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The C-terminal region of *Schizosaccaromyces pombe* proliferating cell nuclear antigen is essential for DNA polymerase activity

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ABSTRACT **Proliferating cell nuclear antigen (PCNA), the processivity factor (sliding clamp) of DNA polymerases (Pols), plays essential roles in DNA metabolism. In this report, we examined the functional role of the C-terminal region of *Schizosaccaromyces pombe* PCNA both *in vitro* and *in vivo*. The deletion or Ala substitution of the last 9 aa (252–260A), as well as Ala replacement of only 4 aa (252–255A) at the C terminus, failed to substitute for the wild-type PCNA protein for cell growth in *S. pombe*. Two other PCNA mutant proteins, A251V and K253E, exhibited cold-sensitive phenotypes. Several yeast strains harboring mutations, including those at the acidic C-terminal region, showed elevated sensitivity to DNA damage. The ability of the mutant PCNA proteins to stimulate DNA synthesis by Polδ and Polε also was studied *in vitro*. The mutant proteins that did not support cell growth and a mutant protein containing a single amino acid substitution at position 252, where Pro is replaced by Ala, stimulated Polδ and Polε activities poorly. All mutant PCNA proteins, however, were assembled around DNA by the clamp loader, replication factor C, efficiently. Thus, the C-terminal region of PCNA is important for interactions with both Polδ and Polε and for cell survival after DNA damage. The C terminus of sliding clamps from other organisms has been shown to be important for clamp loading as well as polymerase interactions. The relationship between the conserved sequence in this region in different organisms is discussed.**

Proliferating cell nuclear antigen (PCNA) plays a critical role in nucleic acid metabolism and cell-cycle control (1, 2). PCNA, a ubiquitous protein with a highly conserved amino acid sequence, has been identified in all eukaryotes, a number of eukaryotic viruses, and in archaea (3). PCNA is the eukaryotic functional homologue of the β subunit of *Escherichia coli* DNA polymerase (Pol) III holoenzyme and of the phage T4 gene product (gp) 45. These proteins are the processivity factors of their respective Pols and are referred to as DNA sliding clamps. They are ring-shaped proteins (Fig. 1*A*) that encircle DNA and tether the polymerase to the DNA template for processive DNA synthesis (4). The clamps, however, cannot assemble themselves around DNA but are topologically linked to DNA by a protein complex referred to as the clamp loader in an ATP-dependent manner. The clamp loaders of PCNA (replication factor C, RFC), the *E. coli* β subunit (γ complex), and T4 phage gp45 (gp44/62 complex) are similar in function, overall oligomeric organization, and amino acid sequence (5, 6). It also has been suggested that the three clamp loaders have similar three-dimensional structure (7).

PCNA is an essential component of the DNA replication machinery. It also is required for DNA repair and recombination, and in several instances, PCNA has been shown to be involved in RNA transcription (1). In addition, PCNA was shown to interact with a large number of cellular proteins involved in cell-cycle regulatory processes (8). Unlike sequence-specific DNA interacting proteins, the DNA-loaded clamp can slide freely along double-stranded DNA bidirectionally (9). Because of this sliding property and its interactions with many cellular proteins, it has been proposed that PCNA may serve as a moving platform by which interacting proteins can scan duplex DNA for their sites of action (8).

The C-terminal region of the *E. coli* β clamp was shown to be important for binding the polymerase catalytic unit and the γ complex (10). In all species, the C-terminal portion of PCNA contains a stretch of acidic residues that includes 3-6 Glu or Asp residues adjacent to the C terminus (Fig. 1*B*). The three-dimensional structures of the budding yeast and human PCNAs indicate that this region forms a hook-like structure that protrudes from the ring surface with the side chains exposed to the solvent (11, 12) (Fig. 1*A*). This highly acidic region is unique to PCNA and is not present in β or gp45 proteins. However, there are several similarities between PCNA and β in the region preceding the acidic C terminus. In both proteins there is a positively charged amino acid followed by a hydrophobic residue as well as a Pro residue in this short amino acid stretch (Fig. 1*B*). Based on these observations and the structural similarities to the β subunit, it was proposed that the acidic stretch in PCNA could play a role in cell-cycle regulation events specific to eukaryotic cells while the preceding region could participate in the interaction with the polymerase and/or the clamp loader (1, 3).

This study describes a mutational analysis of the C-terminal region of *Schizosaccaromyces pombe* (sp) PCNA. Amino acid substitutions and deletions were used to determine the role of the C-terminal region in governing the various activities of PCNA. The mutant proteins were examined for their effects on DNA replication *in vitro*, and on growth and UV sensitivity *in vivo*. Mutations in the acidic stretch at the C terminus did not affect cell growth but rendered cells UV sensitive. Several mutations in the preceding region, however, were lethal. Furthermore, it is shown here that these mutations reduced the efficiency of the mutant PCNAs in supporting the Polδ and Polε holoenzyme replication activities without affecting the clamp-loading activity of RFC. Thus, this study demonstrates that the C-terminal region of PCNA, similar to its prokaryotic counterpart, plays an important role in clamp-polymerase interactions.

