A Single Amino Acid Change (E85K) in Human PCNA That Leads, Relative to Wild Type, to Enhanced DNA Synthesis by DNA Polymerase δ past Nucleotide Base Lesions (TLS) as Well as on Unmodified Templates†

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ABSTRACT: Human proliferating cell nuclear antigen (hPCNA) containing a single amino acid substitution at position 85, that of lysine for glutamate (E85K), was compared to wild-type (wt) hPCNA for its ability to promote DNA synthesis by purified DNA polymerase δ (pol δ) both on unmodified templates and past chemically defined template base lesions (translesion synthesis; TLS). Significant enhancement (up to 4−5-fold or greater) was seen but depended both on the exact PCNA/pol δ ratio tested and on the specific nature of the template (e.g., unmodified versus lesion-containing; chemical nature of the template base lesion). These results suggest that human PCNA, either mutated to contain lysine (K) at position 85 or bearing similar primary mutations, would promote more secondary mutagenesis in cells and/or tissues where PCNA is normally expressed at low levels relative to pol δ. Over an entire lifetime, such secondary mutagenesis could be biomedically significant.

DNA polymerase δ (pol δ)[1] is a eukaryotic enzyme essential for both DNA replication and repair (for a review, see ref *1*). Proliferating cell nuclear antigen (PCNA) acts as an auxiliary factor to increase the binding of pol δ to template-primer DNA by at least ∼2000-fold (*2*). We suggest that this dramatically increases the processivity of pol δ-catalyzed DNA synthesis. Processivity is defined as the number of nucleotides incorporated by the polymerase per binding event. We also showed that, in the PCNA·pol δ· template-primer complex, PCNA resides "behind" pol δ while DNA synthesis takes place in "front" (*3*).

The stimulation of pol δ by PCNA, although essential, appears costly. PCNA also acts on purified pol δ to promote several relatively unfavorable events, including both nucleotide analogue incorporation/nucleotide misincorporation (*4*) and DNA synthesis past many otherwise blocking template base lesions (*5*, *6*). In the presence of unmutated (wild type; wt) human PCNA, calf thymus pol δ is 1−2 orders of magnitude more error-prone than pol δ alone. It was speculated that this putative "mutator" effect of PCNA arises from the fundamental mechanism of polymerase stimulation, namely, the PCNA-mediated enhancement of the binding of pol δ to DNA template-primers, and is hence unavoidable. Recent mutagenesis results with *Drosophila* PCNA corroborate this suggestion (*7*).

As a corollary, we suggested that certain mutations in human PCNA (primary mutations) might enhance PCNA function and thereby enhance this putative mutator effect, leading to what would be, in essence, secondary mutations. In other words, such PCNA "gain-of-function" primary mutations would be expected to enhance pol δ activity as well as processivity and, concomitantly, to enhance DNA synthesis beyond template base lesions (translesion synthesis; TLS). This last property, if manifest in vivo, would lead to secondary mutations and, hence, to a mutator phenotype. It was shown by others that yeast cells harboring PCNA primary mutations indeed displayed a mutator phenotype (*8*−*12*). PCNA appeared to promote mutagenesis by a variety of mechanisms, all distinct from those we propose here. It was also suggested by others that pol δ was involved in bypass of template lesions in living human cells (*13*, *14*).

In our laboratory, a novel screening assay was developed to identify human PCNA (hPCNA) mutants that promoted enhanced TLS by calf thymus pol δ (*15*). Although several single amino acid substitution mutations were identified in this way, one molecule, human PCNA containing lysine at position 85 instead of glutamate (E85K hPCNA), was expressed in large quantities and purified to apparent homogeneity for detailed biochemical comparison with wt hPCNA. We found that E85K hPCNA promoted enhanced pol δ activity on at least one model substrate as well as enhanced processivity on another and, as expected, enhanced TLS, particularly at relatively low PCNA/pol δ ratios. This last, varied TLS at various PCNA/pol δ ratios, has clear

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[1] Abbreviations: pol δ, DNA polymerase δ; PCNA, proliferating cell nuclear antigen; hPCNA, human proliferating cell nuclear antigen; TLS, translesion synthesis; E85K, glutamate at position 85 mutated to lysine; mAb, monoclonal antibody; wt, wild type; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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implications for tissue-specific effects. These implications are discussed.

EXPERIMENTAL PROCEDURES

Many of the materials and much of the methodology were detailed previously (*2*−*4*, *6*, *15*−*18*). Oligonucleotides were 5′-end-labeled with T4 polynucleotide kinase (New England BioLabs) in the presence of [γ-^{32}P]ATP (ICN Biomedical or NEN Research Products). Unlabeled nucleotides and deoxynucleotides were from Invitrogen. Acrylamide and methylenebis(acrylamide) were from Eastman Organic Chemicals; they were further purified for SDS−PAGE of proteins by adsorption of impurities to activated charcoal and for urea−PAGE of synthetic oligonucleotides by adsorption to an ion-exchange resin. All other chemicals were of reagent grade and were used without further purification.

