Schizosaccharomyces pombe Proliferating Cell Nuclear Antigen Mutations Affect DNA Polymerase δ Processivity

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We introduced nine site-directed mutations into seven conserved fission yeast proliferating cell nuclear antigen (PCNA) residues, Leu2, Asp63, Arg64, Gly69, Gln201, Glu269, and Glu260 to Ala to enable to stimulate DNA synthetic activity and to enhance the processivity of calf thymus DNA polymerase δ holoenzyme similar to calf thymus PCNA. Mutations of Leu2 to Val or Arg64 to Ala, either singly or as a double mutant, yielded PCNA mutant proteins that had reduced capacity in enhancing the processivity of DNA polymerase δ but showed no deficiency in stimulation of the ATPase activity of replication factor C. S. pombe strains sustained by these two mutant-pcna alleles had moderate defects in growth and displayed elongated phenotypes. These cells, however, were not sensitive to UV irradiation. Together, these in vitro and in vivo studies suggest that the side chains of Leu2 and Arg64 in one face of the PCNA trimer ring structure are two of the several sites involved in tethering DNA polymerase δ for processive DNA synthesis during DNA replication.

Proliferating cell nuclear antigen (PCNA) is a multifunctional protein in the cell. It was originally identified as the auxiliary protein for DNA polymerase δ during DNA replication (1, 2). In vitro, it is able to enhance the DNA synthetic processivity of both DNA polymerases δ and ε (3, 4) and is essential for synthesizing full-length SV40 DNA replication products (5–11). PCNA has also been found to be required for DNA excision repair and to be an essential component of the in vitro reconstituted DNA repair system (12–14). Furthermore, PCNA has been shown to associate with D-type cyclins and to interact with the cyclin kinase inhibitor p21. The interaction between PCNA and p21 leads to inhibition of DNA replication in vitro (15–18), and this interaction has been shown to specifically inhibit the repair of DNA damage caused by either alkylating agents or by ultraviolet radiation (19). PCNA has also recently been found to physically bind flap endonuclease I, which is a 5′-flap DNA endonuclease and a nick-specific 5′-exonuclease (20), and to stimulate flap endonuclease I activity. This interaction is thought to be important for lagging strand synthesis and is implicated in a broad range of DNA metabolism in which the flap endonuclease I nuclease family is involved (21).

The β-subunit of Escherichia coli DNA pol III holoenzyme is a functional homologue of PCNA (2, 22, 23). Budding yeast PCNA and the β-subunit of E. coli DNA pol III holoenzyme are structurally similar, one exception being that the β-subunit is composed of three globular domains forming a dimeric closed ring, whereas PCNA is composed of only two domains, thus forming a trimeric six-domain ring with a central cavity large enough to encircle DNA (24, 25). Structural data have suggested that PCNA forms a homotrimer torus that encircles DNA and interacts with DNA polymerase δ, tethering it for processive DNA synthesis. These structural data also suggest that the protein loops between the six domains are important for interacting with other proteins for various DNA metabolic processes (24, 25).

There is substantial conservation in the primary sequences of PCNA proteins from human, mouse, rice, Drosophila, and budding and fission yeast (reviewed in Ref. 26). Double alanine scan mutagenesis of charged amino acid residues and random mutagenesis of budding yeast PCNA (POL30) have yielded varying degrees of growth defects and DNA damage sensitivity in vivo and defects in protein folding and protein-protein interactions in vitro. The pol30 mutant phenotypes have indicated distinct roles of budding yeast PCNA in DNA replication and repair (27). In addition, structure-function analysis of human PCNA has recently been described. Several amino acids were identified to be involved in stimulation of replication factor C (RF-C) ATPase activity or in enhancing the processivity of DNA synthesis by polymerase δ in vitro. Results of the human PCNA study suggest that some residues on the outer surface of PCNA function in protein-protein interactions (26).

To test if conservation of several residues in the protein loop regions of PCNA are essential for biological function, we isolated the gene and cDNA of fission yeast PCNA and overexpressed functionally active recombinant PCNA protein in bacteria. In this study, we analyzed nine site-directed mutations in seven conserved residues of fission yeast PCNA for their function in enhancing DNA polymerase δ processivity in vitro and their biological effects in vivo.

EXPERIMENTAL PROCEDURES

Isolation of Schizosaccharomyces pombe PCNA Gene and cDNA—Degenerate oligonucleotides were generated based on conserved sequence regions of PCNA from human, mouse, and budding yeast and used as polymerase chain reaction primers to amplify fission yeast genomic DNA. The resulting polymerase chain reaction products
were used to screen a genomic and cDNA library of *S. pombe* (28, 29). An EcoRI genomic fragment containing the entire gene of PCNA was isolated, and a full-length cDNA clone was obtained from multiple overlapping cDNA fragments.