MATERIALS AND METHODS

Materials. Labeled dNTPs and rNTPs were obtained from Amersham Pharmacia. Unlabeled dNTPs were from Pharma-

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Abbreviations: Pol, DNA polymerase; RFC, replication factor C; h, human; sp, *Shizosaccharomyces pombe*; sc, *Saccharomyces cerevisiae*; PCNA, proliferating cell nuclear antigen; ss, single-stranded.

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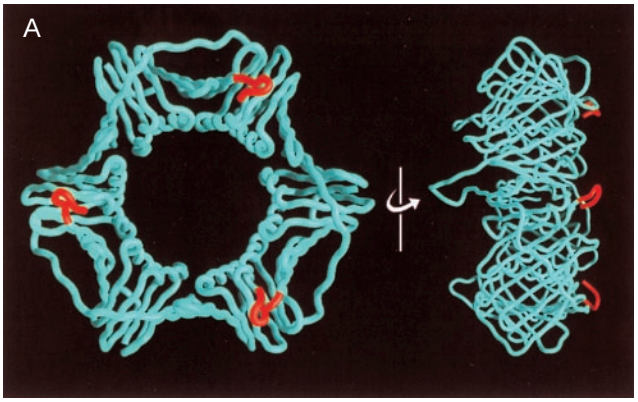

B

PCNA

	251								260
S. pombe	A	P	K	I	G	E	E	D	E E *
S. cerevisiae	A	P	K	F	N	D	E	E	*
Human	A	P	K	I	G	E	D	E	E G S *
X. laevis	A	P	K	I	E	D	E	E	A S *
D. melanogaster	A	P	K	I	E	D	N	E	T *

β subunit

E. coli	M	P	M	R	L	*
B. subtilis	L	P	V	R	T	Y *
P. putida	M	P	M	R	L	*
P. mirabilis	M	P	M	R	L	*
S. coelicolor	M	P	V	R	L	S G *

Consensus

PCNA:	A	P	-	K	I	D/E_{3-5}
β subunit:	M	P	M	R	L	

FIG. 1. The C-terminal region of PCNA. (*A*) Backbone trace representation of the polypeptide backbone of yeast PCNA. Two views of scPCNA are shown in which the C-terminal 7 aa are colored red. (*B*) Alignment of the C-terminal amino acid sequences of PCNA and the β subunit from different organisms. The consensus between the prokaryotic and eukaryotic clamps also is shown.

cia-LKB. Single-stranded (ss) M13 mp19 was from GIBCO/BRL. Poly(dA)$_{4500}$ was from Life Sciences (St. Petersburg, FL), and oligo(dT)$_{12–18}$ and *E. coli* ss-DNA binding protein were from Pharmacia-LKB. Bio-Gel A15 m resin was from Bio-Rad. Singly primed M13 ssDNA, poly(dA)$_{4000}$:oligo(dT)$_{12–18}$ (nucleotide ratio of 20:1) and singly nicked pBluescript vector DNA were prepared as described (13).

Preparation of Proteins. SpRFC and spPolδ were prepared as described (14). Human (h) Polε, a generous gift of Helmut Pospiech of Ouke University, Oulu, Finland, was a hydroxylapatite fraction isolated from HeLa cells as described by Syväoja and Linn (15), except that the ammonium sulfate precipitation step was omitted. The material was estimated to be 5–10% pure.

All spPCNA derivatives used here were cloned into the pQE9 vector (Qiagen, Chatsworth, CA) containing a six-histidine tag at the amino terminus. Wild-type and mutant PCNAs were prepared as described (16) with slight modifications. PCNA proteins were overexpressed by growing 2 liters of *E. coli* cells M15[pREP4] (Qiagen) harboring the different plasmids at 37°C in LB broth. When the culture reached an OD$_{600}$ of 0.7, protein expression was induced by incubating in the presence of 1 mM isopropyl β-D-thiogalactoside for 5 hr. Cell pellets were resuspended in 25 ml of loading buffer (5 mM imidazole/0.5 M NaCl/20 mM Tris·HCl, pH 7.5) and sonicated. After clarification by centrifugation for 20 min at 36,000 × *g*, the extract was mixed with 2 ml of Ni-chelate (Invitrogen) for 2 hr at 4°C with gentle shaking. After washing with 5 column vol of wash buffer (50 mM imidazole/0.5 M NaCl/20 mM Tris·HCl, pH 7.5), bound protein was eluted with 5 ml of elution buffer (0.5 M imidazole/0.5 M NaCl/20 mM Tris·HCl, pH 7.5). Proteins were dialyzed overnight at 4°C against 1 liter of buffer A (20 mM Tris·HCl, pH 7.5/100 mM NaCl/2 mM DTT/0.5 mM EDTA/10% glycerol). Protein concentrations were determined by Bradford assay (Bio-Rad) using BSA as the standard and stored at −70°C.