Proteins. DNA polymerase δ (composed of two subunits, p125 and p50) was purified to apparent homogeneity from calf thymus according to published procedures (*17*, *19*). cDNA coding for either wt (sequence of ^{361}GCGCCGGCAATGAAGATATCATT around the critical position of the coding strand of PCNA cDNA; the nucleotide to be mutated is underlined) or E85K (sequence of ^{361}GCGCCGGCAATAAAGATATCATT around the critical position; the mutated nucleotide is underlined) mutant human PCNA was cloned into pQE30, a bacterial expression vector conferring an N-terminal His tag; both proteins were purified from an *Escherichia coli* extract essentially as previously described (*20*) but as recently modified for His-tagged macromolecules (*15*). Mouse monoclonal anti-PCNA antibody (mAb) PC10 was from Oncogene Sciences (Uniondale, NY). Protein structure data were analyzed using PyMOL software (DeLano Scientific).

Immunoblot Analyses. Immunoblot analysis using the Odyssey IR imaging system was essentially as specified by the manufacturer (LI-COR Biosciences, Lincoln, NB) using mouse mAb PC10 for detection of PCNA. Standard blotting and blot processing procedures were otherwise used (e.g., see ref *21*). After sedimentation, virtually the entirety of each fraction was first SDS-denatured, then concentrated by precipitation with trichloroacetic acid, resolubilized, and finally subjected to SDS−PAGE and immunoblot analysis.

Nucleic Acids. Oligonucleotide templates and primers of defined sequence were synthesized using conventional phosphoramidite chemistry by Dr. F. Johnson and colleagues (Stony Brook). Before use, all primers were purified by standard denaturing PAGE (*22*). Where indicated, templates contained, at defined positions, a modified tetrahydofuran moiety (model abasic site) (*23*) or an 8-oxodeoxyguanosine residue (*24*). A single template contained, at most, one such modified base. DNase I "activated" DNA was prepared essentially as described (*17*, *25*).

DNA Polymerase Incubations. Assays of pol δ activity were performed essentially as previously described (*17*) and explicitly as detailed in the figure legends. Briefly, the standard reaction mixture (50 μL final volume) contained 40 mM Bis-Tris, pH 6.7, 2 mM dithiothreitol, 10% glycerol, 40 μg/mL bovine serum albumin, 40 μM each deoxyribonucleoside triphosphate, 2 mM adenosine monophosphate (AMP), and 2 mM ethylenediaminetetraacetic acid (EDTA). Additional details are provided in the figure legends. Sample incubation was initiated by addition of $MgCl_2$ to a final concentration of 8 mM, after which each was incubated for 10 min at 37 °C. Each reaction also contained ∼1 ng of purified pol δ. Hence the molar ratio of PCNA:pol δ, assuming native masses of 90 and 175 kDa, respectively, ranged from ∼4.3- to 142-fold. In other words, at all PCNA:pol δ ratios where data were analyzed fully, PCNA was present in molar excess. Other components were as indicated on the figures. Before incubation and except where indicated, the primer strands of nucleic acid template-primers were 5′-^{32}P-labeled with bacteriophage T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Primers and templates were annealed essentially as previously described (*26*). After incubation, reaction products (5 μL from each reaction; 10% of each reaction) were subjected to standard denaturing PAGE (*22*); they were visualized and quantified with a Molecular Dynamics 445 SI PhosphorImager using ImageQuant software (Molecular Dynamics).

Sedimentation. For sedimentation velocity analyses, linear 3.75 mL 20−40% glycerol gradients were formed over 0.1 mL of 80% glycerol in 11 × 60 mm Beckman polyallomer ultracentrifuge tubes. All solutions contained, in addition to glycerol, 40 mM Bis-Tris, pH 6.8, 1 mM dithiothreitol, 6 mM $MgCl_2$, and 40 μg/mL BSA. Samples of 200 μL each and each containing 4.0 ng of highly purified PCNA (0.02 μg/mL or 0.7 nM, a concentration in the range of the lower concentrations of PCNA used in our enzymologic assays) were loaded and subjected to centrifugation at 54000 rpm for 16.3 h at 4 °C using a SW60.1 rotor. Fractions were collected from the bottom of each tube and subjected to standard trichloroacetic acid precipitation, iodoacetamide treatment, and SDS−PAGE. PCNAs, both wild type and the E85K mutant, showed similar reactivity with mAb PC10 and were detected (after acid precipitation, iodoacetamide treatment, and SDS−PAGE) by immunoblot analysis. Sedimentation standards were detected by Coomassie blue staining.

