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PCNA trimer instability inhibits translesion synthesis by DNA polymerase η and by DNA polymerase δ

Lynne M. Dieckman and M. Todd Washington*

Department of Biochemistry, University of Iowa College of Medicine, Iowa City, IA 52242-1109

Abstract

Translesion synthesis (TLS), the process by which DNA polymerases replicate through DNA lesions, is the source of most DNA damage-induced mutations. Sometimes TLS is carried out by replicative polymerases that have evolved to synthesize DNA on non-damaged templates. Most of the time, however, TLS is carried out by specialized translesion polymerases that have evolved to synthesize DNA on damaged templates. TLS requires the mono-ubiquitylation of the replication accessory factor proliferating cell nuclear antigen (PCNA). PCNA and ubiquitin-modified PCNA (UbPCNA) stimulate TLS by replicative and translesion polymerases. Two mutant forms of PCNA, one with an E113G substitution and one with a G178S substitution, support normal cell growth but inhibit TLS thereby reducing mutagenesis in yeast. A re-examination of the structures of both mutant PCNA proteins revealed substantial disruptions of the subunit interface that forms the PCNA trimer. Both mutant proteins have reduced trimer stability with the G178S substitution causing a more severe defect. The mutant forms of PCNA and UbPCNA do not stimulate TLS of an abasic site by either replicative Pol δ or translesion Pol η . Normal replication by Pol η was also impacted, but normal replication by Pol δ was much less affected. These findings support a model in which reduced trimer stability causes these mutant PCNA proteins to occasionally undergo conformational changes that compromise their ability to stimulate TLS by both replicative and translesion polymerases.

Keywords

DNA replication; DNA repair; DNA polymerase; translesion synthesis; mutagenesis

1. Introduction

Replicative DNA polymerases have evolved to synthesize DNA on non-damaged templates during normal DNA replication. In general, these enzymes catalyze the template-directed incorporation of nucleotides with high fidelity. For example, eukaryotic DNA polymerase δ (Pol δ), a member of the B-family of polymerases, is responsible for the majority of lagging strand synthesis during normal DNA replication [1–3]. It also plays important roles in base excision repair, nucleotide excision repair, mismatch repair, and double strand break repair [4]. Yeast Pol δ is a hetero-trimer comprised of a catalytic subunit (Pol3) and two accessory subunits (Pol31 and Pol32) [5]. A low resolution structure of Pol δ shows that the protein is

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*To whom correspondence should be addressed: M. Todd Washington, Department of Biochemistry, 4-403 Bowen Science Building, University of Iowa, Iowa City, IA 52242-1109, Phone: 319-335-7518, Fax: 319-335-9570, todd-washington@uiowa.edu.

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elongated with Pol31 acting as a bridge to connect the Pol3 and Pol32 subunits [6]. The X-ray crystal structure of the catalytic Pol3 subunit shows that it possesses three domains: an N-terminal domain, a polymerase domain, and an exonuclease domain for proofreading [7]. The polymerase domain contains fingers, thumb, and palm sub-domains similar to those found in other B-family DNA polymerases. In the absence of the proofreading function, Pol δ incorporates nucleotides with error frequencies of 10^{-4} to 10^{-5} [8–10]. Kinetic analysis shows that this high fidelity arises at multiple steps along the reaction pathway, including both the initial nucleotide-binding step and the subsequent nucleotide-incorporation step [8].

Despite the remarkable catalytic activities of replicative DNA polymerases, most of these enzymes are unable to efficiently incorporate nucleotides opposite template DNA damage. Consequently, cells possess several specialized translesion DNA polymerases, which have evolved to replicate through DNA lesions [11–18]. For example, eukaryotic DNA polymerase η , a member of the Y-family of DNA polymerases, is responsible for bypassing template thymine dimers and 8-oxoguanine (8-oxoG) lesions [19–21]. This enzyme is a monomer, and X-ray crystal structures show that it possesses two domains: a polymerase domain and a polymerase-associated domain [22–25]. The polymerase domain contains fingers, thumb, and palm sub-domains similar to those found in other Y-family polymerases. It also contains an active site that is larger than those of replicative polymerases, and this larger active site allows Pol η to readily accommodate thymine dimers [23, 24]. Kinetic analyses show that the mechanisms of nucleotide incorporation opposite non-damaged templates, opposite thymine dimers, and opposite 8-oxoG lesions are identical [26–28]. Thus these forms of DNA damage present no barrier to nucleotide incorporation by Pol η .

Translesion synthesis (TLS) is the process by which DNA polymerases replicate through DNA lesions by directly using the damaged DNA as a template. Although replicative DNA polymerases can carry out TLS in a few contexts, most TLS is accomplished by translesion polymerases. In these cases, the replicative polymerase is replaced at the replication fork by a translesion DNA polymerase. This polymerase-switching event is facilitated by a key replication accessory factor, proliferating cell nuclear antigen (PCNA). In addition to its role in TLS, PCNA participates in a wide range of functions, including DNA replication, base excision repair, nucleotide excision repair, mismatch repair, recombination, chromatin remodeling, and cell cycle regulation [29–33]. PCNA directly regulates the activities of both replicative and translesion DNA polymerases. For instance, it increases both the processivity and catalytic efficiency of DNA synthesis by replicative Pol δ [8, 34]. Similarly, it increases the catalytic efficiency of DNA synthesis by translesion Pol η [35]. During TLS, PCNA is mono-ubiquitylated on Lys-164 by the Rad6-Rad18 complex [36]. Ubiquitin-modified PCNA (UbPCNA) plays an important role in TLS, as translesion polymerases preferentially interact with UbPCNA through their ubiquitin-binding elements [37].

