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Tyrosine 114 is essential for the trimeric structure and the functional activities of human proliferating cell nuclear antigen

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In order to study the effect of trimerization of proliferating cell nuclear antigen (PCNA) on its interaction with DNA polymerase (pol) δ and its loading onto DNA by replication factor C (RF-C) we have mutated a single tyrosine residue located at the subunit interface (Tyr114) to alanine. This mutation (Y114A) had a profound effect on PCNA, since it completely abolished trimer formation as seen by glycerol gradient sedimentation and native gel electrophoresis. Furthermore, the mutant protein was unable to stimulate DNA synthesis by pol δ and did not compete effectively with wild-type PCNA for pol δ, although it was able to oligomerize and could to some extent interact with subunits of functionally active PCNA. We thus conclude that PCNA molecules that are not part of a circular trimeric complex cannot interact with the pol δ core. Furthermore, the mutant protein could not be loaded onto DNA by RF-C and did not compete with wild-type PCNA for loading onto DNA, indicating that PCNA trimerization may also be a prerequisite for its recognition by RF-C. The adverse effects caused by this single mutation suggest that trimerization of PCNA is essential for the monomers to keep their overall structure and that the structural changes imposed by trimerization are important for interaction with other proteins.

Keywords: DNA replication/PCNA/protein–protein interaction/site-directed mutagenesis/trimerization.

Introduction

Proliferating cell nuclear antigen (PCNA; reviewed in Hübscher *et al.*, 1995) was first discovered as a human auto-antigen in patients with lupus erythematosus (Miyachi *et al.*, 1978). It was independently discovered by two-dimensional electrophoresis as a protein abundant in S phase cells and named cyclin (Bravo and Celis, 1980), but soon thereafter PCNA and cyclin were found to be identical (Mathews *et al.*, 1984). PCNA was later identified as an auxiliary protein for DNA polymerase (pol) δ with a functional similarity to the *Escherichia coli* pol III β subunit (Tan *et al.*, 1986). Direct comparison of this auxiliary factor for pol δ and PCNA showed that the proteins were in fact the same (Prelich *et al.*, 1987a). The role of PCNA as an essential DNA replication factor was established soon thereafter in studies of the *in vitro* SV40 DNA replication system (Prelich *et al.*, 1987b). More recently PCNA was found to be involved in nucleotide excision repair (Shivji *et al.*, 1992).

The function of PCNA in DNA replication and repair is to form a sliding clamp with replication factor C (RF-C), tethering pol δ or pol ε to the 3′-hydroxyl end of a growing DNA chain (Lee and Hurwitz, 1990; Burgers, 1991; Stillman, 1994). Various studies have shown that RF-C can load PCNA onto primed single- or double-stranded DNA in an ATP-dependent manner. The PCNA–RF-C complex then presumably locates at the 3′-hydroxyl end of a primer after sliding along the double-stranded DNA and anchors at the primer–template junction (Podust *et al.*, 1995). In addition to its interactions with pols and RF-C, PCNA has been found to interact directly with various proteins involved in cell cycle regulation, namely D-type cyclin–cyclin-dependent kinase (CDK) complexes (Xiong *et al.*, 1992; Matsuoka *et al.*, 1994), the CDK inhibitor p21 (Zhang *et al.*, 1993; Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994) and the p53-regulated protein Gadd45, which also interacts with p21 (Smith *et al.*, 1994). Unlike the function of PCNA as an auxiliary factor for pols δ and ε, the role of PCNA in cell cycle control remains unclear so far.

The recently published crystal structure of yeast PCNA shows that the protein forms a homotrimeric ring with a negative electrostatic potential on the outside and a positive charge distribution lining a hole through which a DNA strand can thread (Krishna *et al.*, 1994). The N- and C-terminal halves of PCNA form two domains which fold identically. Interestingly, the overall structure of the PCNA trimer strongly resembles the β subunit homodimer of the *E.coli* pol III holoenzyme. The overall folding pattern of the two domains of the PCNA monomer is almost identical to that of the three domains of the β subunit monomer. The β subunit has a similar role in *E.coli* DNA replication as PCNA in eukaryotes, suggesting that the two proteins have common evolutionary roots, in spite of their difference in number of domains and lack of significant amino acid sequence identity.

We were interested to obtain a PCNA mutant in which the monomer/trimer equilibrium would be shifted towards the monomer in order to see if the trimeric structure of PCNA is necessary for its interaction with other proteins, particularly with pol δ and RF-C. A mutant defective in trimerization would also allow us to test if RF-C can aid PCNA trimer formation. We have therefore chosen Tyr114 as a candidate for mutational analysis, since the corresponding residue of *Saccharomyces cerevisiae* PCNA is located at the monomer interface in strand βI_1 and is predicted to be exposed to the solvent in the monomer, but buried in the trimer (Krishna *et al.*, 1994). Furthermore, this residue is highly conserved in PCNA from various species, being either identical or substituted by a structur-

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ally similar Phe residue. This conservation is also apparent in the structurally analogous location of the C-terminal domain of PCNA and the corresponding locations in the three domains of the *E.coli* pol III β subunit (Krishna *et al.*, 1994). Lastly, a mutation of the adjacent residue Ser115 of yeast PCNA to Pro causes a cold-sensitive phenotype and has a strong effect on trimerization (Krishna *et al.*, 1994). Ala was chosen as a substitute for Tyr114 because it would neither be expected to alter the main chain conformation to a significant extent nor have adverse electrostatic effects. In addition to the mutational analysis, we developed an assay to analyse the stability of the functionally active trimer complex. For that purpose a 23 amino acid peptide containing a poly(His) stretch was fused to the N-terminus of wild-type PCNA (wt PCNA), which could subsequently be bound to Ni–NTA resin. This assay allowed us to analyse the dissociation and association of PCNA monomers quantitatively. In this paper we describe the effects of the Tyr114 mutation on the trimerization of PCNA and its implications for the interaction with pol δ and RF-C.