A full-length streptococcal fragment containing the M13 origin and named pMP1. This construct was used for generating site-directed mutants of *S. pombe* PCNA. A BamHI-Sall fragment containing either the wild type or mutant cDNAs as cassettes from site-directed mutagenesis reactions was constructed into pQE9, which contains a six-histidine tag (His6) at the amino terminus, and plasmids named pQE9x. An expression vector pREP181 (LEU2, Ars1) was constructed into the bacterial expression vector pQE9 and the yeast shuttle plasmids named pQE9/x. An expression vector pREP181 (LEU2, Ars1) was constructed into the expression vector pREP181 for *in vivo* expression of protein. Cell cultures (500 ml) were grown in 2 l HI-N media and 200 g/ml bovine serum albumin, 1 M MgCl2, 8 mM MgCl2, 0.2 mg/ml bovine serum albumin, 1 mM dithiothreitol, 50 mM NaCl, 100 ng of single primed M13 mp18 DNA, poly(dA)300-oligo(dT)16 (20:1 nucleotide ratio), 0.3 units of purified calf thymus polymerase (1), and PCNA wild type or mutant protein as indicated were incubated at 37°C for 20 min. Reactions were terminated by a PhosphorImager (Molecular Dynamics).

**Proteins—** Proteins—200 μg of wild type or mutant PCNA proteins purified by Ni2+–nitrilotriacetic acid affinity column chromatography from pQE9/PCNA or pQE9x transformed M15 cell lysates was applied onto a Superdex 200 HR10/30 gel filtration column. Fractions of 500 μl were collected and the absorbance at 280 nm and OD260 was monitored. Reactions were monitored until the dNTP concentration reached 80% of the initial concentration. Fractions of 1 ml of 0.5 M LiCl/1 M formic acid. Conversion of ATP to ADP was quantitated by an isopropyl-1-thio-β-D-galactopyranoside assay (36, 37). The reaction mixture (10 μl) contained 40 mM Bis-Tris, pH 6.8, 2% glycerol, 40 μg/ml bovine serum albumin, 1 mM dithiothreitol, 6 mM MgCl2, 40 μM [α-32P]dUTP (320 cpmp/μmol), 200 nM poly(dA)20-digoldT16 (20:1 nucleotide ratio), 0.3 units of purified calf thymus polymerase (1), and PCNA wild type or mutant protein as indicated were incubated at 37°C for 20 min. Reactions were terminated by the addition of 1 ml of 20 mM sodium pyrophosphate and 5 mg/ml carrier DNA. Acid-insoluble radioactive DNA products were quantitated (35).

**Processivity Measurement—** The processivity of calf thymus polymerase was measured in the presence of calf thymus PCNA and wild type or mutant S. pombe PCNA was measured on poly(dA)20-digoldT16 using the DNA trap method (36, 37). The reaction mixture (50 μl) contained 100 mg/ml bovine serum albumin, 0.2 M MgCl2, 200 μM poly(dA)20-digoldT16 (20:1 nucleotide ratio), 0.3 units of purified calf thymus DNA polymerase δ, and 0.2 μg of either wild type or mutant PCNA. Reactions were finished by incubation with dTTP for 1 min at room temperature, and DNA was subsequently added. Included as controls for each set of incubations were reactions with no dTTP substrate added, reactions with dTTP substrate but no DNA trap added, and reactions with dTTP substrate and DNA trap added simultaneously. The processivity was measured as the mean number of residues added by a DNA polymerase δ to a primer terminus per cycle of nucleotide addition as described in Refs. 36 and 37.

**ATPase Assay—** The assay for RF-C ATPase was described as (38) with minor modification. The reaction mixture (30 μl) contained 400 mM Tris-HCl, pH 7.8, 8 mM MgCl2, 2 mm MgCl2 bovine serum albumin, 1 mM dithiothreitol, 50 mM NaCl, 10 ng of single primed M13 mp18 DNA, 850 ng of E. coli SSB, 100 μM [α-32P]ATP, and PCNA as indicated. Reactions were started by the addition of 25 ng of S. pombe PCNA and incubated at 37°C for 30 min. Reactions were terminated by the addition of 10 μl of 50 mM EDTA and 1% SDS. Products were analyzed on POLYGRAM cel 300 polyethyleneimine thin-layer chromatography in 0.5 M LiCl/1 M formic acid. Conversion of ATP to ADP was quantitated by a PhosphorImager (Molecular Dynamics).

**Microscopy—** Cells were ethanol fixed and stained with DAPI for microscopic analysis as described (39, 40).

UV Radiation Measurements—UV sensitivity analysis was performed by plating a known density of mid log phase cells onto appropriate agar plates and exposing cells to UV irradiation at different