Mass Spectroscopy. Samples were run on a Voyager-DE STR (Applied Biosystems, Framingham, MA) MALDI-TOF mass spectrometer system operated in the linear mode. Proteins were dissolved in a 50% solution of ANC/0.3% TFA containing sinapinic acid (5 mg/mL) and dried on a sample plate. A nitrogen laser operating at 337 nm with a 3 ns pulse rate was employed. The accelerating voltage was set to 25 kV, and a delay of 750 ns was used to accelerate ions into the flight tube of the mass spectrometer. The mass scale was calibrated with bovine serum albumin (400 fmol/μL), and ∼100 laser shots were used to produce each spectrum. These experiments were repeated twice.

RESULTS

Structure, Purity, and Immunoreactivity of both wt and Mutant hPCNA. Shown (Figure 1A,B) are X-ray cystallographically determined structures of human PCNA reported by Gulbis et al. (*27*). Views both end-on (Figure 1A) and rotated 90° to display a side view (Figure 1B) are presented. On each, the location of the E85K mutation is indicated. From the end-on view (Figure 1A), it can be appreciated that E85K affects the "inner ring" of PCNA, that region of the molecule thought to be in intimate contact with the double-stranded DNA template-primer situated behind the polymerase. It is also close to the monomer−monomer

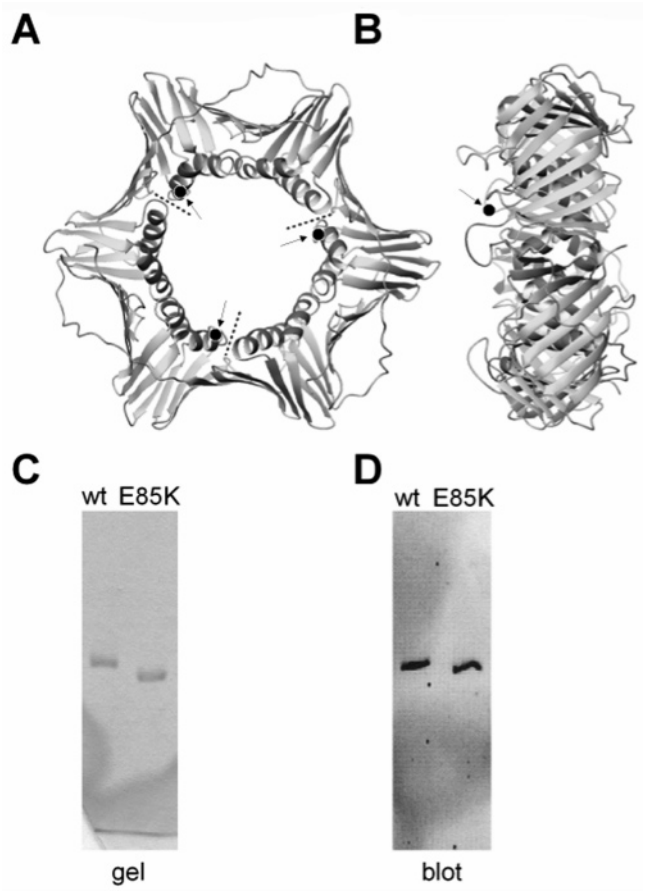

FIGURE 1: X-ray crystal structure showing both the (A) cross section and (B) side view of PCNA and including position of E85K (●), In (A), broken lines are drawn between different but adjacent subunits in the crystal. (C) Coomassie blue staining after SDS−10% PAGE showing the purity of both wt and E85K mutant PCNA. Also see Figure 6C of Zaika et al. (*15*). Migration relative to size standards indicated an apparent mass of ~36 kDa. Each lane was loaded with 140 ng of protein. (D) Immunoblot of samples subjected to SDS−10% PAGE in parallel with those shown in (C). Each lane was loaded with 4 ng of PCNA, either wt or the E85K mutant, as indicated.

interface. From the side view (Figure 1B), it is clear that the E85K mutation is located on the "back" side of PCNA, facing away from the direction of polymerase-catalyzed DNA synthesis.

Both wt and E85K-containing human PCNA were engineered for bacterial expression and purified to near homogeneity from *E. coli* extracts. After purification, samples of each were subjected to SDS−PAGE followed by Coomassie blue staining. The purity of each was demonstrated, although slightly different SDS−PAGE mobilities were seen for the wt versus mutant polypeptides (Figure 1C). This mobility difference was reported previously (*15*). By mass spectroscopy, each polypeptide was similar [30075.6 Da for the wt; 30055.3 Da for the mutant; 30083.8 Da predicted for the His-tagged wt construct used, assuming a subunit mass of 29261 Da (*28*)] (not shown; representative results from a single experiment are reported). Each showed similar reactivity with mouse mAb PC10 (Figure 1D).