Two mutant forms of yeast PCNA, one with a G178S substitution and the other with an E113G substitution, have been identified that support normal cell growth but inhibit TLS thereby reducing mutagenesis in yeast [38–40]. It remains unclear how these mutant forms of PCNA inhibit TLS. The X-ray crystal structures of both of these mutant PCNA proteins have been determined, and both of these amino acid substitutions are located at the subunit interface that forms the PCNA trimer [41, 42]. We have re-examined these structures and noticed substantial disruptions to the subunit interface. This suggested that these mutant PCNA proteins may have defects in trimer stability compared to the wild-type PCNA protein. We found that both mutant PCNA proteins had reduced trimer stability with the G178S substitution causing a more severe defect. We also found that mutant forms of PCNA and UbPCNA did not stimulate TLS of an abasic site by either replicative Pol δ or translesion Pol η . Normal replication by Pol η was also impacted, but normal replication by Pol δ was much less affected. These findings support a model in which reduced trimer

stability causes these mutant PCNA proteins to occasionally undergo conformational changes that compromise their ability to stimulate TLS by both replicative and translesion DNA polymerases.

2. Materials and methods

2.1. Protein expression and purification

The wild-type and E113G mutant PCNA proteins from *S. cerevisiae* were over-expressed as N-terminal His6-tagged proteins and purified from *E. coli* as previously described [41]. The wild-type and E113G mutant UbPCNA proteins from *S. cerevisiae* were over-expressed and purified from *E. coli* using the split-fusion strategy as previously described [43]. *S. cerevisiae* replication factor C (RFC) was over-expressed and purified from *E. coli* as previously described [44]. *S. cerevisiae* Pol η and Pol δ were over-expressed and purified from *S. cerevisiae* as previously described [8, 26].

2.2. DNA and nucleotide substrates

To measure DNA polymerase activity, a 68-mer oligodeoxynucleotide with the sequence 5'-GAC GGC ATT GGA TCG ACC TCX AGT TGG TTG GAC GGG TGC GAG GCT GGC TAC CTG CGA TGA GGA CTA GC with biotins on both ends was used as the template strand. The X represents the position of a non-damaged G or an abasic site. For steady state kinetic studies, a 31-mer oligodeoxynucleotide with the sequence 5'-GGT AGC CAG CCT CGC ACC CGT CCA ACC AAC T was used as a primer. For the processivity assays, a 31-mer oligodeoxynucleotide with the sequence 5'-TCG CAG GTA GCC AGC CTC GCA CCC GTC CAA C was used as a primer. The primer strands were 5'-³²P-end-labeled with T4 polynucleotide kinase and (γ -³²P)ATP. The primer and template strands were annealed at 200 nM in 25 mM TrisCl, pH 7.5, and 100 mM NaCl at 90°C for 2 min and slowly cooled to 30°C. Solutions of each of the four dNTPs (10 mM) were obtained from New England Biolabs and stored in 5 μ l-aliquots at -80°C.

2.3. PCNA trimer stability assays

To assay for PCNA trimer stability, we used non-denaturing polyacrylamide gel electrophoresis (PAGE) and size exclusion chromatography. For non-denaturing PAGE, the wild-type and mutant PCNA and UbPCNA proteins (0.05 to 5 mg/ml) were incubated in 60 mM TrisCl, pH 6.8, 0.01% bromophenol blue, and 10% glycerol for 5 min. The protein samples were then run on a TrisCl pre-cast 4–20% gradient non-denaturing polyacrylamide gel (Bio-Rad) at a constant 25 mA using 25 mM Tris, pH 8.3, and 0.2 M glycine as a running buffer at 4°C. Protein bands were visualized by coomassie staining. For size exclusion chromatography, PCNA proteins were diluted to various concentrations (0.005 to 0.1 mg/ml) and loaded onto a 120 ml HiLoad 16/60 Superdex 200 PG column (GE Healthcare). The column was calibrated with the following molecular weight standards (Bio-Rad): thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B₁₂ (1.35 kDa).

2.4. Enzyme-linked immunosorbent assays

The wells of a 96 well EIA/RIA plate (Corning) were coated with 1 μ g of Pol η in PBS (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4, 137 mM NaCl, 2.7 mM KCl) for one hour. The wells were then washed three times with PBS, 0.2% Tween-20, blocked for 30 min. with PBS with 5% milk, and washed again. Various amounts of wild-type or mutant PCNA or UbPCNA proteins or bovine serum albumin (BSA) (0.5 μ g to 20 μ g) in PBS with 5% milk were added to the wells and incubated for one hour, followed by washing. For the wild-type PCNA and E113G mutant PCNA proteins, a 1:500 dilution of rabbit polyclonal anti-PCNA antibody in PBS with 5% milk was added to the wells and incubated for 30 min. For the

wild-type and E113G mutant UbPCNA proteins, a 1:200 dilution of rabbit polyclonal anti-His tag antibody (Santa Cruz Biotechnology) was used. Wells were washed before adding a 1:10,000 dilution of goat anti-rabbit antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch) in PBS with 5% milk for 30 min. The plate was washed, and 0.8 mg/ml of O-phenylenediamine (Aldrich) in 0.05 M phosphate-citrate buffer with 0.03% sodium perborate (Sigma) was added. Absorbance at 450 nm was measured after 5 to 35 min. with an iMark microplate reader (Bio-Rad). The BSA control absorbance values were subtracted from the absorbance of each sample at the corresponding protein concentration. All steps were performed at 25°C.

