of inclusion bodies was done identically followed by solubilization in buffer D. The soluble fraction was loaded onto 10 ml of ProBond resin (Invitrogen), the bound protein was renatured on the column at room temperature by a slow gradient to buffer E and eluted with a gradient to 500 mM imidazole in buffer F.

Expression and purification of human Fen1

Fen1 was expressed in *E.coli* JM109(DE3). A 500 ml culture in LB (with 100 μg/ml Amp) was grown at 30°C to an OD_{600} of ~0.6 and then IPTG was added to a final concentration of 0.4 mM. After 3 h induction, the cells were harvested, resuspended in 45 ml of buffer G and disrupted with a French press. Insoluble material was removed by centrifugation for 20 min at 40 000 *g* and the supernatant loaded onto a 20 ml SP-Sepharose column (Pharmacia). Proteins were eluted with a 400 ml gradient to 0.6 M KCl in buffer G, collecting 10 ml fractions. Fen1-containing fractions were pooled and dialysed against buffer H. The dialysed pool was passed through a Mono Q HR5/5 column, and the Fen1-containing flow-through fraction was then dialysed against buffer I. The dialysed flow-through was loaded onto a MonoS FPLC column (Pharmacia) and eluted with a steep 5 ml gradient to 1 M KCl in buffer I, collecting 250 μl fractions. The peak fraction was >99% pure, as judged by SDS–PAGE, and highly concentrated (~8 mg/ml). Under standard Fen1 assay conditions (Harrington and Lieber, 1994), 5 ng of peak fraction Fen1 cleaved ~2 fmol of flap substrate corresponding to a specific activity of 400 U/μg.

Expression and purification of human Ligase I

Escherichia coli BL21(DE3) was freshly transformed with the pET11/HligI(2–919) plasmid. Starter cultures were grown at 37°C in LB with 100 μg/ml Amp and used to inoculate 1.6 l of culture in LB with 100 μg/ml Amp. At OD_{600} = 0.64, the temperature was reduced to 24°C and IPTG added to a final concentration of 1 mM. After 1 h, PMSF (200 mM in DMSO) was added to a final concentration of 0.5 mM and the expression was allowed to continue for 1 h. The cells were then rapidly harvested and resuspended in 50 ml of buffer J containing 2.5 μg/ml Aprotinin, 2.5 μg/ml Leupeptin and 5 mM β-mercaptoethanol. The resuspended pellet was passed twice through a French press to disrupt cells, and insoluble material was removed by centrifugation at 40 000 *g* for 20 min The crude extract was loaded onto a Ni^{2+}-charged 1 ml Hi-Trap chelating column, equilibrated in buffer J, washed with 5 ml of buffer J and eluted with a 5 ml gradient to 500 mM imidazole in buffer J, collecting 0.5 ml fractions. Lig I-containing fractions were pooled and diluted with 2 vol buffer K and loaded onto a Mono S HR5/5 column. After washing with 5 ml buffer K, the bound Lig I protein was eluted with a 10 ml gradient to 1 M NaCl in buffer K, collecting 300 μl fractions. The full-length Lig I eluted at the end of the gradient, as judged by SDS–PAGE, and was immediately aliquoted and stored in liquid N_2.

Native gradient PAGE

The electrophoresis was performed in 8–25% polyacrylamide gel using the Phast System (Pharmacia) according to the manufacturers protocol.

PCNA stimulation of Pol δ on poly(dA)/(dT)$_{16}$

The stimulation of Pol δ by PCNA mutants was assayed in BDB buffer with 6 mM $MgCl_2$, 25 μM [^{3}H]dTTP (420 cpm/pmol), 0.5 μg poly(dA)/oligo(dT) base ratio 10:1, or 40:1 as indicated, 0.05–0.25 U Pol δ as indicated, and variable amounts of PCNA and interacting proteins in a final volume of 25 μl. The reactions were incubated for 30 min at 37°C and precipitated with 10% trichloroacetic acid (TCA). Acid-insoluble radioactivity was quantified by scintillation counting as described (Hübscher and Kornberg, 1979).

Product analysis with Pol δ on poly(dA)/(dT)$_{16}$

Pol δ product analysis was performed using a reaction mixture containing BDB buffer with 6 mM $MgCl_2$, 28 μM [α-^{32}P]dTTP (5000 cpm/pmol), 0.5 μg poly(dA)/$(dT)_{16}$ base ratio 40:1, 0.05 U Pol δ and PCNA as indicated, in a final volume of 18 μl. Reactions were incubated for 15 min at 37°C and then stopped by adding 12 μl of gel loading buffer, heated for 3 min at 95°C and the products separated on a 7% polyacrylamide gel (acrylamide/Bis–acrylamide ratio, 75:2) in TBE with 7 M Urea. The [^{32}P]-labelled reaction products were visualized by autoradiography.