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**Table I**

| Strain Genotype Source | S. pombe strains used in this study |
|------------------------|-----------------------------------|
| KG2 h – ade6-M216 leu1–32 ura4-D18 his3-D1 | This study |
| KG3 h – ade6-M216 leu1–32 ura4-D18 his3-D1 | This study |
| rad7D LEU2 | A. M. Carr |
| KG23 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/his3-D1 | This study |
| MAP112 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/pnc1A::his3+ | This study |
| MAP200 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/pnc1A::his3+ | This study |
| MAP201 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/pnc1A::his3+ | This study |
| MAP205 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/pnc1A::his3+ | This study |
| MAP206 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/pnc1A::his3+ | This study |
| MAP226 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/pnc1A::his3+ | This study |
| MAP300 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/pnc1A::his3+ | This study |
| MAP301 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/pnc1A::his3+ | This study |
| MAP302 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/pnc1A::his3+ | This study |
| MAP303 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/pnc1A::his3+ | This study |
| MAP304 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/pnc1A::his3+ | This study |
| MAP305 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/pnc1A::his3+ | This study |
| MAP306 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/pnc1A::his3+ | This study |
| MAP326 h – ade6-M216/ade6-M210 leu1–32/leu1–32 ura4-D18/ura4-D18 his3-D1/pnc1A::his3+ | This study |
were introduced into 7 conserved residues, Leu2, Asp63, Arg64, of the PCNA molecule, whereas the side chain of Asp63 is due, Phe, instead of Leu is located at this position. Asp63 and rice. In mammalian cells and served among PCNA proteins of fission and budding yeast and rice. In mammalian cells and Drosophila, a hydrophobic residue, Phe, instead of Leu is located at this position. Asp63 and Arg64 are located in the protein loop between β-sheets βE1 and βF1 of budding yeast PCNA (25). Asp63 and Arg64 are conserved among PCNAs of mammalian cells, rice, Drosophila, and fission yeast, whereas budding yeast has a polar residue, His, in place of the charged residue, Arg. Gly69 is not located in a protein loop region but is in β-sheet βF1. Gly69 is highly conserved among PCNAs of all species. Mutation of this residue in budding yeast PCNA to Asp has been shown to suppress cold-sensitive alleles of CDC44, which encodes the large subunit of budding yeast replication factor C (41). Thus, Gly69 of S. pombe PCNA was selected for similar mutation to Asp to test the mutational effect. Gly69 is conserved only between budding and fission yeast. PCNA from most other species has the similar size residue Glu instead of Gln at this position. Gly69 is located in the second domain of the protein loop between βE2 and βF2. Glu259 and Glu260 are located at the carboxyl terminus. Leu2 was conservatively changed to Val, and Gly69 was changed to Asp like that in budding yeast (41), whereas Asp63, Arg64, Gly201, Glu259, and Glu260 were all mutated to Ala to abolish both the length and charge of the side chains in order to test their function. A double mutation of the two adjacent charged residues Glu259 and Glu260 was generated to test the combinational charge effect of these two carboxyl terminus residues. The three-dimensional structure of budding yeast PCNA indicates that residues Leu2, Asp63, and Arg64 are located in one region of the PCNA homotrimer. The side chains of Leu2 and Arg64 are oriented toward the outer rim of one face of the PCNA molecule, whereas the side chain of Asp63 is orientated in a different direction (Fig. 1). Thus, a double mutant L2V/R64A was also generated to test the combinational effect of mutation of both Leu2 and Arg64.

RESULTS

Mutation of S. pombe PCNA—Nine site-directed mutations were introduced into 7 conserved residues, Leu2, Asp63, Arg64, Gly69, Gln201, Glu259, and Glu260 of S. pombe PCNA. The location of these seven residues in the PCNA structure are depicted in Fig. 1. Leu2 is located at the amino terminus and is conserved among PCNA proteins of fission and budding yeast and rice. In mammalian cells and Drosophila, a hydrophobic residue, Phe, instead of Leu is located at this position. Asp63 and Arg64 are located in the protein loop between β-sheets βE1 and βF1 of budding yeast PCNA (25). Asp63 and Arg64 are conserved among PCNAs of mammalian cells, rice, Drosophila, and fission yeast, whereas budding yeast has a polar residue, His, in place of the charged residue, Arg. Gly69 is not located in a protein loop region but is in β-sheet βF1. Gly69 is highly conserved among PCNAs of all species. Mutation of this residue in budding yeast PCNA to Asp has been shown to suppress cold-sensitive alleles of CDC44, which encodes the large subunit of budding yeast replication factor C (41). Thus, Gly69 of S. pombe PCNA was selected for similar mutation to Asp to test the mutational effect. Gly69 is conserved only between budding and fission yeast. PCNA from most other species has the similar size residue Glu instead of Gln at this position. Gly69 is located in the second domain of the protein loop between βE2 and βF2. Glu259 and Glu260 are located at the carboxyl terminus. Leu2 was conservatively changed to Val, and Gly69 was changed to Asp like that in budding yeast (41), whereas Asp63, Arg64, Gly201, Glu259, and Glu260 were all mutated to Ala to abolish both the length and charge of the side chains in order to test their function. A double mutation of the two adjacent charged residues Glu259 and Glu260 was generated to test the combinational charge effect of these two carboxyl terminus residues. The three-dimensional structure of budding yeast PCNA indicates that residues Leu2, Asp63, and Arg64 are located in one region of the PCNA homotrimer. The side chains of Leu2 and Arg64 are oriented toward the outer rim of one face of the PCNA molecule, whereas the side chain of Asp63 is orientated in a different direction (Fig. 1). Thus, a double mutant L2V/R64A was also generated to test the combinational effect of mutation of both Leu2 and Arg64.