Results

Mutant and fusion derivatives of PCNA

An oligonucleotide primer was designed to mutate the coding sequence of the cloned human PCNA gene so that Tyr114 would be substituted by Ala. After screening clones by *Sca*I restriction and partial sequencing a clone (Y114A) with the designed mutation was selected. The entire coding sequence of the Y114A clone was sequenced and no other mutations were found.

Previously we constructed a plasmid to produce PCNA which could be ^{32}P-labelled *in vitro* at its N-terminus (ph-PCNA; Podust *et al.*, 1995). This allowed us to analyse assembly of the auxiliary proteins RF-C and PCNA directly onto DNA. In order to monitor the effect of the Y114A mutation on assembly of the sliding clamp we additionally constructed a fusion protein (ph-PCNA–Y114A) containing both the artificial phosphorylation site from ph-PCNA (Podust *et al.*, 1995) and the Y114A mutation mentioned above (see Materials and methods).

To detect dissociation and reassociation of PCNA trimers we added a sequence encoding a 23 amino acid leader peptide containing a poly(His) stretch to the PCNA gene. The leader peptide endowed the recombinant PCNA with two new properties. First, the metal chelating properties of the poly(His) leader were used for purification of the fusion protein and for assaying PCNA subunit exchange (see below). Second, this slightly larger form of PCNA could be resolved from wt PCNA and mutant PCNA Y114A on SDS gels, thus allowing a simple quantitation of the different forms of subunits retained on Ni–NTA resin in subunit exchange assays.

All PCNA derivatives listed above were predominantly present in the soluble fraction of crude extracts upon expression in *E.coli*. The fusion parts and designations are documented in Figure 1.

Mutant PCNA Y114A cannot stimulate pol δ in vitro

First, the stimulatory effect of PCNA Y114A on pol δ was tested using poly(dA)·oligo(dT) as template. In this assay PCNA can load onto DNA via the end and does not require RF-C (RF-C-independent assay; Weiser *et al.*, 1991). PCNA Y114A was completely inactive in this assay, while his-PCNA was fully active, stimulating pol δ even more strongly than wt PCNA (Figure 2). An explanation of this increased activity is not obvious, but the same phenomenon has been described for another N-terminal modification of PCNA, namely ph-PCNA (Podust *et al.*, 1995). Furthermore PCNA Y114A did not compete effectively with wt PCNA for pol δ. A slight inhibition of DNA synthesis was detected only in the presence of 25-fold excess of the mutant to wt PCNA (data not shown). PCNA Y114A was next tested on a singly primed M13 DNA template in the presence of RF-C (RF-C-dependent assay; Podust *et al.*, 1992) and was again found to be totally inactive, as in the RF-C independent assay (data not shown). Another variant of mutant PCNA having Tyr114 substituted by Ser instead of Ala was also isolated and found to be inactive in stimulation of pol δ activity when tested in the RF-C-independent and RF-C-dependent assays (data not shown).

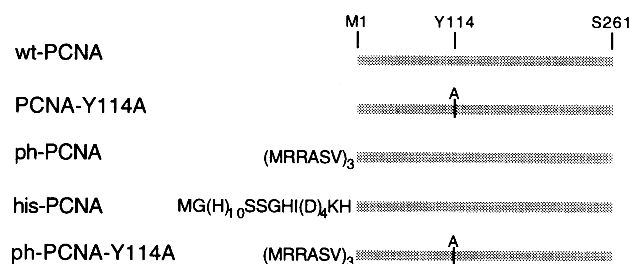

Fig. 1. PCNA constructs used in this study. wt, wild-type, ph-, phosphorylatable, his-, histidine tag. The other letters represent single letter amino acid abbreviations. For more details see text.

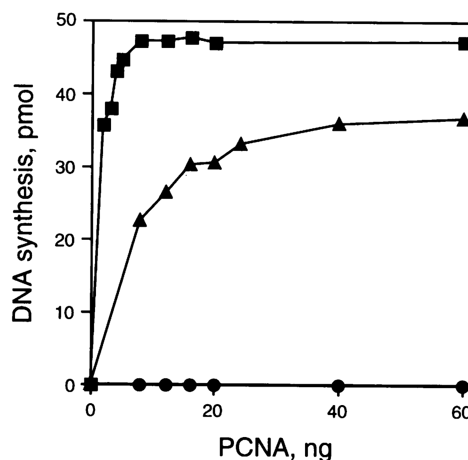

Fig. 2. PCNA Y114A cannot stimulate pol δ. Pol δ activity on the poly(dA)·oligo(dT) template was tested in the presence of different amounts of wt PCNA (▲), his-PCNA (■) and PCNA Y114A (●). The reaction mixture contained 50 mM bis-Tris, pH 6.5, 0.25 mg/ml BSA, 1 mM DTT, 6 mM $MgCl_2$, 10 mM KCl, 25 μM [^{3}H]dTTP (400 c.p.m./pmol), 0.5 μg poly(dA)·oligo(dT) (base ratio 10:1), 0.3 U pol δ and variable amounts of PCNA as indicated in a final volume of 25 μl. The reactions were incubated for 10 min at 37°C, followed by analysis of the acid-insoluble fraction.