We think it important to note that direct comparison of Coomassie blue stain versus immunoblot mobilities cannot be reliably performed on the basis of the data in Figure 1C,D; it is made more complicated by the slight distortion of the

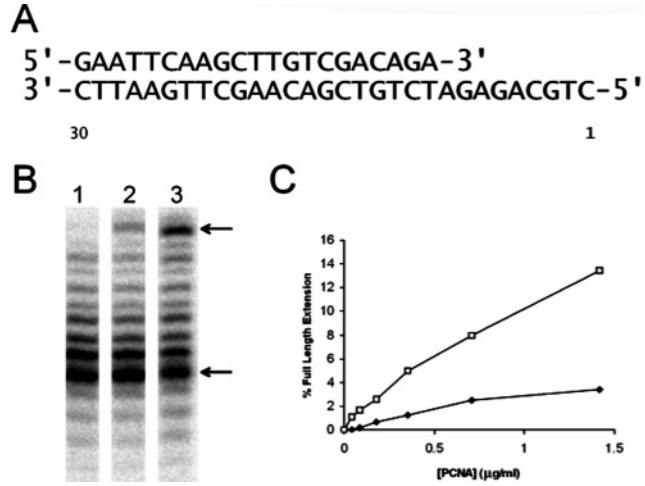

FIGURE 2: Effect of both wt and E85K hPCNA on activity with an unmodified template. (A) The sequence as well as the annealed structure of the 30−21-mer template-primer substrate is shown. The 21-mer primer was 5′-^{32}P-labeled for detection. Activity was monitored by primer extension after standard urea−PAGE and PhosphorImager analysis. (B) Representative primary data from the experiment plotted in (C) are shown, both without PCNA (lane 1) and after addition of either 1.42 μg/mL wt (lane 2) or 1.42 μg/mL E85K hPCNA (lane 3). (C) Graphical quantification comparing stimulation of purified calf thymus pol δ by either wt (◆) or E85K hPCNA (□). Only the two species indicated by arrows in (B) were quantified; quantification was performed for all amounts of PCNA added as indicated.

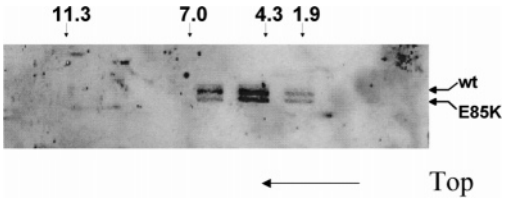

FIGURE 3: Cosedimentation of wt and E85K hPCNA. Positions of sedimentation standards (*S*-values) are as indicated by down-pointing arrows above the immunoblot shown. Positions of standard proteins were determined by SDS−PAGE and Coomassie blue staining. hPCNA, both wt and E85K mutant, was detected by immunoblot analysis with mAb PC10 after SDS−10% PAGE. Only the blot region of interest is shown.

bands shown, particularly evident upon immunoblot examination (Figure 1D). In our experience, there is no difference between Coomassie blue mobility and immunoblot mobility, for either wt or E85K PCNA (not shown). To compare SDS−PAGE migration of wt versus E85K-containing hPCNA, subjecting both to electrophoresis in the same lane of a polyacrylamide gel is far more effective (see Figure 3). The two can clearly be distinguished as shown (Figure 3).

Stimulation of Calf Thymus DNA Polymerase δ by wt versus E85K Mutant hPCNA on Unmodified (Nonlesion-Containing) Template-Primers. To explore the in vitro activities of wt versus E85K hPCNA, assays with highly purified calf thymus pol δ (two-subunit form) were performed. We determined the ability, as a function of PCNA concentration, of a constant amount of pol δ to extend a 5′-^{32}P-labeled 21-mer primer annealed to an unmodified (nonlesion-containing) 30-mer synthetic oligonucleotide template. Since ~1.0 ng of pol δ was included in each 50 μL incubation, PCNA was always present in considerable molar excess, even at the lowest concentration of PCNA used (a

minimum of more than 4-fold assuming a mass for the PCNA trimer of ~90000 Da). Results indicated that, using an appropriately primed, unmodified synthetic oligonucleotide template (structure shown in Figure 2A) and at all levels of PCNA tested, E85K hPCNA was considerably more effective than wt at stimulating the activity of calf thymus pol δ (Figure 2B,C). Stimulation by the E85K mutant above wt hPCNA was consistently about 4-fold. Representative results from a single analysis are shown. To ensure reproducibility, experiments were repeated three times.