Wild Type and Mutant PCNAs Can Form a Homotrimer Ring—Wild type and mutant PCNA proteins were expressed in E. coli and affinity purified to near homogeneity. Although the cdNA of S. pombe PCNA has a predicted molecular mass of 29 kDa, the recombinant PCNA resolves as a 36-kDa protein in SDS gel analysis. The purified proteins were further verified by immunoblotting with antibodies against S. pombe PCNA (Fig. 2). To test if the wild type or mutant PCNA proteins had any significantly physical alterations, these were analyzed by gel filtration for trimer formation. The recombinant wild type PCNA protein and all of the PCNA mutant proteins eluted mainly as trimers with a molecular mass of 90 kDa with minor quantities of the protein as monomer with a molecular mass of 29 kDa (Fig. 3, upper panel). SDS gel analysis of gel filtration fractions of the wild type and mutant PCNA proteins L2V, D63A, R64A, L2V/R64A, Q201A, E259A, E260A, and E259A/E260A showed the majority of protein in homotrimer form with the peak fraction of the protein eluting between fractions 27 and 29 (Fig. 3, lower panel, and data not shown). These results strongly suggest that the mutations introduced into these invariant residues either as single or as double mutants have no significant effect on homotrimer formation of the PCNA protein. Furthermore, all these eight PCNA proteins were expressed with a his-tag at the amino terminus. The ability of these PCNA proteins to form the homotrimer ring in solution strongly suggests that the amino-terminal his-tag does not affect the homotrimer ring formation (Fig. 3).

Results

Mutations L2V, R64A, and L2V/R64A Have Reduced Ability to Stimulate DNA Polymerase δ—The ability of heterospecies PCNA to stimulate calf thymus DNA polymerase δ in vitro has been previously demonstrated (42–44). Using poly(dA)-oligo(dT) as primer template, in vitro DNA synthetic activity of the highly purified two-subunit calf thymus DNA polymerase δ holoenzyme was analyzed either in the absence of PCNA, with calf thymus PCNA, or with wild type S. pombe PCNA. Wild type S. pombe PCNA was able to stimulate calf thymus DNA polymerase δ similar to calf thymus PCNA (Fig. 4A). Mutants D63A, Q201A, E259A, and E260A and the double mutant E259A/E260A were all able to stimulate DNA synthesis by polymerase δ at levels similar to that of wild type S. pombe.
PCNA (Table II). In contrast, mutants L2V and R64A showed approximately 50% of the wild type PCNA’s ability to stimulate DNA polymerizing activity of polymerase δ. The double mutant, L2V/R64A, had synergistic effects resulting in less than 10% of the wild type PCNA’s ability in stimulating DNA polymerizing activity of polymerase δ (Fig. 4B and Table II). The deficiency of mutant PCNAs L2V, R64A, and L2V/R64A to stimulate DNA polymerase δ is specific to Leu2 and Arg64 in this particular region of PCNA because mutant D63A, despite being a highly conserved residue adjacent to Arg64 and located in the same protein loop, had wild type PCNA capacity in stimulating DNA polymerase δ activity (Table II). These results suggest that the side chains of Leu2 and Arg64 but not the side chain of Asp63 are specific in stimulating the DNA synthetic activity of DNA polymerase δ. Lastly, mutant G69D was unable to stimulate DNA synthetic activity of polymerase δ beyond that of the holoenzyme alone. The abilities of each PCNA mutant to stimulate polymerase δ DNA synthetic activity are summarized in Table II.

Mutants L2V, R64A, and L2V/R64A Have Reduced Capacity to Enhance DNA Polymerase δ Processivity but Are Not Deficient in Stimulating the ATPase Activity of RF-C—The processivity of DNA polymerase δ was measured to test whether the deficiency of PCNA mutants L2V, R64A, and L2V/R64A to stimulate polymerase δ DNA synthesis activity was due to their inability to enhance DNA polymerase δ processivity. Using poly(dA)300-oligo(dT)16 as primer template, polymerase δ processivity was analyzed by a DNA trap method either in the absence of PCNA or in the presence of calf thymus PCNA, wild type S. pombe PCNA, or S. pombe mutant PCNAs L2V, R64A, L2V/R64A, and D63A (Fig. 5). In the absence of added PCNA, DNA polymerase δ incorporated merely 3 nucleotides per binding of the primer terminus. In the presence of calf thymus PCNA, polymerase δ was able to incorporate >30 nucleotides per binding event of the primer terminus. With wild type S. pombe PCNA, DNA polymerase δ showed a processivity of >20 nucleotides. In contrast, with mutant L2V, polymerase δ showed a greater than 50% reduction of its processivity as compared with the wild type PCNA with only 9 nucleotides incorporated per primer binding event. With mutant R64A, DNA polymerase δ had a 25% reduction in its processivity incorporating 15 nucleotides per primer binding event. With the double mutant, L2V/R64A, DNA polymerase δ showed a processivity of 5 nucleotides incorporated per primer binding event, which was nearly that of polymerase δ holoenzyme alone with no PCNA added. Again, the reduced capacity of mutants L2V and R64A and the double mutant L2V/R64A to enhance polymerase δ processivity is specific, because mutant D63A enhanced polymerase δ processivity similar to wild type S. pombe PCNA with >20 nucleotides incorporated per primer binding event. These results, quantitatively summarized in Table III, strongly suggest that the side chains of Leu2 and Arg64 play a role in tethering DNA polymerase δ for processive DNA synthesis in vitro.