Wild-type and mutant spPCNA, containing a cAMP-dependent protein kinase recognition motif at the N terminus, were constructed by ligating an oligonucleotide encoding the kinase recognition motif to the *Bam*HI site of the various PCNA vectors. Proteins were purified as described above.

DNA Replication Assays. The replication of singly primed ssM13 DNA (9 fmol) or poly(dA)$_{4000}$:oligo(dT)$_{12–18}$ was carried out in reaction mixtures (20 μl) containing spRFC (0.1 unit, approximately 10 fmol) spPolδ (50 fmol), and spPCNA (at levels indicated in figure legends), as described (13). The RFC and ATP-independent replication of poly(dA)$_{4000}$:oligo(dT)$_{12–18}$ by Polδ was carried out in the presence of bicine-Tris buffer, pH 6.8 as described (14).

Replication of singly primed ssM13 DNA by hPolε was carried out in reaction mixture (10 μl) containing 25 mM Hepes buffer (pH 7.5), 0.5 mM DTT, 6 mM magnesium acetate, 200 μg/ml BSA, 6 fmol of singly primed ssM13 DNA, 250 ng *E. coli* ss-DNA binding protein, 150 mM sodium glutamate, 100 μM each of dTTP, dGTP, and dCTP, 25 μM of [α^{32}P]-dATP (8,000–10,000 cpm/pmol), 1 mM ATP, 0.1 unit of spRFC (or hRFC), 0.17 unit of hPolε [assayed with poly(dA):oligo(dT); ref. 15] and PCNA as indicated. Reactions were incubated at 37°C and then scored for DNA synthesis and the size of DNA products formed by alkaline agarose gel electrophoresis.

PCNA Loading Assay. PCNA containing a cAMP-protein kinase recognition site at its N terminus was phosphorylated with [γ^{32}P]-ATP as described (17). The spRFC-catalyzed loading of PCNA onto DNA was carried out in reaction mixtures (50 μl) containing 4% glycerol, 20 mM Tris·HCl (pH 7.5), 0.1 M NaCl, 8 mM magnesium acetate, 5 mM DTT, 40 μg/ml BSA, 0.5 pmol of singly nicked pBluescript vector DNA, 1 mM ATP, 1 unit of spRFC, and 1 pmol of ^{32}P-PCNA. After incubation at 37°C for 10 min, the reaction mixture was filtered through a 5-ml Bio-Gel A15 m (Bio-Rad) column at 4°C to resolve PCNA bound to DNA (eluted in the excluded volume) from free PCNA (eluted in the included volume). Eight drop fractions (240 μl) were collected and the presence of PCNA was quantitated by Cerenkov counting.

Construction of spPCNA Mutant Strains. Wild-type and mutant spPCNAs were cloned as a *Bam*HI–*Sal*I cassette into the expression vector pREP181 (LEU2, ars1$^+$) (16). The *S. pombe* diploid strain, MAP112 (*h*$^+$/*h*$^-$ *ade6*$^-$*M210/ade6-M216 leu-32/leu-32 ura4-D18/ura4-D18 his3-D1/his3-D1 pcn1*$^+$/*pcn1*Δ::*his3*$^+$), containing a complete deletion of one copy of the PCNA coding sequence by replacement with the *his3*$^+$ gene, was transformed with the expression plasmid pREP181 containing either wild-type (pREP181-pcna$^+$) or mutant PCNA (pREP181-mutant PCNA) and sporulated in medium lacking leucine and histidine. Leucine and histidine prototrophic haploids were selected. Haploid cells, containing the chromosomal Δpcna sustained by either the pREP181-pcna$^+$ or the various pREP181-mutant PCNAs, were analyzed.

Growth of spPCNA Mutant Strains. Fission yeast strains were grown as described (18). Edinburgh minimal medium was used as growth medium in all experiments with appropriate nutritional selection and cultures were grown to midlog at 30°C unless otherwise indicated. Cell number was determined by

hemocytometer count. For growth analysis, cell number was determined at defined time intervals, normalized to cell number at time zero and expressed as N/N_0.

Measurements of UV Sensitivity of spPCNA Mutants. UV sensitivity analysis was performed by plating a known density of midlog phase wild type and cells carrying the mutants PCNAs in duplicate onto appropriate agar plates, and exposing one set of the samples to UV irradiation with increasing doses by using a Stratagene Stratalinker. Irradiated and nonirradiated cells then were incubated at 30°C or 34°C for 3–4 days and surviving colonies were counted. Relative survival is expressed as the percent average number of colonies on UV-irradiated versus nonirradiated plates. A known UV-sensitive strain, rad26Δ (19), was included as a positive control.