Curiously the effect of wt versus E85K hPCNA on calf thymus pol δ was not seen with DNase I activated DNA. We think it noteworthy that activated DNA is generally not used to demonstrate stimulation of pol δ by PCNA, since this substrate contains only limited and highly variable lengths of single-stranded template DNA. Stimulation is hence minimal and very difficult to reproduce among different laboratories. Nevertheless, with this otherwise commonly used substrate, wt and E85K hPCNA both stimulated pol δ activity substantially (as much as 5-fold or more) and behaved similarly at several concentrations (not shown). PCNA is thought to function as a trimer. Our results with DNase I activated DNA hence exclude trivial explanations, such as effects of mutation on the PCNA monomer/dimer/trimer equilibrium, for the difference between wt and E85K hPCNA; such explanations (mechanisms) would be expected to affect activity on all substrates similarly (also see below for results with other template-primers).

Upon Ultracentrifugation under Conditions Used in Our Assays, both wt and hPCNA Containing the E85K Mutation Behave Similarly. We also used ultracentrifugation to compare the native (nondenatured) proteins directly; upon sedimentation through linear glycerol gradients, both wt and E85K hPCNA behave similarly (Figure 3). When subjected to sedimentation at extremely low protein concentrations, similar to those used in reactions with purified pol δ, PCNA sedimented at about 4.3 S (not shown). Indeed, it appeared from initial analyses that wt hPCNA sedimented slightly faster than its mutant counterpart. Because wt and E85K hPCNA migrate slightly differently upon 1D SDS−PAGE (*15*), we were able to sediment both together in the same ultracentrifuge tube and separately detect them subsequently. Results of this analysis (Figure 3) confirm the fact that wt sediments slightly ahead of E85K hPCNA. We noted that the recovery of E85K mutant PCNA was reproducibly somewhat less than seen for wt. This observation is of uncertain significance. Representative results from a single analysis are shown. This experiment was repeated twice.

The Biochemical Mechanism by Which both wt and hPCNA Containing the E85K Mutation Stimulate Purified Calf Thymus DNA Polymerase δ. To elucidate further, the biochemical mechanism by which, relative to wt, E85K hPCNA leads to increased stimulation of calf thymus pol δ, we investigated effects on polymerase processivity using a poly(dA)-oligo(dT) template-primer. Processsivity is defined as the number of nucleotides incorporated each time the polymerase binds its template-primer. wt PCNA is thought to stimulate pol δ by enhancing polymerase processivity through enhanced template-primer binding (see ref *29*; also see refs *2*, *17*, and *30*−*32*). As with the above investigation of pol δ activity on 30−21-mer (Figure 2), catalysis was monitored by denaturing PAGE using an extension of 5′-