2.5. Polymerase processivity assays

Reactions were performed in 40 mM Tris-Cl, pH 7.5, 8 mM MgCl₂, 150 mM NaCl, 1 mM DTT, and 100 µg/ml BSA at 30°C. Annealed DNA substrates were incubated with a 10-fold molar excess of streptavidin for 5 min to block the ends of the DNA to prevent PCNA dissociation. Wild-type or mutant PCNA or UbPCNA protein (90 nM) was loaded onto 20 nM of the DNA substrate with 25 nM RFC and 500 µM ATP. Either Pol η or Pol δ (40 nM) was added to each reaction and pre-incubated for 20 min. Reactions were initiated by the addition of 200 µM of each dNTP and 1 mg/ml of salmon sperm DNA and quenched after 1 min for Pol δ or 2 min for Pol η by the addition of 10 volumes of 80% deionized formamide, 10 mM EDTA, pH 8.0, 1 mg/ml xylene cyanol, and 1 mg/ml bromophenol blue. The products were then analyzed on a 15% polyacrylamide sequencing gel containing 8 M urea and the intensities of the labeled gel bands were determined using the Storm 860 (GE Healthcare). Percent of polymerases that incorporated at least *N* nucleotides was calculated by dividing the sum of the intensities of all gel bands resulting from *N* nucleotide incorporations or greater by the sum of the intensities of all gel bands resulting from one nucleotide incorporation or greater.

2.6. Polymerase activity assays

Reactions were performed using the same buffer conditions as described in the polymerase processivity assays, and the wild-type and mutant PCNA and UbPCNA proteins were loaded on the DNA substrates as described in the processivity assays. For nucleotide incorporation by Pol η opposite a non-damaged template residue, different concentrations of dCTP (2 to 200 µM) were mixed with the PCNA-loaded DNA substrate (20 nM) and Pol η (2 nM). The reactions were quenched at various times up to 15 min. For nucleotide incorporation by Pol η opposite a template abasic site, different concentrations of dGTP (2 to 200 µM) were used, and the reactions were quenched at various times up to 20 min. For nucleotide incorporation by Pol δ opposite a non-damaged template residue, different concentrations of dCTP (2 to 200 µM in the absence of any PCNA; and 0.5 to 50 µM in the presence of the wild-type or mutant PCNA or UbPCNA proteins) were mixed with the PCNA-loaded DNA substrate (20 nM) and Pol δ (2 nM). The reactions were quenched at various times up to 2 min. For nucleotide incorporation by Pol δ opposite a template abasic site, different concentrations of dATP (2 to 200 µM in the absence of any PCNA, and 0.5 to 50 µM in the presence of the wild-type or mutant PCNA or UbPCNA proteins) were used, and the reactions were quenched at various times up to 15 min. The products were then analyzed on a 15% polyacrylamide sequencing gel containing 8 M urea and the intensities of the labeled gel bands were determined using the Storm 860 (GE Healthcare). The rate of product formation was plotted as a function of incoming dNTP concentration, and the V_{max} and K_m parameters were obtained from the best fit of the data to the Michaelis-Menten equation. All experiments were carried out at least three times to ensure reproducibility.

3. Results

3.1. The E113G and G178S mutant PCNA proteins have altered subunit interfaces

Two variant forms of PCNA, the E113G and G178S mutant PCNA proteins, support normal cell growth but inhibit TLS thereby reducing mutagenesis in yeast. These amino acid substitutions are located on β -strand I₁ and β -strand D₂, respectively, which constitute the PCNA subunit interface. The X-ray crystal structures of both of these mutant PCNA proteins have been determined [41, 42]. However, the precise impact of these amino acid substitutions on the structure of the subunit interface was not described in these previous studies. Here we have re-examined these structures and noticed substantial disruptions to this interface (Fig. 1 and Table S1 in Supplemental Material). In the case of the wild-type PCNA protein, there are seven backbone hydrogen bonds between β -strands I₁ and D₂ with the distances between the amide nitrogen atoms and the corresponding carbonyl oxygen atoms ranging from 2.8 to 3.1 Å. In the E113G mutant PCNA protein, there are only five backbone hydrogen bonds between these β -strands. This is because the backbone of β -strand I₁ has shifted so that the distances between two of the amide nitrogen atoms and the corresponding carbonyl oxygen atoms are now 4.5 and 5.9 Å, which is too large to form hydrogen bonds. In the G178S mutant PCNA protein, there are only three backbone hydrogen bonds between these β -strands. This is because the position of the backbone of β -strand I₁ has shifted dramatically so that the distances between four of the amide nitrogen atoms and the corresponding carbonyl oxygen atoms now range from 4.1 to 9.3 Å, which again is too large to form hydrogen bonds.

3.2. Trimer stability of the E113G and G178S mutant PCNA proteins

Because the E113G and G178S mutant PCNA proteins had fewer hydrogen bonds at the subunit interface, we examined the stability of the mutant PCNA trimers. Non-denaturing polyacrylamide gel electrophoresis (PAGE) showed that the wild-type PCNA protein formed a stable trimer under all concentrations tested (0.05 to 5 mg/ml) (Fig. 2). The E113G mutant PCNA protein formed a stable trimer at the higher concentrations (0.2 to 5 mg/ml). At lower concentrations (0.05 to 0.1 mg/ml), however, the gel bands were consistently less intense than those of the wild-type protein and were streaking. This suggested that although the mutant protein was primarily a trimer, it was less stable than the wild-type PCNA trimer. By contrast, the G178S mutant PCNA protein was a monomer under all concentrations tested.