RESULTS

The Importance of the C-Terminal Region of PCNA for Its Function *in Vivo*. Various amino acid substitutions and deletions at the C-terminal region of spPCNA were constructed. These mutants were examined for their ability to support cell growth and their effect on cell sensitivity to UV irradiation. Plasmids carrying the wild-type or mutant forms of PCNA were introduced into a yeast strain containing a null PCNA gene as described in *Materials and Methods*. As shown in Table 1, three of the mutants did not support cell growth whereas two others were cold sensitive. The nonviable mutants had major alterations at their C terminus; no single amino acid substitution resulted in a severe growth defect. Replacement of the last 9 aa in PCNA with Ala (252–260A, resulting in the presence of 10 Ala residues at the C terminus), or only 4 aa (252–255A, which precedes the acidic stretch) (Fig. 1*B*) abrogated its ability to support cell growth. Deletion of the last 9 aa also prevented the mutant PCNA from substituting for the wild-type protein. The latter mutant, however, when overexpressed in *E. coli*, was not soluble and readily aggregated. Thus, the inability of this mutant to support cell growth may be the result of misfolding within the yeast cell. Two mutants demonstrated a cold-sensitive phenotype (Table 1); the cells did not grow at 15°C. Each of these mutants contained a single amino acid substitution (A251V and K253E) located within the region encompassed by the nonviable mutant (252–255A). Interestingly, Ala substitution at position 253 had no effect on cell growth (Table 1) or on *in vitro* DNA synthesis catalyzed by Polδ and Polε (described below). Thus, a major charge alteration at this position is needed to detect an effect (cold sensitivity) on PCNA function. All other point mutants in the C terminus of PCNA supported growth under all conditions examined (Table 1).

Table 1. Summary of phenotypes of PCNA mutants

Mutation	Allele	Growth*	UV response†
Wild type	*pcn1*$^+$	+++	+++
A251V	*pcn1–29*	CS	+
P252A	*pcn1–32*	+++	+++/++
K253E	*pcn1–30*	CS	−
K253A	*pcn1–31*	+++	+
I254A	*pcn1–33*	+++	+++/++
G255A	*pcn1–34*	+++	+++/++
E257A	*pcn1–21*	+++	+++
D258A	*pcn1–22*	+++	+++
E259A	*pcn1–23*	+++	+++‡
E260A	*pcn1–24*	+++	++‡
EE259,260AA	*pcn1–25*	+++	++‡
252–260A	*pcn1–35*	Inviable	N/A
256–260A	*pcn1–36*	+++	+
252–255A	*pcn1–37*	Inviable	N/A
252–260Δ§	*pcn1–38*	Inviable	N/A

*Growth as determined at 34°C, 30°C, and 15°C. +++ = wild-type growth at all temperatures, CS = cold-sensitive growth at 15°C.
†+++ = wild-type resistance, ++ = intermediate, + = high, and − = severe defects in response to UV irradiation. N/A = not applicable.
‡Data from ref. 16.
§This PCNA mutant protein was not soluble when overexpressed in *E. coli*.

In addition to its role in chromosomal DNA replication, PCNA plays an important role in DNA repair (8). Therefore, the various mutants were analyzed for their effect on cell viability after UV irradiation. Three mutants that were most defective in response to DNA damage were the two cold-sensitive mutants (A251V and K253E; Table 1, Fig. 2) and a mutant in which the entire C-terminal acidic stretch was replaced by Ala (256–260A; Table 1, Fig. 2). Several other mutants showed various degrees of sensitivity to UV irradiation (Table 1, Fig. 2).

Mutations at the C-Terminal Region of PCNA Affect Polδ Holoenzyme Activity *in Vitro*. Purified spPCNA mutant proteins, overexpressed in *E. coli*, were analyzed for their ability to support processive DNA synthesis catalyzed by spPolδ. The activity of Polδ was studied by using several *in vitro* replication assays. These assays included the use of singly primed ssM13 DNA and poly(dA):oligo(dT) as templates in the presence of RFC. A third assay used poly(dA):oligo(dT) as template in the absence of RFC. The latter assay, which depends on the intrinsic threading and sliding properties of PCNA (20), examines the effects of PCNA on the polymerase without complications introduced by the activity of the clamp loader.