Similar to the E. coli pol III β-subunit, PCNA requires loading onto chromosomal DNA by the accessory protein RF-C in order to interact with polymerase δ for processive DNA synthesis in vivo. In vitro studies have shown that ATP hydrolysis promotes the interaction between PCNA and RF-C for this loading (10, 45). To test if mutations in Leu2 and Arg64 affect the ability of PCNA to be loaded onto DNA by RF-C, we assayed L2V, R64A, and the double mutant L2V/R64A for their ability to stimulate RF-C ATPase activity. Wild type S. pombe PCNA was found to stimulate the ATPase activity of budding yeast RF-C similar to budding yeast PCNA. Mutants L2V, R64A, and L2V/R64A stimulated the ATPase activity of RF-C similar to wild type fission and budding yeast PCNA (Fig. 4C). These results suggest that mutations in Leu2 and Arg64 of fission yeast PCNA do not affect the interaction of PCNA with RF-C but do specifically affect PCNA’s interaction with DNA polymerase δ.

Recombinant pcnaΔ Is Able to Complement a Null pcna Strain—A diploid strain MAP112 containing one null allele of...
Fig. 4. Stimulation of DNA polymerase α DNA synthetic activity by wild type and mutant S. pombe PCNA proteins. A, wild type S. pombe PCNA is able to stimulate calf thymus DNA polymerase α DNA synthesis comparable with calf thymus PCNA. Reactions were carried out as described under "Experimental Procedures" with increasing amounts of either calf thymus (CT) PCNA (closed diamond), S. pombe PCNA (open square), or buffer (open triangle) as indicated. After incubation for 20 min at 30°C, reactions were terminated, and the extent of [32P]dTMP incorporation was determined. B, mutant S. pombe PCNAs, L2V, R64A, and L2V/R64A have reduced capacity in stimulating polymerase α DNA synthesis. Reactions with increasing amounts of wild type PCNA (open square) or each mutant PCNA protein, L2V (open circle), R64A (open square), and L2V/R64A (open triangle) added as indicated were performed as described under "Experimental Procedures." C, mutant S. pombe PCNAs, L2V, R64A, and L2V/R64A are not deficient in stimulating the DNA-dependent ATPase activity of RF-C. Reactions with increasing amounts of wild type S. pombe (open triangle) or S. cerevisiae (closed triangle) PCNA or with S. pombe mutant PCNA proteins L2V (closed square), D63A (open square), R64A (closed circle), or L2V/R64A (open circle) added as indicated were performed as described under "Experimental Procedures."
PCNA by replacement of pcna<sup>+</sup> with the his3<sup>+</sup> marker was generated (see “Experimental Procedures”). After sporulation in medium lacking histidine, only spores prototrophic for histidine and containing Δpcna germinated. Germinating spores were stained with DAPI, examined microscopically and compared with the parental wild type strain (Fig. 6A). Germinating spores with Δpcna displayed an elongated cell division cycle (cdc) phenotype similar to that previously described (46) (Fig. 6B).

To test whether the wild type recombinant pcna<sup>+</sup> isolated in this study was functional in vivo, recombinant pcna<sup>+</sup> was constructed into an expression vector containing the LEU2 selectable marker (pREP181) in which expression is controlled from a weakened nmt1 promoter (29, 47, 48). pREP181/pcna<sup>+</sup> was transformed into the diploid MAP112. Upon sporulation and selection for histidine and leucine prototrophs, a haploid strain MAP300 was generated with Δpcna sustained by pREP181/pcna<sup>+</sup>. MAP300 had a normal growth rate (see results described below) and phenotype compared with the parental wild type strain (Fig. 6C). This indicates that the recombinant pcna<sup>+</sup> isolated in this study is functional in vivo.

Table II
Summary of PCNA mutant effects on DNA polymerase δ holoenzyme synthetic activity in vitro

| Mutant      | DNA polδ Activity |
|-------------|-------------------|
| L2V         | 46                |
| D63A        | 90–100            |
| R64A        | 46                |
| Q201A       | 0.3               |
| E259A       | 90–100            |
| E260A       | 90–100            |
| L2V/R64A    | 8–10              |
| E259A/E260A | 90–100            |

Fig. 5. Enhancement of DNA polymerase δ processivity by wild type and mutant S. pombe PCNA proteins. Processivity of DNA polymerase δ with wild type and mutant S. pombe PCNAs was performed as described under “Experimental Procedures.” In each set, lane 1 is the control with no DNA polymerase δ (buffer alone); B, control with DNA polymerase δ alone and no PCNA added; C, DNA polymerase δ with calf thymus PCNA; D, DNA polymerase δ with wild type S. pombe PCNA; E, DNA polymerase δ with S. pombe PCNA mutant L2V; F, DNA polymerase δ with S. pombe PCNA mutant R64A; G, DNA polymerase δ with S. pombe PCNA double mutant L2V/R64A; H, DNA polymerase δ with S. pombe PCNA mutant D63A; and I, wild type S. pombe PCNA alone with no DNA polymerase δ added.
MAP112 was independently transformed with each pREP181/mutant pcna. Sporulation and selection for leucine and histidine prototrophs yielded haploid strains with Δpcna sustained by pREP181/mutant pcna. These haploid strains are summarized in Tables I and IV.

