Different types of interaction between PCNA and PIP boxes contribute to distinct cellular functions of Y-family DNA polymerases

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ABSTRACT

Translesion DNA synthesis (TLS) by the Y-family DNA polymerases Polη, Polλ and Polκ, mediated via interaction with proliferating cell nuclear antigen (PCNA), is a crucial pathway that protects human cells against DNA damage. We report that Polη has three PCNA-interacting protein (PIP) boxes (PIP1, 2, 3) that contribute differentially to two distinct functions, stimulation of DNA synthesis and promotion of PCNA ubiquitination. The latter function is strongly associated with formation of nuclear Polη foci, which co-localize with PCNA. We also show that Polκ has two functionally distinct PIP boxes, like Polη, whereas Polλ has a single PIP box involved in stimulation of DNA synthesis. All three polymerases were additionally stimulated by mono-ubiquitinated PCNA in vitro. The three PIP boxes and a ubiquitin-binding zinc-finger of Polη exert redundant and additive effects in vivo via distinct molecular mechanisms. These findings provide an integrated picture of the orchestration of TLS polymerases.

INTRODUCTION

Translesion DNA synthesis (TLS), a DNA damage tolerance mechanism, is a crucial biological function that protects cells from various genotoxic agents. Particularly in humans, DNA polymerase η (Polη), a Y-family DNA polymerases (1), plays an important role in preventing cell death and mutagenesis after ultraviolet (UV) light irradiation, and malfunction of Polη causes the inherited genetic disorder, xeroderma pigmentosum variant (XP-V) (2–4).

Interactions between proliferating cell nuclear antigen (PCNA) and the three Y-family human DNA polymerases (Polη, Polλ and Polκ) are critically involved in regulation of TLS. Polη and Polκ are known to contain two PCNA-interacting protein (PIP) boxes (PIP1 and PIP2) in their central and C-terminal regions, respectively, whereas Polλ is known to contain only one functional PIP box (PIP1, in the central region) (5–12). In DNA-damaged cells, PCNA is mono-ubiquitinated at residue K164 by the RAD6–RAD18 complex (13–15), and poly-ubiquitinated by additional factors including UBC13, MMS2, and RAD5/HLTF or SHPRH (13,16–19). Each of the three Y-family DNA polymerases described above has one or two copies of the ubiquitin-binding domain (UBD), called UBZ (ubiquitin-binding zinc-finger) in Polη and Polκ and UBM (ubiquitin-binding motif) in Polλ (6). These findings support the notion that mono-ubiquitination of PCNA plays a key role in switching from replicative DNA polymerase stalled at a site of DNA damage to a DNA polymerase (such as Polη, Polλ or κ) capable of carrying out TLS (14–15,20–21). However, this idea is still controversial (5), and more recent publications report that Polη and Polκ are able to carry out TLS independently of PCNA ubiquitination in some circumstances (22–24).

The intracellular functions of the various motifs of Polη are monitored in two ways: formation of nuclear foci containing Polη co-localized with PCNA and complementation of UV sensitivity of XP-V cells. Mutations in PIP2 strongly impair the localization of Polη, indicating that PIP2 plays a crucial role in the accumulation of this protein in replication foci (23,25–27). However, ubz mutants also failed to accumulate in replication foci (6,26), and accumulation is barely detectable in a human cell line expressing the PCNAK164R mutant instead of endogenous PCNA (28).

References

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and in a PCNAK16R knock-in murine cell line (23), demonstrating that the PIP2–PCNA interaction itself is not sufficient for foci formation. Recently, Durando and colleagues reported an additional function of PIP2 of Polh, namely, that Polh promotes mono-ubiquitination of PCNA in a PIP2-dependent manner (29). It remains unclear how the two PIP2-mediated activities, co-localization of Polh with PCNA and promotion of PCNA mono-ubiquitination, are linked at the molecular level.

The pip2 and ubz mutants of Polh exhibit pronounced defects in foci formation, but retain the capacity to complement UV sensitivity of XP-V cells; however, the levels of complementation activity vary amongst studies by different groups (5–6,25–26,30), including one report that showed no role for PIP2 in survival after UV irradiation (27). Even a pip2ubz double mutant (25,26) and a mutant in which PIP2 and UBZ were deleted (30) still exhibited significant levels of complementation activity. By contrast, the pip1pipp2 double mutant and a mutant lacking the entire PIP1, UBZ and PIP2 domains were severely defective in complementation (5,30).

The biochemical activities of the motifs of human Polh have been studied in vitro. In primer extension assays, both PIP1 and PIP2 contributed to some extent to the stimulation of DNA synthesis in the presence of PCNA (5,8,30). Using a reconstitution system to investigate switching between Polδ and Polh at DNA lesions, we demonstrated that the weak enhancement of the recruitment of Polh to the 3′-end of primer DNA by the UBZ domain depended on the mono-ubiquitination of PCNA (21). These biochemical properties of mutant proteins defective for PIP1, PIP2 or UBZ are well correlated with their abilities to complement UV sensitivity of XP-V cells, but not with their abilities to promote accumulation into the replication foci.