To test further the trimer stability of these mutant PCNA proteins, we used size exclusion chromatography (Fig. 3 and Table S2 in Supplemental Material). At all concentrations tested (0.005 to 0.1 mg/ml), the wild-type PCNA protein elutes from the size exclusion column at a volume of approximately 74 ml, which corresponds to the 90-kDa trimer. At all concentrations tested, the E113G mutant PCNA protein was primarily a trimer. However, at the lower concentrations (0.005 and 0.01 mg/ml), a significant proportion (20 % and 7 %, respectively) eluted from the column at a volume of approximately 86 ml, which corresponds to the 30-kDa monomer. This result is consistent with a previous study showing decreased trimer stability of the E113G mutant PCNA protein [42]. By contrast, at all concentrations tested, the G178S mutant PCNA protein was primarily a monomer. This result seems to be at odds with the X-ray crystal structure of the G178S mutant protein showing the mutant protein to be a trimer [41]. It should be noted that the formation of this was due to the extremely high concentration of protein in the crystalline state. Taken together, the native PAGE and size exclusion chromatography results show that both mutant proteins form less stable trimers than does the wild-type protein. Furthermore, the trimers formed by the G178S mutant PCNA protein were far less stable than those formed by the E113G mutant PCNA protein.

In cells, PCNA is mono-ubiquitylated on Lys-164 during TLS. In order to study these mutant proteins in the more biologically relevant context of ubiquitin-modified PCNA (UbPCNA), we produced both wild-type and mutant UbPCNA proteins using the split-fusion method [43]. This method, which involves co-expressing the UbPCNA as two polypeptide fragments that self-assemble *in vivo* to form ring-shaped trimers, was used previously to determine the X-ray crystal structure of UbPCNA [43]. Moreover, the split-fused UbPCNA functions the same as genuine UbPCNA *in vitro* [43]. We used native PAGE to examine the stability of the wild-type and mutant UbPCNA trimers (See Fig. S1 in Supplemental Material). The wild-type UbPCNA protein formed a stable trimer under all concentrations tested (0.01 to 5 mg/ml). At lower concentrations (0.01 to 0.1 mg/ml), the E113G mutant UbPCNA protein was not a trimer. However, at higher concentrations (0.5 to 5 mg/ml), the E113G mutant UbPCNA protein was a trimer. At all concentrations tested (0.5 to 5 mg/ml), the G178S mutant UbPCNA proteins was completely aggregated indicating that it is highly unstable. Because of the extreme instability of the G178S mutant PCNA and UbPCNA proteins, all subsequent biochemical studies were performed only with the E113G mutant UbPCNA protein.

3.3. Interactions of the E113G mutant PCNA protein with DNA polymerases

One possible way that the E113G substitution in PCNA prevents TLS is by interfering with the ability of PCNA to interact with translesion polymerases. To examine this possibility, we have used enzyme-linked immunosorbent assays (ELISAs) to determine if the E113G mutant PCNA protein can bind Pol η , the prototypical translesion polymerase. Pol η was immobilized in the wells of a microtiter plate, and various concentrations of the wild-type or E113G mutant PCNA protein was added (Fig. 4A). The absorbance measured is proportional to the amount of PCNA bound to the immobilized Pol η . There was no difference in binding observed between the wild-type and mutant PCNA proteins. We carried out similar experiments with the wild-type or E113G mutant UbPCNA proteins (Fig. 4B). There was also no observed difference in binding in this case. Therefore, the E113G substitution does not affect the ability of either PCNA or UbPCNA to bind the translesion polymerase Pol η .

3.4. Impact of the E113G mutant PCNA protein on the activity of Pol η

Although the E113G mutant PCNA protein interacts with the translesion polymerase Pol η , the mutant PCNA protein may be unable to enhance the catalytic activity of Pol η . To test this possibility, we measured the affect of the E113G substitution on the ability of PCNA and UbPCNA to increase activity of Pol η by examining DNA synthesis on non-damaged DNA templates in the presence of an excess of unlabeled competitor DNA (Fig. 5A). Under these conditions, little DNA synthesis by Pol η was observed. However, in the presence of the wild-type PCNA and UbPCNA proteins, DNA synthesis by Pol η was clearly visible with some of the products extending to the end of the DNA template. Significantly less Pol η activity, however, was observed with the E113G mutant PCNA and UbPCNA proteins. Thus, the E113G mutant PCNA and UbPCNA proteins are unable to fully enhance the catalytic activity of Pol η .

Because these experiments were carried out in the presence of an excess of unlabeled competitor DNA, it was possible to examine the affect of the E113G substitution on the ability PCNA and UbPCNA to increase the processivity of Pol η . Processivity is a measure of the number of nucleotides a DNA polymerase incorporates per DNA-binding event. For example, in the presence of the wild-type and the E113G mutant PCNA proteins, 10 to 20 % of the total extension products resulted from at least four nucleotide incorporations (Table S3 in Supplemental Material). In the presence of the wild-type and the E113G mutant UbPCNA proteins, 20 to 30 % of the extension products resulted from at least four

nucleotide incorporations. Overall, these results show UbPCNA increases the processivity of Pol η to a somewhat greater extent than does PCNA. Moreover, these results show that despite a clear effect on the ability of these mutant proteins to enhance the catalytic activity of Pol η , they do not significantly affect the processivity of Pol η .