Strains MAP300, MAP301, MAP304, MAP305, and MAP326 were grown in medium lacking thiamine to allow full expression of each of the mutant-pcnas. Microscopic examination of DAPI-stained cells showed that MAP300 sustained by wild type pcna and MAP304 cells sustained by the mutant pcna-4 allele (with D63A mutation) (see Table IV) had wild type phenotypes (Fig. 7, A and B). Mutant strains MAP301, MAP305, and MAP326, sustained by pcna-1 (with L2V mutation), pcna-5 (with R64A mutation), and pcna-26 (with L2V/R64A mutations) alleles, respectively, displayed elongated phenotypes with cells on average approximately 5–10 μm longer than cells sustained by either wild type pcna or pcna-4 (Fig. 7, compare C, D, and E with A and B).

Strain MAP306, sustained by mutant allele pcna-6 (with G69D mutation), failed to grow beyond one or two cell divisions and displayed a phenotype similar to Δpcna (Fig. 6B), generating highly elongated cells (depicted by arrow in Fig. 7F). Thus, mutation of S. pombe PCNA Gly69 to Asp not only yields mutant protein unable to stimulate polymerase δ DNA synthetic activity in vitro (Table II) but also fails to sustain growth of cells containing Δpcna in vivo.

Cells Sustained by Mutant-pcna That Are Defective in Enhancing DNA Polymerase δ Processivity in Vitro Exhibit Moderate Growth Defects but No Altered Sensitivity to UV—We analyzed the growth rate of the mutant strains that are defective in enhancing DNA polymerase δ processivity and display elongated phenotypes. MAP300 (with pcna sustained by pREP181/pcna1) and the parental strain KG3 had generation times of ~2.5 h at 30 °C, whereas MAP301 (with L2V mutation), MAP305 (with R64A mutation), and MAP326 (with L2V/R64A mutations) had reproducible generation times 40 min longer than MAP300 or KG3 (Fig. 8). In contrast MAP304, sustained by the pcna-4 allele (with D63A mutation), had a growth rate similar to MAP300 (data not shown). These results indicate that strains sustained by mutant pcna alleles encoding PCNA proteins defective in their ability to enhance polymerase δ processivity in vitro not only display elongated phenotype but also have moderate growth defects in vivo.

Because PCNA has been shown to play a role in DNA repair in vitro, strains MAP301, MAP304, MAP305, and MAP326 were also tested for their sensitivity to UV irradiation, a DNA damaging agent. MAP301, MAP304, MAP305, and MAP326 showed no significant difference in UV sensitivity compared with MAP300 or the parental strain (Fig. 9). Under the same conditions, rad26 showed high UV sensitivity.

**DISCUSSION**

To begin to understand how PCNA interacts with replication factors for S phase progression, we used the S. cerevisiae PCNA structure (25) as a guiding model and performed structure-function analyses of S. pombe PCNA both in vitro and in vivo.

Rationale for the Mutations Introduced—Structural data of the E. coli pol III β-subunit and budding yeast PCNA suggest that residues in the protein loops are most likely involved in protein-protein interactions, whereas residues in either the β-sheets or α-helices are important for maintenance of the ring structure of these two molecules (24, 25). We introduced nine site-directed mutations into seven residues located in the protein loop regions of S. pombe PCNA. The rationale for the
mutations are targeted to residues in the protein loop regions that are conserved among PCNA proteins of different phylogenetic species. Furthermore, the mutations introduced must not alter the global physical homotrimer ring structure of PCNA. Leu\textsuperscript{2}, Asp\textsuperscript{63}, and Arg\textsuperscript{64} have been chosen for mutational analysis because these three residues are conserved and clustered in one region of PCNA (Fig. 1). Because Leu\textsuperscript{2} is located at the amino terminus, which might be important for the homotrimer ring formation of PCNA, Leu\textsuperscript{2} was conservatively changed to another hydrophobic residue, Val, to test length functionality of the leucine side chain. Based on the structure data of budding yeast PCNA (25), Asp\textsuperscript{63} and Arg\textsuperscript{64} side chains protrude in different directions, so these two residues were mutagenized to Ala to test not only the functionality of the side chains but also the effect of side chain directionality on protein interactions.

Gly\textsuperscript{69} is located within \(\beta F1\), and mutation of Gly\textsuperscript{69} to Asp yielded a mutant that was unable to stimulate the DNA synthetic activity of polymerase \(\delta\) (Table II). In addition, ectopically expressed G69A mutant PCNA was unable to sustain growth of null pcna cells, displaying a severely elongated phenotype similar to null pcna cells, suggesting a loss of function mutation (Fig. 7F). Mutant G69D protein expressed in bacteria was mostly insoluble, and the quantities of soluble G69D protein were insufficient to be analyzed for structural integrity by gel filtration. However, the in vivo phenotype of this mutant suggests that mutation of Gly\textsuperscript{69} to Asp in \(\beta F1\) causes some structural perturbation resulting in nonfunctional PCNA protein. In budding yeast, the \(\text{pol}30–36\) allele (G69D mutation) is able to suppress a cold-sensitive mutation of CDC44 (cdc44-cs) but does not affect growth of budding yeast cells independent of cdc44-cs (41). Our in vivo and in vitro results suggest that the pcna-6 allele (G69D) in \(S.\ pomb\) e is lethal. Sequence analysis