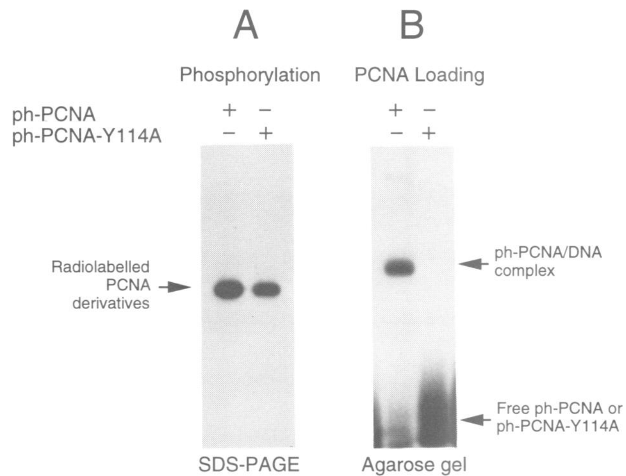

Fig. 3. RF-C does not interact with the PCNA Y114A mutant in the clamp assembly assay. The technique to phosphorylate PCNA *in vitro* at an artificially added phosphorylation site at the N-terminus has recently been described (Podust *et al.*, 1995). This derivative of PCNA (termed ph-PCNA) can, after radiolabelling, be used to follow the process of PCNA loading onto DNA. The analysis is carried out on agarose gels, where the labelled PCNA derivative migrates with the DNA, if loaded. (**A**) Phosphorylation of ph-PCNA and ph-PCNA–Y114A *in vitro*. The phosphorylation mixture (10 μl) contained 20 mM Tris–HCl, pH 7.5, 1 mM DTT, 12 mM $MgCl_2$, 0.1 M NaCl, 1.5 U cAMP-dependent protein kinase catalytic subunit, 10 μCi [γ-^{32}P]dATP (3000 Ci/mmol) and 250 ng ph-PCNA or ph-PCNA–Y114A. The mixtures were incubated for 20 min at 37°C. Aliquots (1 μl) of both reaction mixtures were loaded onto 12.5% SDS gels, electrophoresed and autoradiographed. (**B**) Loading of PCNA onto gapped double-stranded DNA. The reaction mixture (25 μl) contained 40 mM triethanolamine–HCl, pH 7.5, 0.2 mg/ml BSA, 10 mM $MgCl_2$, 1 mM ATP, 100 ng gapped circular double-stranded DNA, 30 ng RF-C and 15 ng [^{32}P]ph-PCNA or [^{32}P]ph-PCNA–Y114A. The mixtures were incubated for 3 min at 37°C, fixed with glutaraldehyde and analysed by agarose gel electrophoresis as described (Podust *et al.*, 1995).

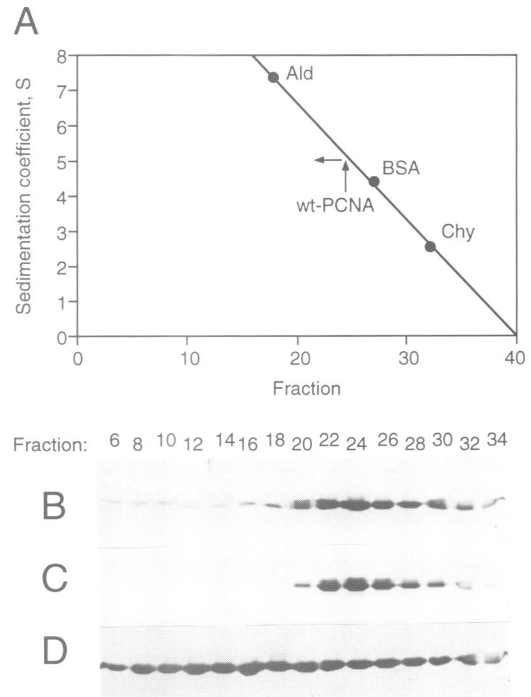

Fig. 4. Glycerol gradient centrifugation of three forms of PCNA. Samples (100 μl, containing 50–130 μg protein) of wt PCNA, his-PCNA and PCNA Y114A were loaded onto a 4 ml 15–30% (v/v) glycerol gradient in buffer I and subjected to centrifugation at 55 000 r.p.m. in a TST 60.4 rotor at 4°C for 18 h. The samples were separated into 100 μl fractions from the bottom of the tube. Aldolase (7.35 S), BSA (4.4 S) and chymotrypsinogen A (2.54 S) were sedimented in the same run and sedimentation profiles were analysed by the Bradford assay. A calibration curve was drawn by plotting the centres of each peak (**A**). Aliquots of 25 μl of every second fraction from 6 to 34 were analysed by SDS–PAGE and stained with Coomassie Blue. (**B**) wt PCNA; (**C**) his-PCNA; (**D**) PCNA Y114A.

RF-C does not interact with the PCNA Y114A mutant in a clamp assembly assay

The interaction of DNA, RF-C and PCNA results in the assembly of a functional sliding clamp which precedes assembly of the pol δ (or pol ε) holoenzyme complex. Therefore, we next tested the ability of RF-C to load ph-PCNA–Y114A onto DNA in the presence of ATP. Both ph-PCNA and ph-PCNA–Y114A could be efficiently phosphorylated *in vitro* with [γ-^{32}P]ATP and cAMP-dependent protein kinase (Figure 3A). However, only [^{32}P]ph-PCNA and not [^{32}P]ph-PCNA–Y114A could be loaded onto DNA by RF-C (Figure 3B). A 50-fold excess of the PCNA–Y114A mutant did not inhibit [^{32}P]ph-PCNA loading onto DNA, indicating that RF-C did not recognize and was not sequestered by the mutant protein (data not shown). Further evidence was obtained by pre-incubating [^{32}P]ph-PCNA Y114A with wt PCNA in order to produce chimeric forms of PCNA oligomers by subunit exchange. Even though PCNA Y114A can participate in subunit exchange (see below and Figure 6), no loading of the chimeric [^{32}P]ph-PCNA–Y114A/wt PCNA by RF-C onto DNA was observed (data not shown).