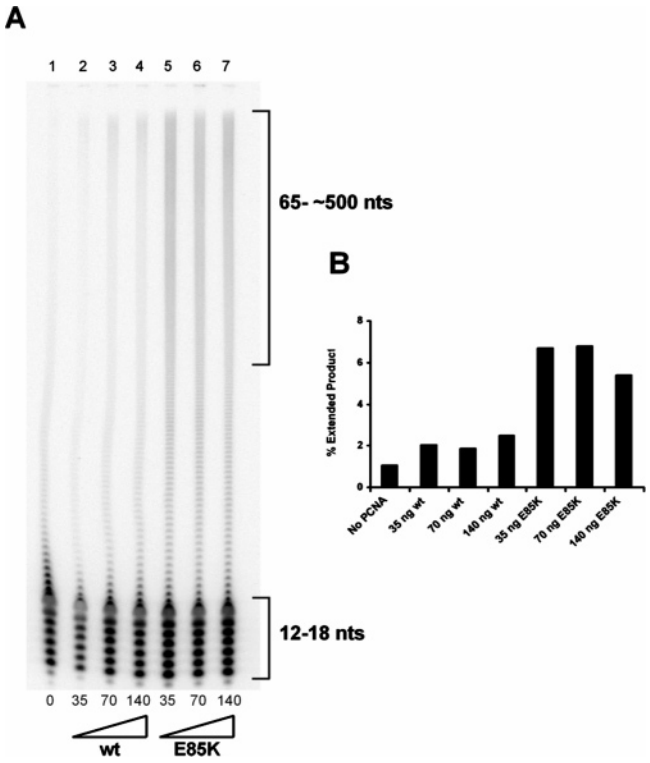

FIGURE 4: Effect of both wt and E85K hPCNA on pol δ processivity with a poly(dA)·oligo(dT) template-primer. All assays were performed with $(dA)_{\sim 500}$ annealed to $(dT)_{12-18}$ at a molar ratio (nucleotide) of 40:1. (A) After routine incubation in a final volume of 50 μL, 5 μL of each sample was subjected to standard urea−PAGE and PhosphorImager analysis. Individual incubations contained the following (per 50 μL total): lane 1, no PCNA; lane 2, 35 ng of wt hPCNA; lane 3, 70 ng of wt hPCNA; lane 4, 140 ng of wt hPCNA; lane 5, 35 ng of E85K hPCNA; lane 6, 70 ng of E85K hPCNA; lane 7, 140 ng of E85K hPCNA. (B) Data shown in (A) were quantified by first determining the amount of radioactivity in each of the two bracketed regions and then plotting, for each incubation condition, product in the 65−~500 nt region divided by product in the 65−~500 nt region plus unextended primer (in the 12−18 nt region). nt, nucleotide. PCNA added to each incubation was as indicated.

^{32}P-labeled primers with unlabeled dNMPs. Processivity was reflected by the average size of the product synthesized since incubations all contained a vast molar excess of template annealed to primer relative to both pol δ and PCNA. In addition, because end-labeled primers were used for detection, our analyses were unbiased; all products, both shorter and longer, were visualized with equal sensitivities. Processivity enhancement is dependent on the concentration of PCNA added; that can be seen for the wt protein (Figure 4A, lanes 2−4). Comparison with results obtained with E85K hPCNA (Figure 4A, lanes 5−7) suggests that processivity increases occur at significantly lower PCNA concentrations than with wt. This impression was obtained by visual inspection. To confirm, data shown in Figure 4A were quantified, thereby normalizing for the amount of material loaded in each lane, and plotted (Figure 4B). An identical conclusion was drawn; i.e., relative to wt, E85K hPCNA dramatically enhances the processivity of pol δ. Representative results from a single analysis are shown. This experiment was repeated twice.

Stimulation of Calf Thymus DNA Polymerase δ by wt versus E85K Mutant hPCNA on Primed, Lesion-Containing Templates. The behavior of wt versus E85K hPCNA on calf

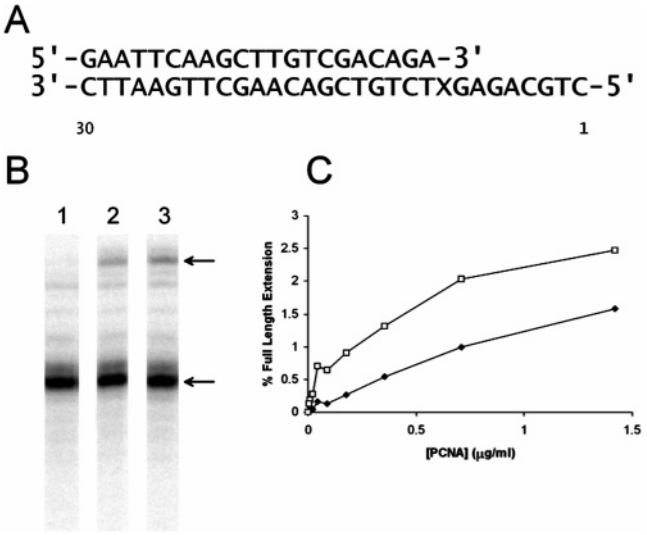

FIGURE 5: Effect of both wt and E85K hPCNA on TLS with 8-oxo-dGMP in the template. See the legend to Figure 2. (A) The 30−21-mer template-primer was used as substrate. X designates the 8-oxo-dGMP residue. The 21-mer primer was 5′-^{32}P-labeled, and primer extension activity was monitored by PhosphorImaging after standard urea−PAGE. (B) Representative data from the experiment plotted in (C), both without PCNA (lane 1) and after addition of either 1.42 μg/mL wt (lane 2) or 1.42 μg/mL E85K hPCNA (lane 3). (C) Stimulation of purified calf thymus pol δ by either wt (◆) or E85K hPCNA (□). Only the species indicated by arrows in (B) were quantified.