Here, we describe the molecular functions of Polh’s PIP1, PIP2 and UBZ domains, together with the newly found PIP3, in UV tolerance, and present findings that resolve the controversies raised in previous reports. Our key finding is that Polh has two functionally distinct types of PIP box, one that stimulates DNA synthesis, and another that promotes PCNA mono-ubiquitination and accumulation into replication foci. Both PIP functions, together with the UBZ domain, are redundantly required for survival after UV irradiation. Additionally, we show that Polh has two PIP boxes with different functions, whereas Polh has only one PIP box involved in stimulation of DNA synthesis. Taking the results of previous reports together with the in vivo and in vitro data obtained in this study, we propose a model for the cellular functions of the various motifs of Polh’s/s/κ in orchestrating TLS.

MATERIALS AND METHODS

Proteins

Expression plasmids were constructed as follows. Human POLH, POLI (encoding 740 amino-acid residues) (11) and POLK were cloned into pET20b(+) (Novagen) to obtain untagged proteins, and into pET15b (Novagen) to obtain N-terminally histidine-tagged proteins. Plasmids for expression of Polh, Polhubz and His-Polh in E. coli were described previously (21,31). A truncated gene encoding PolhΔC (32) was cloned into pET21a(+) (Novagen). A gene encoding UBCH5cS22R was cloned into pET15b. Mutations were created by PCR, and nucleotide sequences were verified after cloning.

E1, RAD6-(His-RAD18)2, RAD6-(RAD18AC2)2, ubiquitin, RPA, PCNA, RFC, His-Polh, Polh and their mutants were purified as described previously (21,31,33–35). Column chromatography was carried out at 4 °C on an FPLC system (GE Healthcare Life Science) using columns from GE Healthcare unless otherwise indicated. Protein concentrations were determined by the Bio-Rad protein assay using BSA (Bio-Rad) as the standard.

PolhΔC was purified in the same way as Polh (21), except that HisTrap Phenyl HP was used instead of an Econopack methyl column (Bio-Rad).

A histidine-tagged Polk (His-Polk) and its mutants were purified as follows. BL21 (DE3) harbouring each of the expression plasmids and pMS-tRNA1 (36) was grown in 2 l of Terrific broth (37) supplemented with ampicillin (250 μg/ml) and kanamycin (30 μg/ml) at 15°C. His-Polk was induced with 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 8 h, and then purified by sequential chromatography on Ni2+-charged HiTrap chelating HP, POROS 50 HE (Applied Biosystems), Econopack CHT-II (BIO-RAD) and Superdex 200 columns. The peak fraction containing His-Polk was frozen in liquid nitrogen and stored at −80°C.

Polh and PolhΔp1 were purified as follows. BL21 (DE3) harbouring each of the expression plasmids was grown in 5 l of LB supplemented with ampicillin (250 μg/ml) at 15°C. Polk was induced with 0.2 mM IPTG for 5 h, and then purified by sequential chromatography on HiTrap Capto MMC, Ni2+-charged HiTrap chelating HP, HiTrap SP HP, HiTrap Q HP and Superdex 200 columns. Note that Polh itself (without the His-tag) has weak affinity for the Ni2+-charged HiTrap chelating column. For PolhΔp1, the gel-filtration chromatography step was omitted. The peak fraction containing Polh was frozen in liquid nitrogen and stored at −80°C.

A histidine-tagged Pola (His-Pola) was purified as follows. BL21 (DE3) harbouring the expression plasmid was grown in 3 l of LB supplemented with ampicillin (250 μg/ml) at 15°C. Pola was induced with 0.2 mM IPTG for 8 h, and then purified by sequential chromatography on Ni2+-charged HiTrap chelating HP, HiTrap SP HP, HiTrap Q HP and Superdex 200 columns. The peak fraction containing His-Pola was frozen in liquid nitrogen and stored at −80°C.

His-UBCH5cS22R was purified as follows. BL21 (DE3) harbouring the expression plasmid was grown in 2 l of LB supplemented with ampicillin (250 μg/ml) at 15°C. His-UBCH5cS22R was induced with 0.2 mM IPTG for 16 h, and then purified by sequential chromatography on Ni2+-charged HiTrap chelating HP and HiTrap SP HP columns. The peak fraction containing His-UBCH5cS22R was frozen in liquid nitrogen and stored at −80°C.

PCNA-ubiquitination assays

PCNA-ubiquitination assays were performed as described (21). Briefly, the reaction mixture (25 μl) contained 20 mM HEPES-NaOH (pH 7.5), 50 mM NaCl, 0.2 mg/ml BSA, 1 mM DTT, 10 mM MgCl2, 1 mM ATP, poly(dA)-oligo(dT)
(GE Healthcare) (100 ng), PCNA (1.0 pmol trimer), E1 (0.85 pmol), RAD6A-(His-RAD18)$_2$ (0.54 pmol trimer), Ub (174 pmol) and DNA polymerases (2.5 pmol unless indicated otherwise). Reaction mixtures were prepared on ice, and then incubated at 30°C for 30 min unless indicated otherwise. The reactions were terminated with sample buffer for SDS-PAGE. Products were analysed by western blotting with anti-PCNA antibody (Santa Cruz Biotechnology, sc-7907). Signals were detected with a Chemi-Lumi LAS4000MiniBiomolecularImager(GEHealthcare), and analysed using ImageQuantTM TL software (GE Healthcare).