Physicochemical analysis of PCNA Y114A

Since the single point mutation resulted in an apparently complete loss of ability to interact with both pol δ and RF-C, we next characterized the possible structural consequences of the Y114A mutation. First, wt PCNA, his-PCNA and PCNA Y114A were analysed by glycerol gradient centrifugation. wt PCNA (Figure 4B) and his-PCNA (Figure 4C) sedimented as expected (Almendral *et al.*, 1987), around 5 S (Figure 4A). PCNA Y114A, on the other hand, was distributed throughout the entire gradient and showed no defined peak (Figure 4D). This result suggested that the mutation altered the nature of oligomerization. To clarify this finding both wt PCNA and PCNA Y114A were next analysed by polyacrylamide gradient gel electrophoresis under non-denaturing conditions. Figure 5 shows that wt PCNA migrated as two bands: one is dominant and very well shaped and its M_r corresponds to the trimer, while the second is diffuse with a M_r corresponding to a dimer. In contrast, PCNA Y114A yielded no band equivalent to the trimer of wt PCNA and was exclusively present as a mixture of forms which appear to represent oligomers. The results of the native PAGE analysis could explain why PCNA Y114A did not resolve into a sharp peak after glycerol gradient centrifugation, as did wt PCNA and his-PCNA (compare Figure 4B, C and D).

A subunit exchange assay can estimate the exchange rate of PCNA monomers into trimers

The results of native gel electrophoresis indicated that the PCNA Y114A mutant retains the ability to form oligomers.

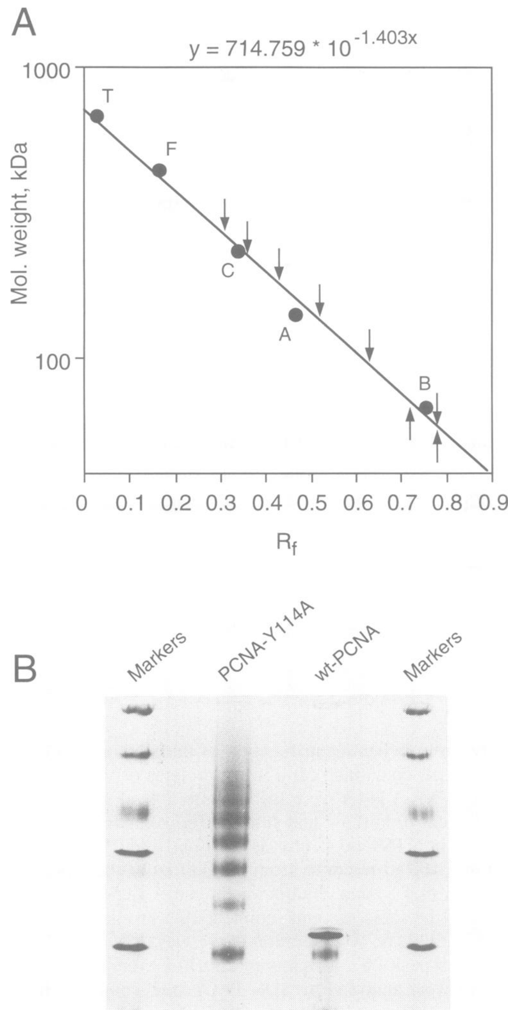

Fig. 5. Native gel electrophoresis of wt PCNA and mutant PCNA Y114A. Samples of 1 µg wt PCNA, PCNA Y114A or high molecular weight markers (Pharmacia) were analysed on 8–25% native gradient gel using the Phast System (Pharmacia) and stained with Coomassie Blue. A calibration curve was drawn by plotting the positions of the markers (**A**): thyroglobulin (669 kDa, T), ferritin (440 kDa, F), catalase (232 kDa, C), aldolase (140 kDa, A) and BSA (67 kDa, B). The two bands found in the lane containing wt PCNA (**B**) are shown by ascending and the bands found in the lane containing PCNA Y114A by descending arrows respectively.