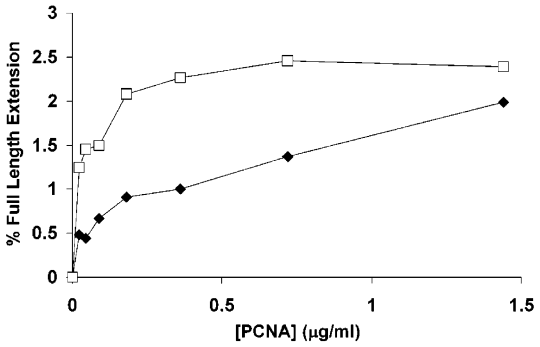

FIGURE 6: Effect of both wt and E85K hPCNA on TLS with the model abasic site in the template. The 30−21-mer template-primer shown in Figure 5A was used as substrate. X designates the abasic site. Stimulation of purified calf thymus pol δ was by either wt (◆) or E85K hPCNA (□); for details, see the legend for Figure 2C.

thymus pol δ was studied with two lesion-containing templates, one with an 8-oxo-dGMP residue in place of an unmodified dNMP (dAMP) at position 9 and the other with a modified tetrahyrofuran residue (the model abasic site hereafter referred to simply as an abasic site) at position 9 of the template. The precise structure of the template-primer used for each experiment is shown (Figure 5A). Results with the primed 8-oxo-dGMP-containing template are shown in Figure 5. Notably extended DNA synthesis beyond a template 8-oxo-dGMP, although highly dependent on PCNA as previously reported (*6*), was stimulated more at the lower PCNA concentrations when we compared the E85K mutant with wt (Figure 5C; 4.4-fold and 5.1-fold stimulation at 0.044 and 0.089 μg/mL PCNA, respectively). At higher PCNA concentrations, this difference between wt and the E85K mutant was substantially less (Figure 5C; 1.6-fold stimulation at 1.42 μg/mL PCNA). Representative results from a single analysis are shown. To ensure reproducibility, experiments were repeated three times.

The experiment shown in Figure 5 was performed with a primer terminated immediately before the template 8-oxo-dGMP. This resulted in analysis of pol δ-catalyzed incorporation from a so-called "standing start" on the DNA template-primer. To study the behavior of a polymerase potentially engaged in DNA synthesis before encountering a lesion, i.e., from a so-called "running start", the same primer was annealed to a 30-mer template containing an 8-oxo-dGMP residue at position 5 (instead of position 9). Results quite similar to those shown (Figure 5) were obtained (not shown).

Results with the primed abasic site-containing template are shown in Figure 6. As with the 8-oxo-dGMP-containing template, DNA synthesis beyond the lesion was stimulated most when we compared the E85K mutant with wt at low PCNA concentrations (Figure 6; 3.2-fold stimulation at 0.044 μg/mL PCNA versus 1.2-fold at 1.42 μg/mL). The experiment shown in Figure 6 was performed with a primer terminated immediately before the template abasic site. Representative results from a single analysis are shown. To ensure reproducibility, experiments were repeated three times. The same primer was annealed to the 30-mer template containing an abasic site at position 5. Results similar to those shown (Figure 6) were obtained (not shown).

DISCUSSION

We here compare wt human PCNA with mutant PCNA expressed from a human PCNA cDNA clone bearing a single nucleotide point mutation (G372A) leading to the single amino acid replacement of lysine for glutamate at position 85 (E85K hPCNA). Glutamate 85 is located on the inner ring of PCNA facing away from both pol δ and the direction of DNA synthesis (Figure 1). On the basis of this location and the chemical nature of the change (negative charge being changed to positive), we would speculate that E85K hPCNA binds more tightly to DNA than does wt and, thereby, forms a more stable pol δ·PCNA·template-primer complex. We suggested this previously (*15*). As with enzyme-catalyzed reactions in general (*33*), a more stable pol δ·PCNA·template-primer complex can lead either to an increase in net activity due to increased enzyme productivity or to an activity decrease due to enzyme trapping in an overly stable enzyme·substrate complex. Specifically in the case of DNA polymerase δ, an overly stable pol δ·PCNA·template-primer complex could result in inhibition of enzyme translocation and diminution of product production. Also unique to a DNA polymerase, such effects may be nucleic acid template-primer-specific. For example, it seems likely that activity could be stimulated on one substrate (such as the 30−21-mer used here; see below) and not on another (e.g., DNase I activated DNA).

In the present study, both E85K and wt hPCNA stimulated the activity of the purified two-subunit form (containing only the 50 kDa subunit and the 125 kDa subunit) of calf thymus pol δ (*17*). Indeed, on the 30−21-mer synthetic oligonucleotide template-primer, we found that mutant E85K hPCNA was substantially more effective than wt; enhancement of product formation by the mutant was relatively similar at all PCNA concentrations tested (Figure 2). Hence we would

consider E85K to be potentially a gain-of-function mutation. Consistent with increased activity stimulation, we found that the mutant E85K hPCNA was also demonstrably more effective than wt at enhancing the processivity of pol δ (Figure 4).