![Fig. 7. Micrographs of strains sustained by mutant-pcna alleles. DAPI stains of cells grown in selective medium are shown. A, KG3 (wild type \(S.\ pomb\) e); B, MAP300 (\(\Delta pcna\) sustained by pREP81/pcna\(^{-}\)); C, MAP301 (\(\Delta pcna\) sustained by pREP181/L2V); D, MAP305 (\(\Delta pcna\) sustained by pREP181/R64A). E, MAP326 (\(\Delta pcna\) sustained by pREP181/L2V, R64A). F, MAP306 (\(\Delta pcna\) sustained by pREP181/G69D). Arrows mark the cells displaying abnormal phenotypes.](image1)

![Fig. 8. Cells containing mutant-pcna with reduced capacity to enhance polymerase \(\delta\) processivity in vitro also display growth defects. Cells sustained by wild type or mutant-pcna alleles were grown in selective medium at 30°C. Cell samples were collected and counted every 2 h. \(N/N_0\) denotes the number of cells at each time point normalized to number of cells of the time 0 cell sample. A, KG3; B, MAP300; C, MAP301; D, MAP305; E, MAP326.](image2)
has indicated that there is no other mutations in the strain besides G69D mutation. Thus, this is contrary to that seen in budding yeast.

Structural Integrity of Mutant PCNAs—Mutations introduced into S. pombe PCNA in this study were targeted to residues that would not be expected to affect the overall structure of PCNA with the exception of G69D. The structural integrity of the mutants was proven by two criteria. First, gel filtration analysis of six single mutant PCNA proteins L2V, D63A, R64A, Q201A, E259A, and E260A as well as the two double mutants E259A/E260A and L2V/R64A showed that all of these mutant PCNA proteins maintained the homotrimer ring structure (Fig. 3 and data not shown). Second, each of these six single mutants and the two double mutants were able to sustain growth of Δpcna cells. These results indicate that the introduced mutations do not perturb the homotrimeric structure of S. pombe PCNA.

Hetero-species Interactions—Several lines of evidence have shown that PCNA is able to interact with protein factors of hetero-species. The primary sequence of S. pombe PCNA is 52% identical to human and rat PCNAs. The disrupted S. pombe PCNA gene can be functionally complemented by ectopically expressed human PCNA (46). Furthermore, Drosophila PCNA with approximately 70% identity to the human protein (49) is able to substitute for human PCNA in SV40 replication in vitro (43). Budding yeast PCNA, with 35% identity to the human protein, is able to enhance the processivity of mammalian DNA polymerase δ in vitro but cannot substitute for human PCNA in SV40 in vitro replication (42). Nonetheless, it has been shown that PCNA proteins and polymerase δ from budding yeast and calf thymus DNA polymerase δ are interconvertible for processive DNA synthesis (42, 50). In this study, we showed that S. pombe PCNA is able to enhance calf thymus DNA polymerase δ enzymatic activity and enhance calf thymus DNA polymerase δ processivity similar to calf thymus PCNA (Figs. 4A and 5). Furthermore, S. pombe PCNA is able to stimulate the DNA-dependent ATPase activity of budding yeast RF-C similar if not identical to budding yeast PCNA (Fig. 4C). These results strongly suggest that particular sites or domains of the PCNA protein are functionally conserved and able to interact with DNA replication and/or repair proteins from hetero-species.

We have overexpressed an enzymatically active catalytic subunit of S. pombe DNA polymerase δ in recombinant baculovirus-infected insect cells. The recombinant S. pombe PCNA described in this study, however, was unable to stimulate the single catalytic subunit of S. pombe polymerase δ. This is similar to the observation in Drosophila, where PCNA is unable to stimulate the DNA polymerizing activity of the single catalytic subunit of polymerase δ (51), and to the findings in mouse (52). In contrast, budding yeast PCNA is able to stimulate the budding yeast polymerase δ single catalytic subunit overexpressed in bacteria (53). Because S. pombe PCNA is unable to stimulate the single catalytic subunit of homo-species polymerase δ, we used calf thymus polymerase δ for our study.