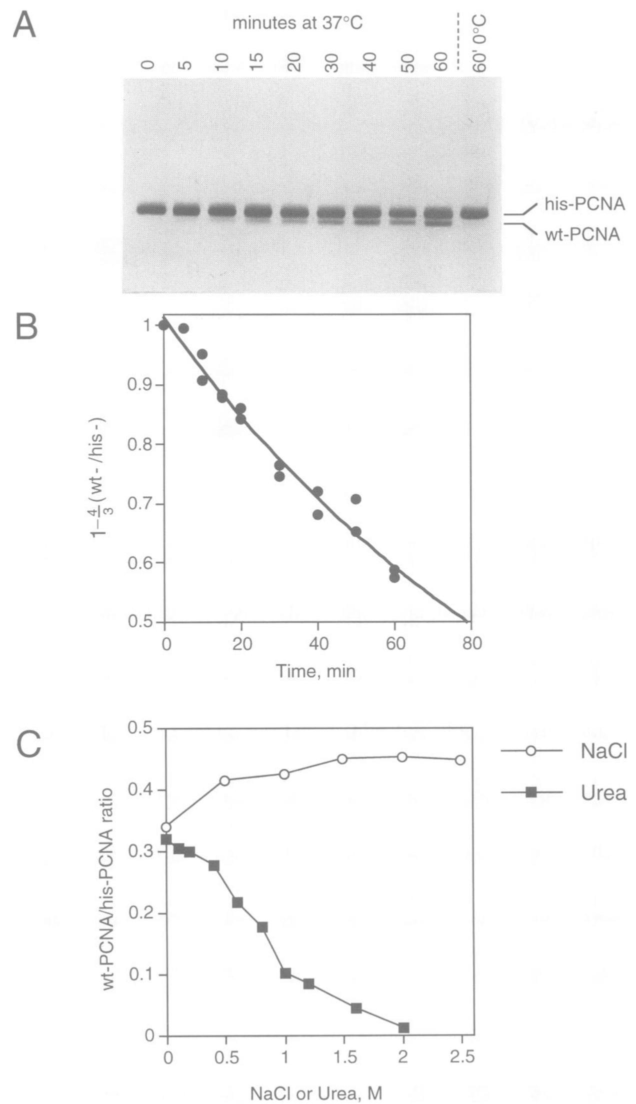

Fig. 6. Subunit exchange kinetics of wt PCNA with his-PCNA. wt PCNA and his-PCNA (3 µg each) were mixed in 50 µl buffer F and incubated for various times at 37°C. The proteins were then adsorbed onto Ni–NTA resin and processed as described in Materials and methods. (**A**) SDS–PAGE analysis of proteins retained by the Ni–NTA resin after incubation of reaction mixtures for various times at 37°C. (**B**) The amounts of wt PCNA were quantified by densitometry and normalized to the amounts of his-PCNA in the same lanes. The obtained values were amended by the value 4/3 (see Results) and plotted as a function of time. The kinetics were treated as a first order reaction using the program Cricket Graph III. (**C**) Stability of chimeric PCNA in the presence of salt or urea. wt PCNA and his-PCNA (30 µg each) were mixed in 0.5 ml buffer F and incubated for 1.5 h at 37°C. The proteins were then adsorbed onto Ni–NTA resin, washed twice with buffer G and the suspension of resin divided into 10 equal samples. The resin in the sample was washed twice with 400 µl buffer G containing increasing concentrations of NaCl or urea. The bound proteins were eluted with 50 µl buffer H and analysed by SDS–PAGE. The amounts of wt PCNA were quantified and normalized to the amounts of his-PCNA in the same lanes.

In order to test whether this interaction is specific we next developed a monomer exchange assay for PCNA. As described in Materials and methods, the assay is based on binding of his-PCNA subunits to Ni–NTA resin. When his-PCNA was incubated with wt PCNA at 37°C a slow exchange of subunits took place, leading to the formation of chimeric complexes. Importantly, no detectable exchange occurred when the two forms of PCNA were incubated together on ice for as long as 60 min (Figure 6A). This assay was used to measure the subunit exchange rate of PCNA. Figure 6A shows that increasing amounts of wt PCNA were co-adsorbed on Ni–NTA with his-PCNA upon incubation at 37°C. The amounts of wt PCNA co-adsorbed with his-PCNA after varying times of incubation were quantified on densitrometry of SDS gels and normalized to the amount of his-PCNA in the same lane. In theory the exchange mixture at equilibrium consists of wt PCNA homotrimer, 2 wt PCNA/1 his-PCNA, 1 wt PCNA/2 his-PCNA and his-PCNA homotrimers in a quantitative ratio of 1:3:3:1 respectively. Only the forms containing at least one subunit of his-PCNA can adsorb to the Ni–NTA resin and therefore the maximal amount of wt PCNA subunits in the adsorbed pool is 75% of the input. Taking this

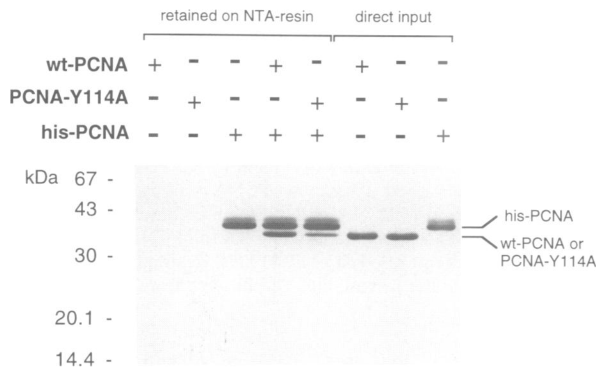

Fig. 7. Subunit exchange of wt PCNA or mutant PCNA Y114A with his-PCNA. wt PCNA or mutant PCNA Y114A as indicated was mixed with his-PCNA (3 μg each) in 50 μl buffer F and incubated for 1.5 h at 37°C. The proteins were then adsorbed onto Ni–NTA resin and processed as described in Materials and methods. The right-most three lanes contained 0.6 μg wt PCNA, PCNA Y114A or his-PCNA loaded directly onto the polyacrylamide gel and served as quantitative controls (direct input). The relative amounts of wt PCNA and PCNA Y114 retained on Ni–NTA by interaction with his-PCNA (lanes 4 and 5) were estimated by analysis of a digitized image of the dried gel using ImageQuant software (Molecular Dynamics) and were standardized for the amount of his-PCNA in the respective lanes.