The mutant protein in which the last nine residues were replaced by Ala (252–260A) was not viable *in vivo* and also had the most dramatic effect on the *in vitro* Polδ replication activity in all three assays (Fig. 3, lanes 11 and 12). Other mutants had various effects on the activity of Polδ. By using singly primed ssM13 DNA as template, three mutants showed defects in supporting Polδ-catalyzed DNA synthesis. Two of these mutants contained substitutions of four and five residues in the C terminus (252–255A, Fig. 3, lanes 13 and 14 and 256–260A, Fig. 3, lanes 9 and 10), while in the other, a single amino acid substitution at position 252 was made [Pro is replaced by Ala (Fig. 3, lanes 1 and 2)]. This amino acid is located within the 252–255A mutant, suggesting that the Pro residue may play an important role in the stimulation of Polδ by PCNA. Furthermore, as shown in Fig. 3, the effect of these mutants appears to be on the stability of the polymerase clamp complex and/or on the formation of the complex, leading to the alteration of processivity of the polymerase. Although the total nucleotide incorporation was not dramatically reduced, the amounts of full-length product formed (7.25 kb) were reduced when the

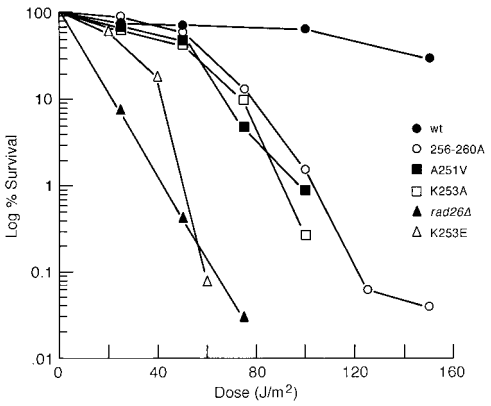

FIG. 2. Sensitivity of spPCNA mutants to UV irradiation. PCNA mutant strains were treated with increasing levels of UV, and survival rates were compared with those of the wild-type (wt) strain. A known UV-sensitive strain (rad26Δ) was used as a control.

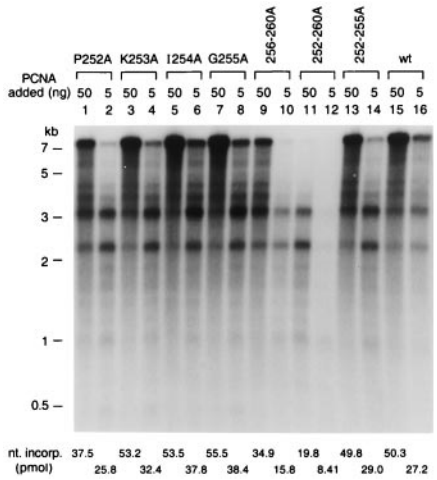

FIG. 3. Influence of spPCNA on the replication of primed DNA template by spPolδ. Replication of singly primed ssM13 DNA. Reaction mixtures (20 μl) were as described in *Materials and Methods* with the indicated levels of PCNA and 9.2 fmol of ssM13 DNA. wt, wild type.

reactions were performed with more limiting levels of PCNA (5 ng) (Fig. 3, lanes 2, 10, 12, and 14).

The PCNA mutants that showed reduced activity with singly primed ssM13 DNA also were analyzed by using poly(dA)$_{4000}$:oligo(dT)$_{12–18}$ as template. ssM13, as a "natural template," contains secondary structure. Thus, if a PCNA mutant was to bind weakly to the polymerase, the clamp and the polymerase could dissociate upon encountering hairpinned secondary structures present in the template DNA. To reduce this complication, we used poly(dA):oligo(dT), a homopolymer template devoid of secondary structure. The results obtained with poly(dA):oligo(dT) in the presence or absence of RFC (the latter assay carried out at pH 6.8), however, were similar to those found with singly primed ssM13 DNA (data not presented). The mutant in which the last 9 aa were replaced by Ala was the least active. The other three mutants (252–255A, 256–260A, and P252A) were less active compared with the wild-type protein but to different extents. Though less poly(dT) synthesis was observed, particularly with mutant PCNA 252–260A, the size of the products formed (between 0.2 and 1 kb, averaging ≈0.5 kb) were the same as those observed with wild-type PCNA. All other PCNA mutants examined in Fig. 3 were as active as the wild-type protein in the poly(dA):oligo(dT) assays (data not shown). These results suggest that the C-terminal mutant PCNAs are defective in formation of the Polδ-PCNA complex and upon encountering pause sites in natural templates are more prone to dissociate than the complex formed with wild-type PCNA.

Mutations at the C-Terminal Region of PCNA Do Not Affect Its Assembly Around DNA by RFC. The experiments described in Fig. 3 suggest that all PCNA mutants can be assembled onto DNA by RFC. This conclusion is based on the observation that in the presence of RFC all mutant proteins stimulated Polδ activity. In Fig. 4, an assay was used to analyze directly the loading efficiency of the various PCNA mutants by RFC. The loading of ^{32}P-labeled PCNA onto singly nicked pBluescript vector DNA by RFC can be followed after a sizing column step that separates ^{32}P-PCNA complexed with DNA from free ^{32}P-PCNA. The technique uses a gel-filtration column containing beads with large pores so that free proteins (^{32}P-PCNA) elute in the included fractions, resolved from proteins bound to the large plasmid DNA, which elutes in the excluded fractions. Based on the results described in Fig. 4, approximately similar amounts of PCNA were loaded onto the DNA