Leu²⁶ and Arg⁶⁴ Are Involved in Enhancing DNA Polymerase δ Activity and Processivity in Vitro—We identified two residues, Leu²⁶ and Arg⁶⁴, that are specifically involved in interacting with polymerase δ for processive DNA synthesis but not with RF-C (Figs. 4, B and C, and 5 and Tables II and III). According to the structural data of S. cerevisiae PCNA (25), the side chain directions of Leu²⁶ and Arg⁶⁴ are also specific for interaction with polymerase δ, because mutation of Asp⁶³, adjacent to Arg⁶⁴ but with its side chain oriented in a different direction than Arg⁶⁴, did not affect the processivity of polymerase δ (Fig. 5 and Tables II and III). This suggests that the face of PCNA with the protruding Leu²⁶ and Arg⁶⁴ side chains is involved in the functional interaction between polymerase δ and PCNA. These results are supported by in vitro structure-function analysis of human PCNA by Fukuda et al., which identified residues on the outer surface and carboxyl- and amino-terminal regions of PCNA that are responsible for interaction with multiple DNA replication factors (26). In the study of human PCNA, mutation of Arg⁶⁴ but not Asp⁶³ to Ala reduced the ability of mutant PCNA to stimulate human DNA polymerase δ activity by 50%. However, neither mutation had demonstrable effects on the ATPase activity of RF-C. Our findings that null pcnA cells sustained by mutant PCNA R64A display abnormal elongated phenotypes and have moderate growth defects, further support the notion that Arg⁶⁴ indeed has a role in interacting with polymerase δ for processive DNA synthesis.

Mutations of Gln²⁰¹, Glu²⁵⁹, and Glu²⁶⁰ and double mutations of Glu²⁵⁹ and Glu²⁶⁰ to Ala did not appear to affect the DNA polymerizing activity of calf thymus DNA polymerase δ (Table II). Because a hetero-species polymerase δ was used in our study, we cannot rule out the possibility that these residues may have species specificity for interacting with polymerase δ. Deletion analysis of a short carboxyl-terminal stretch of human PCNA from Lys²⁵⁴ to Glu²⁶⁰ was found to be necessary for stimulation of RF-C ATPase activity but not for stimulation of DNA polymerase δ activity (26). A double mutation, D256A/ E257A, of acidic residues at the carboxyl-terminal region of budding yeast PCNA equivalent to those that were introduced in our fission yeast study have been analyzed in vivo. A strain containing the mutant allele pol30–22 showed slightly higher sensitivity to UV and methylmethane sulfonate than the wild type strain but had a growth rate similar to wild type. However, this budding yeast PCNA mutant has not been studied in vitro for its effect on polymerase δ activity (27). Preliminary in vivo studies of S. pombe PCNA with single or double mutations of Glu²⁵⁹ and Glu²⁶⁰ to Ala show that mutations in these two residues do not significantly affect growth similar to that ob-

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2 M. P. Arroyo and T. S.-F. Wang, unpublished observation.
erved with the budding yeast pol30–22, mutant (27). To-
gether, these budding and fission yeast results suggest that
these charged residues at the carboxyl-terminal region might
not play a role in S phase progression.

In Vivo Effect of Ectopically Expressed pcna+ and pcna Mu-
ant in Null pcna Cells—Waseem et al. (46) previously re-
ported that neither pcna+ nor POL30 expressed from pREP1
were able to complement Δpcna cells. Moreover, overexpression
of pcna+ rendered cells with increased generation times and
abnormal phenotypes (46). We found that pcna+ isolated in this
study and expressed from a weakened nmt1 promoter (pREP1
or pREP181) is able to complement Δpcna cells under fully
induced conditions (Fig. 6). This indicates that although over-
expression of PCNA from pREP1 at levels approximately 400-
fold greater than endogenous leads to abnormal germination
and inability to rescue Δpcna cells (46), moderate expression
of PCNA at levels 7- to 10-fold over endogenous (47) does not appear
to compromise germination of Δpcna spores nor growth of
Δpcna cells.

Strains MAP301, MAP305, and MAP326, sustained by ec-

topically expressed PCNA mutants that are defective in vitro

for enhancing polymerase δ processivity, have abnormal elon-
gated phenotypes and slower growth rates (Figs. 7 and 8).
These findings suggest that cells sustained by mutant PCNA
proteins that have a reduced capacity to enhance polymerase δ
for processive DNA synthesis in vitro might cause a delay in S
phase progression, thus resulting in longer doubling times and
elongated phenotypes in vivo. The moderate effects in vivo of
Leu2, Arg64, and Leu2/Arg64 mutants suggest that Leu2 and
Arg64 are only two of several residues that interact with po-

lymerase δ for processive DNA synthesis. These in vivo results
reflect the in vitro results that mutations in Leu2 or Arg64
reduce the capacity of mutant PCNA to stimulate DNA poly-
merase δ activity but do not abolish stimulation completely (Fig.
4B and Table II).

The lack of UV sensitivity of MAP301 (with LZV mutation),
MAP305 (with R64A mutation), and MAP326 (with LZV/R64A
mutations) suggests that the observed moderate growth defects
and elongated phenotypes of these mutant strains are primar-
ily due to defects in replication and not to UV irradiation repair
defects.

Conclusion—In this study, we defined two residues in a
region on one face of the S. pombe PCNA trimer ring structure
that are involved in interacting with polymerase δ for proces-
sive DNA synthesis. Correlating with the in vitro findings, S.
pombe cells containing these mutant alleles have elongated
phenotypes and moderate growth defects. Cells with these mu-
tant alleles, however, have no apparent altered sensitivity to
UV. Together, these in vitro and in vivo results strongly sug-
gest that residues Leu2 and Arg64, with their side chains pro-
truding toward one face of the S. pombe PCNA trimer ring,
functionally interact with DNA polymerase δ for DNA replica-
cation in cells.

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