into consideration the normalized amount of wt PCNA was amended with the coefficient 4/3 and plotted against time (Figure 6B). For the calculations the minor portion of PCNA present as dimer (Figure 4) was neglected. The exchange reaction fits first order kinetics, implying that a monomolecular reaction, presumably opening of the trimers, may be the rate limiting step of the exchange. From our data we obtained the value 80 min for the half-time of subunit exchange between wt PCNA and his-PCNA. It should be noted, however, that the addition of the poly(His) tag and adsorption to Ni–NTA may effect the PCNA oligomerization characteristics and the values thus obtained may differ from the *in vivo* situation. To further evaluate the nature of the interactions at the subunit interface we measured the exchange rate in the presence of varying salt, detergent and urea concentrations. The presence of NaCl (0.5–2 M) slowed down the exchange reaction, Nonidet P-40 (up to 0.5%) showed no effect and urea (0.2–0.6 M) increased the exchange of PCNA subunits (data not shown). A second experiment in which pre-exchanged his/wt PCNA chimeras bound to Ni–NTA were washed with various amounts of NaCl and urea showed that salt increases the stability of the trimers, whereas urea drastically reduced the amount of wt PCNA retained in complex with his-PCNA/Ni–NTA (Figure 6C). These results suggested that PCNA monomers are predominantly kept in the complex by hydrogen bonds. This is in agreement with assumptions based on the crystal structure of the PCNA trimer (Krishna *et al.*, 1994).

Interaction of PCNA Y114A with his-PCNA

Finally, the subunit exchange assay as described above was used to see whether the PCNA Y114A mutant can interact with his-PCNA. Neither wt PCNA nor PCNA Y114A showed any non-specific binding to Ni–NTA resin, but only bound to the resin in the presence of his-PCNA (Figure 7). The binding of PCNA Y114A was, however, ~60% less efficient than the binding of wt PCNA, as would be expected for a mutant incapable of proper trimer formation (Figure 7). Unless the unlikely circumstance that this single mutation caused extensive structural changes in both domains of the PCNA molecule occurred one of the monomer interfaces should not be affected by the mutation. The mutant protein should thus be able to form dimers with wt PCNA or his-PCNA or even higher order oligomers, such as open trimers with two of the intact subunits. This seems indeed to be the case, but apparently the stability of the closed trimer is such that the mutant protein cannot compete effectively for the binding sites with his-PCNA.

Discussion

The mechanism of the interaction of PCNA with pol δ, pol ε and RF-C has been studied extensively with purified proteins (reviewed in Hübscher *et al.*, 1995). *In vivo* effects of mutations in PCNA have been described in yeast (McAlear *et al.*, 1994) and *Drosophila* (Henderson *et al.*, 1994), but random or site-directed *in vitro* mutational analyses of the protein have not been described. Studies of the effect of a number of deletions in PCNA on its interaction with D-type cyclins (Matsuoka *et al.*, 1994) and on trimerization and binding to human auto-antibodies (Brand *et al.*, 1994) have been carried out. From these studies and the published structure of PCNA it is apparent that the deletion of a number of residues in any part of the protein except at its termini causes serious distortion of the tertiary structure and is thus of limited importance for analysis of protein–protein interactions. Proteins that bind to exposed residues at the N- and C-termini of PCNA, as was proposed for D-type cyclins (Matsuoka *et al.*, 1994), may be an exception. On the other hand, the resolution of the tertiary structure of PCNA greatly facilitated a mutational approach, since specific residues can be chosen to investigate certain aspects of the diverse functions of PCNA. Here we describe such an approach to analysing the trimerization of PCNA and the importance of trimerization for protein–protein interactions.

The Y114A mutation led to the formation of a protein that did not form trimers, as was seen by glycerol gradient centrifugation and native PAGE. Yet bacterially produced PCNA Y114A remains soluble and resolves into distinct bands on native PAGE. Our results showed that although the mutant protein was to some extent able to oligomerize with his-PCNA, it does so to a much lesser extent than wt PCNA. It is likely that the PCNA Y114A mutant is able to fold nearly correctly and that the differences it displayed compared with wt PCNA were caused by the inability to trimerize due to a local alteration at the monomer interface.

The mutant PCNA Y114A protein has no stimulatory effects on DNA synthesis by pol δ and cannot be loaded onto DNA by RF-C, even when present in a chimeric form with wt PCNA. These results were not surprising, considering the mode of PCNA action. The fact that PCNA Y114A did not compete effectively with wt PCNA for pol δ or with ph-PCNA for RF-C indicated that neither the pol δ core nor RF-C could recognize this protein in solution. Therefore, it is unlikely that RF-C has an important role in aiding trimer formation. A possible explanation for the inability of the mutant PCNA to compete for pol δ would be a low affinity of pol δ for

PCNA not loaded on DNA. A more general explanation would be that trimerization causes a change in the structure of the monomers leading to altered exposure of amino acids in some regions of the protein. The fact that the binding of many human auto-antibodies to PCNA does not tolerate deletions that inhibit trimer formation (Brand et al., 1994) is in agreement with this view. In the experiments described here we focused on the effect of trimerization on interaction with pol δ and RF-C, but it is likely that the binding of other proteins to PCNA likewise depends on the proper conformation of the PCNA trimers. In a preliminary study we found that the PCNA Y114A mutant protein did not interact with GST–p21 bound to glutathione–Sepharose, whereas wt PCNA is readily co-precipitated (Z.O.Jónsson and U.Hübscher, unpublished data).