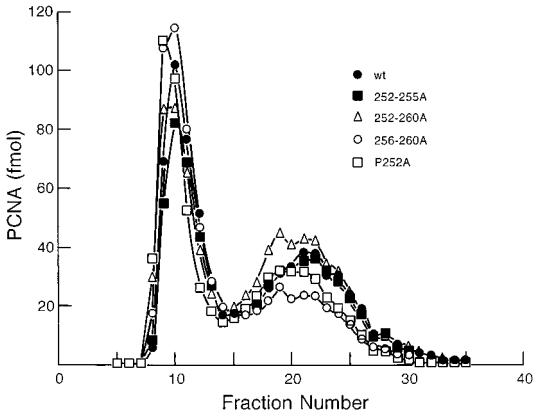

FIG. 4. The loading of spPCNA onto singly nicked pBluescript vector DNA by spRFC. Reaction mixtures (50 μl) were as described in *Materials and Methods*. wt, wild type.

with all PCNA mutants examined as judged by the levels of PCNA present in the excluded volume where DNA is eluted (fractions 8–14). Mutants that are not viable *in vivo* and showed reduced activity *in vitro* were loaded onto DNA as efficiently as wild-type PCNA.

The PCNA loading results support the conclusions derived from the Polδ replication assays (Fig. 3) that the C-terminal portion of PCNA is not essential for the interaction with RFC. Mutations in the C-terminal region of PCNA, even a major alteration such as replacement of the last nine residues by Ala, did not affect the ability of the PCNA mutants to be assembled around DNA.

Mutations at the C-Terminal Region of PCNA Affect Polε Activity *in Vitro*. Polδ is not the only polymerase stimulated by PCNA. The other polymerase essential for chromosomal DNA replication, Polε, also is stimulated by the sliding clamp (21). In contrast to Polδ, however, Polε is stimulated markedly by PCNA at elevated ionic strength (22). The following experiments were designed to determine whether the C-terminal region of PCNA is important for the stimulation of Polε activity. Because highly purified spPolε devoid of Polδ is not available, we used purified hPolε (15).

To determine whether hPolε can efficiently substitute for the corresponding *S. pombe* polymerase, we examined the stimulatory effects of spPCNA and spRFC on hPolε activity. All experiments were carried out in the presence of 0.15 M sodium glutamate, conditions under which hPolε was totally inactive in the absence of RFC and PCNA. As shown in Fig. 5*A*, in the absence of RFC and/or PCNA (lanes 9–11), no DNA synthesis was observed (<1 pmol of nucleotide incorporation). In the presence of spRFC and spPCNA (lanes 7 and 8), the activity of hPolε was dramatically increased. Furthermore, the loading of the spPCNA enabled the polymerase to replicate the entire M13 template (7.25 kb) (lane 7). These experiments demonstrate that a heterologous clamp and clamp loader (from *S. pombe*) can support replication by hPolε. Similar results were previously reported for the stimulation of calf (23) and hPolδ (unpublished observation) by spPCNA. The stimulatory effect by the sliding clamps in heterologous systems probably is the result of the relatively highly conserved structural and amino acids sequence similarities between PCNAs from the different organisms (3). However, the efficiency and processivity of the replication reactions with the heterologous system was lower than that observed in the completely homologous reaction, with hRFC and hPCNA (Fig. 5, lanes 1 and 2).

After establishing that spPCNA stimulated hPolε (Fig. 5*A*), the effect of the various spPCNA mutants on hPolε activity was examined. Several mutant PCNAs showed defects in their ability to stimulate this polymerase. The mutant in which the