We next examined the activity of Pol η on a DNA substrate containing an abasic site at the sixth position in template following the primer terminus (Fig. 5B and Table S3 in Supplemental Material). Again, in the absence of PCNA or UbPCNA, little DNA synthesis by Pol η was observed. With the wild-type and E113G mutant PCNA proteins, 10 to 30 % of the extension products resulted from DNA synthesis up to the abasic site, and 1 to 5 % of the products resulted from incorporation opposite the abasic site. With the wild-type and E113G mutant UbPCNA proteins, 30 to 50 % of the products and 10 to 20 % of the products resulted from incorporation up to and opposite the abasic site, respectively. These results further show that despite a clear effect on the ability of these mutant proteins to enhance the catalytic activity of Pol η , they do not significantly affect the processivity of Pol η .

3.5. Impact of the E113G mutant PCNA protein on the catalytic efficiency of Pol η

To quantitatively examine the affects of the E113G substitution in PCNA and in UbPCNA to increase the activity of translesion Pol η , we used steady state kinetics to measure the catalytic efficiency ($V_{\max}/K_m$) of nucleotide incorporation by Pol η (Fig. 6 and Table S4 in Supplemental Material). For the non-damaged template, the wild-type PCNA protein increases the catalytic efficiency of nucleotide incorporation by 2.7-fold on average and the wild-type UbPCNA protein increases it by 9.5-fold on average. These values are consistent with previous reports [43]. Interestingly, neither the E113G mutant PCNA protein nor the E113G mutant UbPCNA protein increases the catalytic efficiency of nucleotide incorporation by Pol η on non-damaged templates. These results show that the E113G substitution in both PCNA and UbPCNA has a significantly affect on the efficiency of Pol η .

We next examined the impact of the E113G substitutions on the ability of PCNA and UbPCNA to increase the catalytic efficiency of nucleotide incorporation by translesion polymerases when they are confronted with a kinetic barrier, such as a non-cognate lesion. Abasic sites are non-cognate lesions for Pol η and reduce its efficiency of nucleotide incorporation substantially [45]. For the template abasic site, the wild-type PCNA protein increases the catalytic efficiency of nucleotide incorporation by 2.5-fold on average and the wild-type UbPCNA protein increases it by 4.2-fold on average. Similar to what was observed with the non-damaged DNA, neither the E113G mutant PCNA protein nor the E113G mutant UbPCNA protein notably increase the catalytic efficiency of nucleotide incorporation by Pol η on non-damaged templates. Thus in the context of both non-damaged DNA and a non-cognate lesion, the E113G substitution interfered with the ability of PCNA and UbPCNA to increase the efficiency of Pol η . Moreover, this substitution also interferes with the ability of UbPCNA to increase the efficiency of incorporation to a greater extent than PCNA does.

3.6. Impact of the E113G mutant PCNA protein on the activity of Pol δ

Because the E113G substitution interferes with the ability of both PCNA and UbPCNA to increase the efficiency of nucleotide incorporation by the translesion polymerase Pol η , we next examined whether this substitution affects the activity of Pol δ , the prototypical replicative DNA polymerase. We first examined the ability of Pol δ to synthesize DNA on non-damaged DNA templates (Fig. 7A). In all cases (Pol δ alone, Pol δ with the wild-type PCNA protein, Pol δ with the wild-type UbPCNA protein, Pol δ with the E113G mutant PCNA protein, and Pol δ with the E113G mutant UbPCNA protein), robust DNA synthesis

by Pol δ was clearly visible with many of the products extending to the end of the DNA template. Again, because these experiments were carried out in the presence of an excess of unlabeled competitor DNA, it was possible to measure the processivity of Pol δ (Table S5 in Supplemental Material). In all cases, 70 to 80 % of the extension products resulted from at least four incorporations, and 50 to 60 % of the products were full length. Because Pol δ synthesizes with such high processivity under these conditions, we cannot conclude that the E113G mutant PCNA and UvPCNA proteins increase the processivity of Pol δ , but we can conclude that they do not reduce it.

We next examined the activity and the processivity of Pol δ on the abasic site-containing DNA substrate (Fig. 7B and Table S5 in Supplemental Material). In the absence of PCNA, 50 % of the extension products resulted from DNA synthesis up and opposite the abasic site. In all other cases, 70 to 80 % of the extension products resulted from DNA synthesis up to the abasic site, and 60 to 70 % of the products resulted from incorporation opposite the abasic site. In addition, in all cases, 30 to 40 % of the products were full length. Again, because Pol δ synthesizes with such high processivity, we can only conclude that the E113G mutant PCNA and UvPCNA proteins do not reduce the processivity of Pol δ .

3.7. Impact of the E113G mutant PCNA protein on the catalytic efficiency of Pol δ

We next examined whether this substitution interferes with the ability of PCNA and UvPCNA to increase the efficiency of Pol δ (Fig. 8 and Table S6 in Supplemental Material). For the non-damaged template, the wild-type PCNA protein increases the catalytic efficiency of nucleotide incorporation by 10-fold on average and the wild-type UvPCNA protein increases it by 20-fold on average. The E113G mutant PCNA protein increases the catalytic efficiency of nucleotide incorporation by 6.7-fold on average and the E113G mutant UvPCNA protein increases it by 4.8-fold on average. Although the E113G substitution in PCNA and UvPCNA does cause a reduction in the efficiency of nucleotide incorporation by Pol δ , the mutant PCNA and UvPCNA proteins still significantly increase the efficiency of nucleotide incorporation. Thus, the impact of this substitution on the activity of replicative Pol δ on non-damaged DNA templates is quite different from the impact on the activity of translesion Pol η .