Since trimerization of PCNA is so crucial for carrying out its physiological functions, we developed an assay to analyse the dissociation and reassociation of PCNA monomers into trimers. A PCNA derivative carrying a poly(His) fusion was constructed, allowing us to visualize and quantify the dynamics of PCNA trimerization. The results of these experiments provided an estimate of the half-time of subunit exchange in PCNA and some insight into the nature of the interaction between PCNA subunits. The half-time of subunit exchange of 80 min at 37°C showed that PCNA trimers are remarkably stable. Subunit exchange was not greatly affected by salt, but is markedly stimulated by urea, indicating that the subunits in PCNA trimers are mainly held together by hydrogen bonding, as would be expected from the structure of the PCNA trimer (Krishna et al., 1994). Interestingly, the structure of the β clamp of *E.coli* DNA pol III holoenzyme shows a number of ion pairs at the monomer interface (Kong et al., 1992), which might imply some differences in the mechanism of loading onto DNA. Nevertheless, the overall mechanism for clamp loading is likely similar in eukaryotes and bacteria, since the β subunit and PCNA have highly similar structures and the subunits of the clamp-loading *E.coli* γ complex share significant sequence homology with RF-C subunits (O'Donnell et al., 1993).

The extensive sequence conservation in eukaryotic PCNA proteins reflects the important role of PCNA in DNA replication and repair (reviewed in Hübscher et al., 1995). That a single mutation can completely inactivate the protein is, however, surprising. It might be possible that the consequences of a Y114A mutation *in vivo* could be somewhat less dramatic than the *in vitro* effects would suggest, since the adjacent S115P mutation in yeast causes only a cold-sensitive phenotype (Krishna et al., 1994). Our findings show the importance of PCNA trimerization for its function and interaction with pol δ and RF-C. The importance of trimerization should be considered when planning experiments to study the interaction of PCNA with other proteins, such as p21 and Gadd45.

Materials and methods

Nucleic acids

Poly(dA)$_{1000-1500}$ was from Sigma and oligo(dT)$_{12-18}$ from Pharmacia. Poly(dA)/oligo(dT) (base ratio 10:1) was prepared as described (Weiser et al., 1991). Gapped circular DNA was prepared according to Podust et al. (1994). Oligonucleotides for mutagenesis and sequencing of the PCNA gene were from Mycrosynth (Balgach, Switzerland). The plasmid pT7/hPCNA carrying the cDNA of human PCNA was kindly provided by B.Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and the plasmids pET19b and pET23a were from Novagen.

Enzymes and proteins

Calf thymus pol δ (Weiser et al., 1991) and RF-C (Podust et al., 1992) were isolated as described. Human wt PCNA was produced in *E.coli* using the plasmid pT7/hPCNA and purified as described (Fien and Stillman, 1992). *N*-Phosphorylatable wt PCNA (ph-PCNA) was produced and purified according to Podust et al. (1995). Restriction enzymes, T4 polynucleotide kinase, T4 ligase and T4 pol were from Boehringer.

Buffers

The following buffers were used: buffer A, 20 mM KPO_4, pH 7.8, 300 mM NaCl; buffer B, 25 mM Tris–HCl, pH 7.5, 10% (v/v) glycerol, 5 mM dithiothreitol (DTT), 1 mM EDTA; buffer C, 25 mM Tris–HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 0.01% (v/v) Nonidet P-40, 10 mM $NaHSO_3$, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin, leupeptin and pepstatin; buffer D, buffer C containing 10% (v/v) glycerol; buffer E, 25 mM Tris–HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol; buffer F, 40 mM Tris–HCl, pH 7.5, 0.2 mg/ml bovine serum albumin (BSA), 10 mM $MgCl_2$; buffer G, 30 mM imidazole–HCl, pH 7.2, 10% (v/v) glycerol; buffer H, 400 mM imidazole–HCl, pH 7.2, 10% (v/v) glycerol; buffer I, 20 mM Tris–HCl, pH 7.5, 1 mM DTT, 0.3 M NaCl.

Mutagenesis of human PCNA

The human PCNA gene was cloned from pT7/PCNA by PCR and ligated between the *Nde*I and *Bam*HI sites of pET23a (Novagen). The clone obtained (pET/PCNA) was sequenced to confirm the absence of PCR-induced mutations. The mutagenesis was performed by a modified version of the original method for second strand synthesis on uracil-containing ssDNA (Kunkel et al., 1987). Uracil-containing ssDNA was produced by co-infection of *E.coli* CJ236 (*dut-1, ung-1, thi-1, relA-1,* [pCJ105(Cm^r)]) with pET/PCNA and the helper phage M13KO7 (BioRad). After purification of the pET/PCNA ssDNA the complementary strand was synthesized using T4 pol, the mutagenic primer (5′-pCTT-CATTTCAGCGTCTGAAAC-3′) and a selection primer (5′-pA-CTGGTGAATATTCAACCAAG-3′). The latter changes a unique *Sca*I site in the vector *bla* gene to a *Ssp*I site, thus allowing selection of mutants by restriction analysis. Complementary strand synthesis was performed in a final volume of 20 μl containing 20 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM DTT, 1 mM ATP, 500 μM each of the four dNTPs, 0.3 pmol ssDNA template, 3 pmol mutagenic primer, 1 pmol selection primer, 5 U T4 pol and 2 U T4 DNA ligase. After selection in a Dut^+ Ung^+ *E.coli* strain plasmid DNA was isolated and incubated with *Sca*I to identify mutated clones. The PCNA gene insert of the resulting clone was sequenced to verify that only the desired mutation was present. A second clone containing a Ser residue instead of Tyr114 was also isolated (see Results).