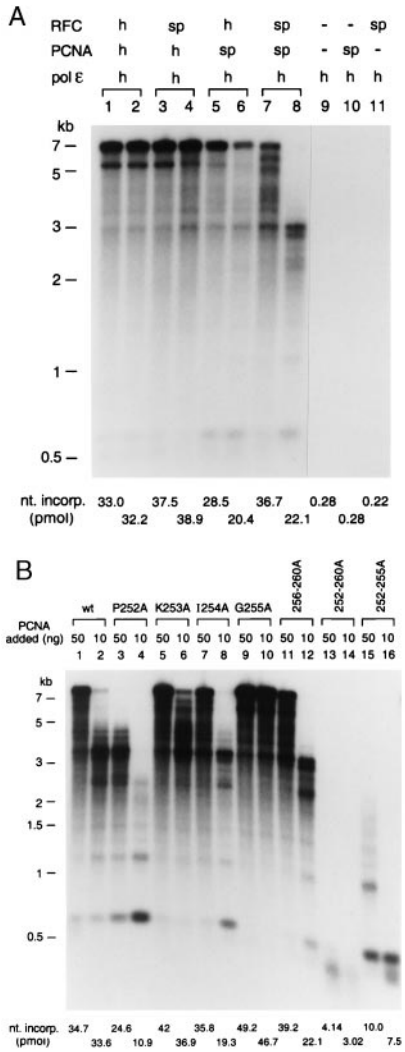

FIG. 5. Replication of singly primed ssM13 DNA by hPolε. (*A*) Replication in the presence of human and *S. pombe* RFC and PCNA. Reaction mixtures (10 μl) containing hPCNA or spPCNA, hPCNA or spRFC, and hPolε were incubated for 45 min at 37°C as described in *Materials and Methods*. The levels of PCNA added were 50 ng (lanes 1, 3, 5, 7, and 10) and 10 ng (lanes 2, 4, 6, and 8). All reactions contained 150 mM sodium glutamate. (*B*) Effect of spPCNAs on the spRFC-dependent replication of M13 DNA by hPolε. Reaction mixtures (10 μl), containing the indicated levels of spPCNAs, 0.1 unit of spRFC, 0.18 unit of hPolε, and 0.15 M sodium glutamate, were incubated at 37°C for 45 min. wt, wild type.

last 9 aa were replaced by Ala (252–260A), the least active with spPolδ (Fig. 3), was also the most defective in supporting replication with hPolε (Fig. 5*B*, lanes 13 and 14). Another spPCNA mutation that severely impaired the ability of the protein to support hPolε replication was 252–255A (Fig. 5*B*, lanes 15 and 16). These two mutants did not substitute for the wild-type protein *in vivo* (Table 1). Two additional mutant PCNAs (256–260A and P252A) showed moderate effects on hPolε activity (Fig. 5*B*, lanes 11 and 12 and lanes 3 and 4, respectively), whereas all other mutants had no major effect on Polε activity. The mutant PCNAs also were examined for their ability to support Polε-catalyzed synthesis of poly(dT) in the presence of poly(dA):oligo(dT) (Table 2). In keeping with the results obtained in the singly primed ssM13 DNA assay, reduced poly(dT) synthesis was observed with mutant 252–260A, 256–260A, 252–255A, and P252A. These results are in keeping with the defective formation of the Polε-PCNA complex with these C-terminal PCNA mutants.

Table 2. Effect of various PCNA proteins on hPolε activity with poly(dA)$_{4000}$:oligo(dT)$_{12\text{-}18}$ as template

PCNA added	dTMP incorp., pmol	
	50 ng	5 ng
Human	46.8	30.0
Sp wild type	39.5	18.4
P252A	21.1	8.61
256–260A	13.0	4.95
253–260A	2.32	1.65
252–255A	4.12	1.80

Reaction mixtures (15 μl), containing 25 mM Hepes (pH 7.5), 0.5 mM DTT, 200 μg/ml BSA, 7 mM magnesium acetate, 20 μM [α^{32}P]-dTTP (16,000 cpm/pmol), 2 mM ATP, 0.13 μg of poly(dA)$_{400}$:oligo(dT)$_{12\text{–}18}$ (20:1), 0.25 μg *E. coli* SSB, 0.2 M sodium glutamate, 0.1 unit of spRFC, and 0.034 unit of hPolε and PCNA as noted, were incubated at 37°C for 30 min. Aliquots were spotted on DE81 paper for processed for nucleotide incorporation. The omission of PCNA, RFC, or both PCNA and RFC resulted in the incorporation of 1.49, 1.11, and 0.99 pmol of dTMP, respectively. The above data have been corrected for dTMP incorporation observed in the absence of PCNA.

DISCUSSION

The DNA sliding clamps from different organisms have very similar three-dimensional structures (3, 4). It was noted, however, that PCNA, isolated from various eukaryotes, has a highly acidic C-terminal region not found in clamps from bacteria and T4 phage. Furthermore, the C-terminal region of PCNA forms a hook-like structure protruding out from the plane of the ring (Fig. 1*A*) (11, 12). This unique structure led to the hypothesis that the C-terminal acidic region could play a role in cell-cycle regulatory processes specific to eukaryotic cells (1, 3). Indeed, two known cell-cycle regulators that are up-regulated after DNA damage, p21 and Gadd45, have been shown to interact in part with the C-terminal acidic portion of PCNA (12, 24). Here we show that cells containing a mutant PCNA protein in which the C-terminal 5 aa of spPCNA were replaced by Ala, as well as other mutant proteins containing single amino acid substitutions in this region, exhibit elevated sensitivity to UV irradiation. This observation supports the idea that the C terminus of PCNA plays a role in cellular response to DNA damage. This region, however, does not appear to be important for cell viability. Mutant proteins in which the last five residues were replaced by Ala, as well as those that contained single amino acid replacements, substituted efficiently for the wild-type protein. The mutant in which the last 5 aa were replaced by Ala, however, showed some reduced activity with Polδ and Polε *in vitro*.