We next examined the impact of the E113G substitutions on the ability of PCNA and UvPCNA to increase the catalytic efficiency of nucleotide incorporation by replicative polymerases when they are confronted with a kinetic barrier, such as a DNA lesion. Abasic sites significantly reduce the efficiency of nucleotide incorporation by Pol δ , but the presence of PCNA increases this efficiency [46]. Thus, we examined whether the E113G substitution interferes with the ability of PCNA and UvPCNA to increase the efficiency of nucleotide incorporation opposite abasic sites by Pol δ . We found that the wild-type PCNA protein increases the catalytic efficiency of nucleotide incorporation by 14-fold on average and the wild-type UvPCNA protein increases it by 20-fold on average. By contrast, the E113G mutant PCNA protein increases the catalytic efficiency of nucleotide incorporation by only 3.2-fold on average and the E113G mutant UvPCNA protein increases it by only 3.6-fold on average. Therefore, the E113G substitution does interfere with the ability of both PCNA and UvPCNA to increase the efficiency of incorporation opposite abasic sites by Pol δ . Moreover, this effect is more pronounced than the effect on nucleotide incorporation opposite non-damaged templates by Pol δ .

3.8. Impact of the G178S mutant PCNA protein on the activity of Pol δ

Finally, because the G178S mutant PCNA protein exists primarily in the monomeric form, we examined whether this substitution interferes with the ability of PCNA to increase the activity of replicative Pol δ on non-damaged DNA templates (Fig. S2 in Supplemental

Material). Interestingly, the G178S mutant PCNA protein does not stimulate the activity of Pol δ to the same degree as does the wild-type or the E113G mutant PCNA proteins. In fact, the amount of DNA synthesis observed in the presence of the G178S mutant PCNA protein is approximately the same as that observed in the absence of PCNA. This is surprising because yeast producing the wild-type PCNA protein and yeast producing the G178S mutant PCNA protein grow at the same rate [38]. This suggests either that the presence of other replication fork components help stabilize the trimeric form of the G178S mutant PCNA protein *in vivo* or that the slower rate of DNA synthesis by Pol δ in the presence of the G178S mutant PCNA protein does not limit the cell cycle in yeast.

4. Discussion

4.1. Inhibition of TLS in yeast by mutant PCNA proteins

Sporadic mutations play an important role in causing a wide range of diseases, such as cancer, autism, diabetes, and schizophrenia [47–51]. Because many mutations results from TLS, it is important to understand how this process can be inhibited. Here we focused on two variant forms of PCNA that block TLS and thereby mutagenesis in yeast without affecting normal replication and cell growth [38–40]. The E113G and the G178S amino acid substitutions are adjacent to one another at the subunit interface of PCNA. Although both substitutions inhibit TLS by translesion polymerases Rev1 and Pol ζ , there is some disagreement regarding their impact on Pol η . It has been suggested that the G178S mutant PCNA protein blocks TLS by Pol η [38], whereas the E113G mutant protein does not [52]. We believe that these differences are overstated. For example, the lack of inhibition of Pol η -dependent TLS by the E113G substitution is based on epistasis analysis showing that the UV sensitivity of strains producing the E113G mutant protein is made greater by the lack of Pol η [52]. However, the difference in UV sensitivities in the absence and presence of Pol η is very slight and is only apparent at the highest dose of UV radiation used in the assay. Moreover, it should be noted that this result only shows that Pol η is functional to some extent in these cells; this result is consistent with a partial defect in Pol η -dependent TLS. In any case, our findings reported here clearly show that the E113G mutant PCNA protein does not support TLS by Pol η *in vitro*. Therefore, E113G and G178S substitutions likely inhibit TLS by the same underlying mechanism.

4.2. Mechanism of TLS inhibition by mutant PCNA proteins

The mechanism by which these two mutant PCNA proteins block TLS in cells remains unclear. It was previously shown that the E113G mutant PCNA protein is ubiquitinated in response to DNA damage [40]. This shows that the inhibition of TLS is not due to an inability of these PCNA mutant proteins to become modified with ubiquitin. It was also reported that the translesion DNA polymerases Rev1 and Pol ζ do not physically interact with the E113G mutant PCNA protein *in vitro* [52]. This lead to the view that the inhibition of TLS is due to the inability of these polymerases to interact with the mutant PCNA protein [52]. It is not known, however, whether these polymerases fail to interact with the mutant UbPCNA protein, which is the biologically relevant state. This is particularly important given that translesion polymerases possess ubiquitin-binding motifs for interacting with the ubiquitin moiety on UbPCNA [37]. In any event, we have shown here that translesion Pol η binds the wild-type and the E113G mutant PCNA proteins with the same affinity. Others have shown that the E113G mutant PCNA protein binds replicative Pol δ [52]. Thus, although a lack of binding may explain the inability of this mutant protein to support TLS by translesion polymerases Rev1 and Pol ζ , it does not explain the inability to support TLS by Pol η and Pol δ that we observed here.

PCNA has been shown to stimulate nucleotide incorporation by Pol η on both non-damaged and damaged DNA templates [35, 41], and UbPCNA stimulates this activity even further [43, 53]. One of the objectives of this study was to assess the impact of the G178S and E113G amino acid substitutions on the ability of PCNA and UbPCNA to stimulate the activity of Pol η . Because of the high instability of the G178S mutant UbPCNA protein, we have focused on the E113G substitution. We showed that the E113G mutant PCNA protein does not increase the efficiency of nucleotide incorporation by Pol η in either the absence or presence of ubiquitylation. This finding has two direct implications. First, the effect of this substitution on the activity of Pol η occurs even with the non-ubiquitylated form of PCNA. Second, the additional ability to stimulate the activity of Pol η afforded to PCNA by its mono-ubiquitylation is entirely eliminated by this substitution. Overall, these findings show that these mutant PCNA proteins blocks TLS by failing to form productive complexes with translesion polymerases that stimulate their activity.