Construction, expression and purification of his-PCNA

The original plasmid pT7/hPCNA contained the PCNA cDNA inserted in the single *Nde*I restriction site (Fien and Stillman, 1992). The *Nde*I restriction fragment containing PCNA cDNA was ligated into the plasmid pET19b (Novagen) digested with *Nde*I. The resulting plasmid encoded human PCNA containing 23 additional amino acids (Met-Gly-His$_{10}$-Ser-Ser-Gly-His-Ile-Asp$_4$-Lys-His-) prior to the first Met of the authentic PCNA. The *E.coli* strain BL21(DE3)pLysS was transformed with the pET/his-PCNA expression construct and grown at 37°C in 400 ml Luria broth to an A_{600} of 0.5. Isopropylthiogalactoside was added to a final concentration of 0.8 mM and growth was continued for 3 h. Cells were harvested by centrifugation and lysed by freezing/thawing in 30 ml buffer A. The mixture was sonicated to decrease the viscosity of the extract and centrifuged for 15 min at 25 000 *g*. The supernatant was mixed with 4 ml Ni–NTA resin (QIAGEN) and stirred for 1 h at 4°C. The resin was washed once with 30 ml buffer A containing 10% (v/v) glycerol and packed into a column. The column was washed with 40 ml buffer A and the proteins eluted with a 50 ml gradient of 0.01–0.5 M imidazole–HCl, pH 7.2 in 10% (v/v) glycerol. Fractions containing his-PCNA were detected by SDS–PAGE. his-PCNA eluted at 0.25–0.3 M imidazole–HCl. Pooled fractions were diluted 2.5-fold with buffer B and loaded onto a DEAE–Sepharose (Pharmacia) column (1.3×2.4 cm) equilibrated with buffer B. The column was washed with 30 ml buffer B containing 50 mM NaCl and the proteins eluted with a 40 ml gradient of 0.05–0.5 M NaCl in buffer B. The his-PCNA peak centred at 0.35 M

NaCl. These fractions were pooled and dialysed against 25 mM Tris–HCl, pH 7.5, containing 50 mM NaCl and 50% (v/v) glycerol. A preparation starting from a 400 ml culture yielded 7 mg purified his-PCNA.

PCNA Y114A expression and purification

The expression of PCNA Y114A was performed as for his-PCNA except that induction was carried out at 32°C. Cells from a 1 l culture were harvested by centrifugation and lysed by freezing/thawing in 40 ml buffer C. The mixture was sonicated, followed by centrifugation for 15 min at 25 000 *g*. The supernatant was loaded onto a 15 ml Q Sepharose column equilibrated in buffer D containing 0.1 M NaCl. The column was washed with 100 ml buffer D and the proteins eluted with a 200 ml gradient of 0.1–0.7 M NaCl in buffer D. Fractions containing PCNA Y114A were detected by SDS–PAGE. Pooled fractions were diluted 3-fold with buffer D and loaded onto a 4 ml hydroxyapatite column equilibrated with buffer D containing 0.1 M NaCl. The column was washed with 20 ml buffer D and the proteins eluted with a 50 ml gradient of 0–0.2 M KPO_4 in buffer D. The PCNA Y114A peak centred at 50 mM KPO_4. The fractions containing PCNA Y114A were pooled and kept in aliquots at –20°C. A preparation starting from a 1 l culture yielded 10 mg purified protein.

Construction, expression and purification of ph-PCNA–Y114A

A construct encoding phosphorylatable PCNA Y114A (ph-PCNA–Y114A) was made by substituting a fragment between the initial *Nde*I site and a unique, internal *Nco*I site of the PCNA gene in pET/PCNA-Y114A with the corresponding fragment from pT7/ph-PCNA (Podust *et al.*, 1995). Expression and purification of ph-PCNA–Y114A was as for PCNA Y114A with the addition of a Mono-Q chromatography step. Hydroxyapatite fractions containing ph-PCNA–Y114A (10 ml total) were pooled and loaded onto a 1 ml Mono-Q column HR-5/5 (Pharmacia) washed with 10 ml buffer E containing 0.1 M NaCl. Protein was eluted with a 30 ml 0.1–0.7 M NaCl gradient in buffer E and 0.5 ml fractions collected. The fraction eluting at 0.45 M NaCl contained the purest ph-PCNA–Y114A and was used for the experiments described in this paper.

PCNA exchange assay

Aliquots of 3 μg wt PCNA (or PCNA Y114A) and 3 μg his-PCNA were mixed in buffer F in a final volume of 50 μl. After incubation (for details see legends to Figures 6 and 7) the mixture was cooled on ice and mixed with 20 μl of a 50% slurry of Ni–NTA resin equilibrated in buffer F. The suspension was incubated at 4°C for 15 min with gentle stirring and the resin washed three times in batch with 400 μl ice-cold buffer G. The supernatant was carefully removed and the resin suspended in 50 μl elution buffer H. The eluted proteins were separated by 12.5% SDS–PAGE, stained with Coomassie blue and quantified using a densitometer (model 300A, Molecular Dynamics).

Glycerol gradient centrifugation

Preparations of PCNA and its derivatives were dialysed against buffer I containing 5% (v/v) glycerol. Samples (100 μl, containing 50–130 μg protein) were loaded onto a preformed 4 ml 15–30% (v/v) glycerol gradient in buffer I. After centrifugation at 55 000 r.p.m. in a TST 60.4 rotor at 4°C for 18 h fractions of 100 μl were collected from bottom to top and analysed by 12.5% SDS–PAGE.

Native gradient PAGE

Electrophoresis was performed in 8–25% polyacrylamide gels using the Phast System (Pharmacia) according to the manufacturer's protocol.

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