At relatively low PCNA concentrations, the mutant E85K hPCNA was significantly more effective than wt at promoting pol δ-dependent DNA synthesis (TLS) beyond either 8-oxo-dGMP or a single modified tetrahydrofuran (a model abasic site) present in the template (Figures 5 and 6, respectively). However, at higher PCNA concentrations, the mutant E85K hPCNA and wt promoted more similar levels of TLS. This illustrates another possible in vitro scenario, i.e., stimulation under one condition (low PCNA concentrations) where complex formation may be rate-limiting but lesser effect where it may not (high PCNA concentrations). In this context, we think it is important to note that in vivo concentrations of both PCNA and pol δ, i.e., in the nuclei of living cells, are likely to be vastly different than those optimized for use in vitro. For example, Morris and Mathews (*34*) determined that there was about 0.19 ng of PCNA per S-phase HeLa cell. Based on a volume for the typical HeLa cell of 2000 μm^3, we calculate that PCNA is present at a concentration of at least 85 mg/mL, several orders of magnitude higher than the concentrations typically used in cell-free assays. On one hand, biological effects predicted from in vitro biochemistry may be minimal or nonexistent in living cells. On the other hand, they may be substantially more dramatic than expected.

We also think it noteworthy that the mutant E85K hPCNA would be predicted to lead to considerably more TLS, and hence secondary mutagenesis, in tissues that naturally expressed relatively low levels of PCNA compared with pol δ. At this time, we are unaware of any documented tissue-specific differences in hPCNA:pol δ ratios. However, thorough immunochemical analyses are currently underway to evaluate this directly. Similarly, E85K hPCNA would be expected to promote substantially more secondary mutagenesis in individuals also harboring pol δ promoter mutations coincidently, thereby leading to increased polymerase expression, either in specific tissues or in general.

One plausible explanation for enhanced secondary mutagenesis promoted by primary PCNA mutations concerns the equilibrium between monomeric but inactive protein and fully active multimers. A PCNA mutation affecting this equilibrium, leading either to increased or to decreased multimerization, would be expected to alter the ratio of PCNA:pol δ at which any given effect (e.g., either enhanced processivity or TLS) would be manifest. Such a mutation would also be expected to be equally apparent on all substrates (templates) tested since the PCNA monomer/mutimer equilibrium would be predicted to be of similar importance to all PCNA-dependent reactions catalyzed by pol δ.

On several bases, we excluded the possibility that alteration of the monomer/trimer equilibrium accounted for the effect of E85K compared to wt hPCNA on in vitro DNA synthesis with unmodified versus lesion-containing templates. (1) Clearly, relative to wt, the E85K mutant of human PCNA affects pol δ differently depending on the nature of the template being copied. (2) Although we noted the proximity of E85K to the monomer−monomer interface (see Figure 1A), the nearest adjacent subunit residue in the crystal is R146 (14.5 Å between the α-carbons of E85 and R146). If there were significant changes in trimer stability when E85 was changed to K, we would indeed predict destabilization and decreased activity. (3) We evaluated the native structure directly under sedimentation conditions quite similar to those used for TLS reactions. Both mutant and wt behaved comparably (Figure 3) and, consistent with the measured PCNA K_D of ∼21 nM (35), were apparently monomeric (*9*, *12*). Since only trimeric PCNA is thought to stimulate pol δ, the monomer to trimer conversion must occur in our assays, perhaps at relatively low efficiency and/or only in the presence of other macromolecules such as pol δ and/or DNA, but presumably with equal efficiencies for wt versus E85K hPCNA.

Mutations in PCNA may be either dominant or recessive. It is thought that PCNA functions as a trimer, and hence, it is possible that an effect such as enhanced TLS could be manifest if only one or two of the three subunits were mutated and the other(s) wt. Clearly, a mutation found to be dominant, i.e., where mixed mutant/wt trimers can form and exert an effect, would be predicted to be much more significant biomedically. Since at the low PCNA concentrations used for our sedimentation analysis both E85K and wt hPCNA are apparently monomeric (Figure 3), results obtained do not address this question. By analyzing much larger amounts of PCNA, insight into this question may be obtained. That sufficient amounts of PCNA can be so analyzed was recently demonstrated (*12*). Mixing during translation and subsequent purification may also more closely mimic in vivo events. Finally, in vivo (genetic) strategies are now being developed to test dominance directly.

During DNA metabolism in living cells, it has been shown that the so-called "clamp-loader" protein, RF-C, is required to load PCNA (the "clamp") onto DNA at the template-primer junction (for a review, see ref *36*; see also ref *37*). Most of our in vitro results were obtained with short linear template-primers (e.g., the 30−21-mer), thus precluding the need for RF-C. It would be particularly interesting to repeat our experiments with a template-primer (e.g., a primed single-stranded circular template) that required interaction between PCNA and a homologous clamp loader.

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