4.3. Inhibition of TLS is caused by PCNA trimer instability

The structures of the G178S and E113G mutant PCNA proteins have been determined [41, 42]. It was suggested based on these structures that the aberrant position of loop J (residues 105 to 110), which is immediately adjacent to the subunit interface, might be responsible for the inability of these mutant proteins to support the activity of translesion polymerases. It was suggested that this loop may be a key binding site for translesion polymerases. However, we have re-examined these structures and noted that the separation of the β -strands constituting the subunit interface is a more substantial change than the movement of loop J. In fact, the separation of these β -strands is directly responsible for the movement of loop J in these mutant proteins. We also found that this structural perturbation causes a decrease in trimer stability, which we believe to be the cause for the inability of the mutant forms of PCNA to facilitate TLS. It should be noted that although these decreases in trimer stability are sufficient to inhibit TLS, they are not severe enough to cause a defect in normal DNA replication and cell growth. In further support of this notion, we found that TLS by replicative Pol δ is also impacted by the E113G substitution as well. It is unlikely that replicative Pol δ and all translesion polymerases share the same key binding site at the subunit interface. It is more likely that the effect of these mutant proteins on TLS is an indirect effect mediated by their trimer instability. Finally, we have carried out random mutagenesis of the PCNA subunit interface, and we found that these mutant PCNA proteins lead to a range of phenotypes. While we found that some of these mutant PCNA proteins cause defects in cell growth, we identified over ten additional mutant proteins that have no impact on cell growth, but block TLS *in vivo* (C. M. Kondratick and M. T. Washington, unpublished data). This further confirms the importance of the integrity of the PCNA subunit interface and trimer stability for TLS.

4.4. Model of TLS inhibition by PCNA trimer instability

To understand how PCNA trimer instability inhibits TLS by both replicative and translesion polymerases alike, we must first consider how the wild-type PCNA protein increases the catalytic efficiency of replicative and translesion polymerases. The catalytic efficiency is determined by the individual steps of the overall polymerase reaction, especially the nucleotide-binding step and the nucleotide-incorporation step. Thus PCNA must either increase the binding affinity for the incoming nucleotide, increase the rate of the nucleotide-incorporation step, or both. The only way that PCNA can have such an effect is by maintaining the polymerases in a more catalytically competent conformational state – *i.e.*, one that either binds the incoming nucleotide tighter, incorporates it faster, or both. Interestingly, PCNA must be able to maintain polymerases in this more catalytically competent state for different periods of time depending on the DNA polymerase and the template. In the case of fast nucleotide incorporation, such as with replicative polymerases

on non-damaged templates, PCNA would only need to maintain the polymerase in the more competent state for a very brief time. In the case of slow nucleotide incorporation, such as with both replicative and translesion polymerases on damaged templates, PCNA would need to maintain the polymerase in this state significantly longer.

Based on these considerations, we propose that a moderate degree of trimer instability is causing the mutant PCNA proteins to occasionally undergo a conformational change that is compromising its ability to maintain the more catalytically competent state of the bound polymerase. This might involve a transient opening of the PCNA ring at the subunit interface, but other possibilities exist. In any event, in cases where PCNA only needs to maintain the competent state for a short period of time (such as a replicative polymerase with non-damaged templates), the likelihood of the mutant PCNA protein undergoing this conformational change and disrupting the competent state of the polymerase is small. Consequently, normal replication is largely unaffected. However, in cases where PCNA needs to maintain the competent state for a longer period of time (such as a replicative polymerase with damaged templates or a translesion polymerase with either non-damaged or damaged templates), the likelihood of the mutant PCNA protein undergoing this conformational change and disrupting the competent state of the polymerase is much greater. While this model nicely explains all of our observations, more work on the structure and dynamics of the wild-type and mutant PCNA proteins and their complexes with DNA polymerases will be necessary to fully understand the mechanism of TLS inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

The abbreviations used are:

dNTP	deoxynucleoside triphosphate
EDTA	ethylenediamine tetracetic acid
ELISA	enzyme-linked immunosorbent assay
PCNA	proliferating cell nuclear antigen
Pol	polymerase
TLS	translesion synthesis
UbPCNA	ubiquitin-modified proliferating cell nuclear antigen
8-oxoG	8-oxoguanine

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Highlights

The E113G and G178S PCNA mutants inhibit translesion synthesis and mutagenesis

Both PCNA mutants have disruptions at the subunit interface causing trimer instability

The E113G mutant does not stimulate translesion synthesis by either Pol δ or Pol η

Replication by Pol η was impacted, but replication by Pol δ was much less affected

Trimer instability affects polymerases when nucleotide incorporation is inefficient

Figure 1. The subunit interface of the wild-type and mutant PCNA proteins

Structures of the subunit interface of the wild-type PCNA protein (1PLQ.pdb), the E113G mutant PCNA protein (3GPM.pdb), and the G178S mutant PCNA protein (3F1W.pdb). β -strand I_1 (residues 110 to 117) is shown in blue and β -strand D_2 (residues 175 to 182) is shown in red. The dashed lines show the positions of the backbone hydrogen bonds that constitute the PCNA subunit interface. The distances between the backbone hydrogen bond donors and acceptors are provided in Table S1 in Supplemental Material.