Stimulation of a holoenzyme complex containing Pol δ and RF-C

The assays with a replication-competent holoenzyme (RC-complex) were carried out as described by Maga and Hübscher (1996). In brief, each reaction contained 25 μl of TDB with 10 mM $MgCl_2$, 1 mM ATP, 40 μM each dATP, dGTP and dCTP, 15 μM [^{3}H]dTTP (150 cpm/pmol), 100 ng of spM13 DNA, 250 ng SSB, 0.07 U of Pol δ and different amounts of wt or mutant PCNA to be tested. The reactions were prepared on ice and 0.01 U of RC-complex was added immediately before incubation at 37°C for 30 min. TCA-insoluble products were quantified as described (Hübscher and Kornberg, 1979). The results were verified using an alternative RF-C dependent assay on poly dA/$(dT)_{16}$ (20:1) as described by Uhlmann *et al.* (1997).

Product analysis with the holoenzyme complex

The primer was end-labelled using T4 PNK and γ-[^{32}P]dATP and subsequently annealed to single-stranded M13 mp11 DNA. Reactions were carried out in 25 μl containing TDB with 10 mM $MgCl_2$, 1 mM ATP, 50 μM each dGTP, dATP, dTTP and dCTP, 500 ng RP-A, 0.1 U Pol δ, 100 ng of labelled spM13 DNA and different amounts of wt or mutant PCNA to be tested. The reactions were prepared on ice and 0.01 U RC-complex was added immediately before incubation at 37°C for 30 min. DNA synthesis was stopped by adding 25 μl gel loading buffer, and the samples were heated for 3 min at 95°C before the products were separated on a 6% polyacrylamide gel (acrylamide/Bis–acrylamide ratio, 38:2) in 7 M Urea and TBE buffer. After drying the gel, the labelled products were visualized by autoradiography.

Fen1 flap endonuclease assays

The assays were performed using the Fen1 substrate oligonucleotides HJ41, HJ42 and HJ43 described by Harrington and Lieber (1994) or the same oligonucleotides modified with a biotin moiety at either the 3′ or 5′ termini. The flap-forming oligonucleotide (HJ42) was labelled by incubating 12.5 pmol in a total of 50 μl reaction containing 20 U T4 PNK with 20 pmol [γ-^{32}P]ATP (3000 ci/mmol) for 45 min at 37°C in PNK forward reaction buffer (Gibco). After heat inactivation for 15 min at 70°C, the reaction was passed over a G-50 nick column (Pharmacia) equilibrated in 10 mM Tris–HCl, pH 8.0, to remove unincorporated ATP. The labelled oligonucleotide was contained in 500 μl of buffer and was subsequently concentrated to 40 μl using a Speed Vac. The labelled HJ42 oligonucleotide (2.5 pmol) was then annealed to equimolar amounts of the 'flap bridge' (HJ41) and 'flap adjacent' (HJ43) oligonucleotides, or biotin end-labelled derivatives of them, at a final concentration of 100 fmol/μl in 20 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$ 50 mM NaCl.

Unless otherwise indicated, the Fen1 assays were performed in 18 μl reactions containing 10 fmol Flap substrate in BDB with 10 mM $MgCl_2$, 250 μg/ml BSA and 5 ng of Fen1 with varying amounts of PCNA and streptavidin added. The reactions were incubated at 30°C for 30 min and then mixed with 12 μl of gel loading buffer, heated for 3 min at 95°C and separated on 16% polyacrylamide gels (acrylamide/Bis–acrylamide ratio, 19:1) containing 7 M Urea and TBE. The [^{32}P]-labelled reaction products were visualized by autoradiography and digitized with a PhosphorImager (Molecular Dynamics) for quantitation with the Image Quant software (Molecular Dynamics).

Activity assays for DNA ligase

Ligase activity was assayed by fluorimetry using the 5′ fluorescein-labelled 25mer and 5′ phosphorylated 25mer oligonucleotides described by Thorbjarnardóttir *et al.* (1995), annealed to a 2-fold molar excess of a complementary 50 bp oligonucleotide. Each assay contained 10 pmol of the fluorescein labelled substrate in 12 μl of BDB buffer (pH 6.5), supplemented with 6 mM $MgCl_2$ and 1 mM ATP, or Lig I buffer (pH 8.0), with varying amounts of PCNA, NaCl and Lig I or T4 ligase. Samples were incubated for 15 min at 37°C, mixed with 8 μl of gel loading buffer and heated for 3 min at 95°C. The ligated products (50 nt) were separated from unligated 25 nt oligonucleotides by electrophoresis on 16% denaturing PAGE. At least five titration points spanning 0.05–10 pmol of the fluorescein labelled oligonucleotide were included in each experiment for quantitative reference. The gels were scanned immediately after running the gels, using a Fluorimager 575 (Molecular Dynamics) and the band intensities quantified using Image Quant.

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