The alignment of the amino acid sequences of bacterial (β subunit) and eukaryotes (PCNA) clamps reveals little sequence identity (3). Therefore, it was interesting to note that the C-terminal region preceding the acidic stretch in PCNA, contains some sequence identity to the bacterial β subunit (Fig. 1*B*). This similarity includes the presence of a Pro residue, a positively charged residue (Arg or Lys) as well as a hydrophobic amino acid (Ile or Leu). In the *E. coli* system, the C-terminal 5 aa are essential for the interactions of β with the γ-complex and Pol III; β containing Ala substitutions in this region did not stimulate the *in vitro* activity of Pol III (10). The similarities in the C-terminal regions of PCNA and β suggest that this domain may be involved in the interactions between PCNA and RFC and/or Polδ and Polε. For these reasons we studied whether the C terminus of PCNA is essential for cell viability *in vivo* and/or involved in clamp loading and polymerase activities *in vitro*.

PCNAs containing Ala in place of the last 9 aa, as well as Ala replacement in the four residues similar to the bacterial clamp, did not support cell growth. Deletion of the C-terminal 9 aa

also abolished cell viability. However, because this mutant PCNA was insoluble when expressed in *E. coli*, and thus may not be soluble in yeast cells, this observation is uninterpretable. Deletion of the C-terminal region in hPCNA also was reported to form an insoluble protein (25).

These mutated PCNAs were examined for their ability to support *in vitro* replication reactions catalyzed by Polδ and Polε. Before interacting with the polymerase, the clamp has to be assembled around DNA by the clamp loader in a reaction that uses ATP hydrolysis. All mutant PCNAs studied were loaded onto DNA with similar efficiency, suggesting that the C-terminal region is not involved in RFC binding. This observation is consistent with previous studies in which mutations at the C-terminal region of *Saccharomyces cerevisiae* (sc) PCNA did not affect its ability to stimulate the ATPase activity of scRFC (26). In humans, however, removal of the C terminus of hPCNA reduced its stimulation of hRFC ATPase activity (25). However, other indications that the C-terminal region of PCNA is not involved in clamp loading come from the observation that p21, which interacts with the C terminus of hPCNA, did not dramatically reduce the ability of PCNA to be assembled around DNA (27, 28).

Several mutant spPCNAs were defective in stimulating the activity of spPolδ and hPolε. The most severe effect was observed with the protein in which the last 9 aa residues were substituted by Ala (252–260A). This low activity is likely to contribute to its inability to substitute for the wild-type PCNA *in vivo*. The other mutant protein not viable *in vivo* was the PCNA 252–255A. Although this mutant showed a reduced activity with Polδ, it was not much different from other mutants (e.g., P252A and 256–260A), which were viable *in vivo*. PCNA 252–255A, however, showed low activity with Polε, suggesting that its effect *in vivo* may be the result of its limited stimulation of this polymerase. The P252A mutant, although viable, showed reduced activity with both Polδ and Polε. No effect on Polδ activity was observed for any other PCNA mutant that contained a single amino acid substitution in this region. Ala substitution of two residues in scPCNA corresponding to the P252 and K256 in spPCNA (Fig. 1*B*) was drastically defective in replication with scPolε as well as showing reduced ability to stimulate scPolδ (26). Similar observations have been reported with hPCNA in the stimulation of hPolε (29). On the other hand, a single amino acid substitution in hPCNA, in which the corresponding Lys was replaced by Ala, had no effect on hPolδ activity (25). Taken together, these observations suggest that the Pro residue at the C terminus plays an important role in the interactions between PCNA and the polymerases. Interestingly, in the *E. coli* β subunit, the substitution of Ala for a Pro in a position corresponding to Pro-252 in spPCNA (Fig. 1*B*), severely reduced the ability of β to stimulate the activity of Pol III (10).

Although the C-terminal region of PCNA plays a role in Polδ stimulation, it is not the only region to which Polδ binds. It has been well established that the interconnector loop between the two globular domains within each PCNA monomer is a major site of interaction with Polδ (30). Polδ, however, is a multisubunit complex with several subunits participating in the interaction and stimulation by PCNA. Thus it is likely that more than one region of PCNA will be involved in the interaction with the polymerase. Similarly, Polε is also a multisubunit polymerase but the subunit(s) that bind PCNA currently is unknown. The identification of the C-terminal region as an important binding site for Polε (ref. 26; this study) may help to identify these subunits.

In this study, we have focused on the role of the C-terminal region of PCNA in governing the activities of Pols and the clamp loader. PCNA also interacts and regulates the activity of other proteins involved in many DNA transactions (8). These PCNA-interacting proteins include FEN-1, DNA ligase 1, DNA methyltransferase, CAF-1, and many others. The conserved C terminus, with its unique hook-like structure, may be important for the interaction with these and other factors. If these proteins indeed interact with the C-terminal region of PCNA, mutations in this domain may contribute to the effects observed *in vivo* in *S. pombe*.

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