Figure 2. Analysis of the wild-type and mutant PCNA proteins by native gel electrophoresis (A) Coomassie stained non-denaturing polyacrylamide gradient gel (4 to 20 %) in which solutions of the wild-type and mutant PCNA proteins (0.05 to 0.2 mg/ml) were run. The positions of the PCNA monomer and PCNA trimer are shown. (B) Coomassie stained non-denaturing polyacrylamide gel in which solutions of the wild-type and mutant PCNA proteins (0.50 to 5.0 mg/ml) were run. The positions of the PCNA monomer and PCNA trimer are shown.

Figure 3. Analysis of the wild-type and mutant PCNA proteins by size exclusion chromatography (A) Elution profiles of a size exclusion chromatography column in which 0.005 mg/ml (●), 0.01 mg/ml (▼), or 0.1 mg/ml (■) solutions of the wild-type PCNA protein were run. The amounts of the monomeric and trimeric species calculated from the area under these curves are provided in Table S2 in Supplemental Material. (B) Elution profiles of 0.005 mg/ml (●), 0.01 mg/ml (▼), or 0.1 mg/ml (■) solutions of the G178S mutant PCNA protein. The amounts of the monomeric and trimeric species are provided in Table S2. (C) Elution profiles of 0.005 mg/ml (●), 0.01 mg/ml (▼), or 0.1 mg/ml (■) solutions of the wild-type

PCNA protein. The amounts of the monomeric and trimeric species are provided in Table S2.

Figure 4. Interaction of the wild-type and mutant PCNA and UbPCNA proteins with Pol η
(A) Results of an ELISA assay showing the interaction of the wild-type PCNA protein (●) and the E113G mutant PCNA protein (○) with Pol η . (B) Results of an ELISA assay showing the interaction of the wild-type UbPCNA protein (●) and the E113G mutant UbPCNA protein (○) with Pol η .

Figure 5. Processive DNA synthesis by Pol η in the presence of the wild-type and mutant PCNA proteins

(A) Autoradiogram of the extension products of Pol η on a non-damaged DNA template in the absence of PCNA (N) or in the presence of the wild-type PCNA protein (P), the wild-type UbPCNA protein (U), the E113G mutant PCNA protein (P*), or the E113G mutant UbPCNA protein (U*). The percentages of extension products at least 4 nt. in length, at least 9 nt. in length, or full length are provided in Table S3 in Supplemental Material. (B) Autoradiogram of the extension products of Pol η on a DNA template containing an abasic site in the absence of and presence of the wild-type and E113G mutant PCNA and UbPCNA proteins. The gel band representing extension products 6 nt. in length, which corresponds to incorporation opposite the abasic site, is indicated by the arrow. The percentages of extension products at least 5 nt. in length, at least 6 nt. in length, or full length are provided in Table S3 in Supplemental Material.

Figure 6. Steady state kinetics of Pol η in the presence of the wild-type and mutant PCNA proteins

(A) The catalytic efficiency ($V_{\max}/K_m$) of nucleotide incorporation by Pol η on a non-damaged template G in the absence of PCNA (N) or in the presence of the wild-type PCNA protein (P), the wild-type UbPCNA protein (U), the E113G mutant PCNA protein (P*), or the E113G mutant UbPCNA protein (U*). The individual $V_{\max}$ and K_m parameters are provided in Table S4 in Supplemental Material. **(B)** The catalytic efficiency of incorporation by Pol η on a template abasic site in the absence of and presence of the wild-type and E113G mutant PCNA and UbPCNA proteins. The $V_{\max}$ and K_m parameters are provided in Table S4 in Supplemental Material.

Figure 7. Processive DNA synthesis by Pol δ in the presence of the wild-type and mutant PCNA proteins

(A) Autoradiogram of the extension products of Pol δ on a non-damaged DNA template in the absence of PCNA (N) or in the presence of the wild-type PCNA protein (P), the wild-type UbPCNA protein (U), the E113G mutant PCNA protein (P*), or the E113G mutant UbPCNA protein (U*). The percentages of extension products at least 4 nt. in length, at least 9 nt. in length, or full length are provided in Table S5 in Supplemental Material. (B) Autoradiogram of the extension products of Pol δ on a DNA template containing an abasic site in the absence of and presence of the wild-type and E113G mutant PCNA and UbPCNA proteins. The gel band representing extension products 6 nt. in length, which corresponds to incorporation opposite the abasic site, is indicated by the arrow. The percentages of extension products at least 5 nt. in length, at least 6 nt. in length, or full length are provided in Table S5 in Supplemental Material.

Figure 8. Steady state kinetics of Pol δ in the presence of the wild-type and mutant PCNA proteins

(**A**) The catalytic efficiency ($V_{\max}/K_m$) of nucleotide incorporation by Pol δ on a non-damaged template G in the absence of PCNA (N) or in the presence of the wild-type PCNA protein (P), the wild-type UbPCNA protein (U), the E113G mutant PCNA protein (P*), or the E113G mutant UbPCNA protein (U*). The individual $V_{\max}$ and K_m parameters are provided in Table S6 in Supplemental Material. (**B**) The catalytic efficiency of incorporation by Pol δ on a template abasic site in the absence of and presence of the wild-type and E113G mutant PCNA and UbPCNA proteins. The $V_{\max}$ and K_m parameters are provided in Table S6 in Supplemental Material.