DNA polymerase assays

DNA polymerase assays were performed as described (35). Briefly, the reaction mixture (25 µl) contained 20 mM HEPES-NaOH (pH 7.5), 50 mM NaCl, 0.2 mg/ml BSA, 1 mM DTT, 10 mM MgCl$_2$, 1 mM ATP, 0.1 mM of each deoxynucleotide (dGTP, dATP, dCTP and dTTP), 19 nmol of ubiquitin (36 nmol), and Pol (500 fmol trimer) and DNA polymerases (25 fmol for Pol C for 5 min with 10 pmol of each deoxynucleotide), and DNA polymerases assays were performed as described (35) using the NeonR transfection system (Invitrogen). Forty-eight hours after transfection, cells were harvested and lysed in 1% SDS in PBS to obtain whole cell lysates (WCL). In the case of fractionation, cells were suspended in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM KCl, 25% glycerol, 0.5% NP-40, 1.5 mM MgCl$_2$, 1× Complete Protease Inhibitor Cocktail (Roche), 1× Phosphatase Inhibitor Cocktail Set II (Calbiochem)), and a portion was withdrawn as WCL. Next, soluble materials (soluble fractions) were separated by centrifugation. The precipitants were resuspended in micrococcal nuclease buffer (20 mM Tris-HCl (pH 7.5), 100 mM KCl, 300 mM sucrose, 0.1% Triton X-100, 2 mM MgCl$_2$, 1 mM CaCl$_2$, 1× EDTA-free Complete protease inhibitor cocktail (Roche)) and incubated with 2.5 U of micrococcal nuclease (Roche) at room temperature for 10 min. After centrifugation, soluble materials were collected as chromatin fractions, and precipitates (insoluble fractions) were resuspended in 1% SDS in PBS and solubilized by sonication. Cellular fractions were analysed by western blotting with anti-PCNA antibody as described previously (28).

Preparation of cellular fractions and western blotting

XP2SASV3 cells were transfected with expression constructs encoding either wild-type or mutant FLAG-Pol$_\text{I}$ using the Neon® transfection system (Invitrogen) and incubated for 24 h. Three hours after 15 J/m$^2$ UVC irradiation, cells were harvested and lysed in 1% SDS in PBS to obtain whole cell lysates (WCL). In the case of fractionation, cells were suspended in lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM KCl, 25% glycerol, 0.5% NP-40, 1.5 mM MgCl$_2$, 1× Complete Protease Inhibitor Cocktail (Roche), 1× Phosphatase Inhibitor Cocktail Set II (Calbiochem)], and a portion was withdrawn as WCL. Next, soluble materials (soluble fractions) were separated by centrifugation. The precipitants were resuspended in micrococcal nuclease buffer (20 mM Tris-HCl (pH 7.5), 100 mM KCl, 300 mM sucrose, 0.1% Triton X-100, 2 mM MgCl$_2$, 1 mM CaCl$_2$, 1× EDTA-free Complete protease inhibitor cocktail (Roche)) and incubated with 2.5 U of micrococcal nuclease (Roche) at room temperature for 10 min. After centrifugation, soluble materials were collected as chromatin fractions, and precipitates (insoluble fractions) were resuspended in 1% SDS in PBS and solubilized by sonication. Cellular fractions were analysed by western blotting with anti-PCNA antibody as described previously (28).

Analysis of co-localization of Pol$_\text{I}$ with PCNA

XP2SASV3 cells were transfected with expression constructs encoding either wild-type or mutant FLAG-Pol$_\text{I}$ using the Neon® transfection system (Invitrogen). Forty-eight hours after transfection, cells were irradiated with 15 J/m$^2$ UVC and incubated for 3 h. Triton-soluble materials were removed by incubation with extraction buffer (0.5% Triton X-100, PBS, 0.4 µg/ml antipain, 0.4 µg/ml aprotinin, 0.2 µg/ml leupeptin, 0.16 µg/ml pepstatin, 0.1 mM EGTA and 0.5 mM phenylmethylsulfonyl fluoride), proteins that bound to the beads were analysed by western blotting with anti-PCNA antibody as described above.
and then the cells were fixed with 3.5% formaldehyde in PBS and permeabilized with 0.1% Triton X-100 and 3.5% formaldehyde in PBS. After sequential treatments with 70% EtOH, 100% EtOH, and acetone on ice, cells were incubated with anti-POLH (Santa Cruz Biotechnology, sc-5592) and anti-PCNA (Santa Cruz Biotechnology, sc-56) antibodies. Alexa Fluor 488- and 594-conjugated secondary antibodies (Invitrogen) were used to visualize the immune-conjugated proteins. Nuclei were visualized by staining with 2 µg/ml Hoechst 33342. Images were collected using an LSM710 confocal microscope (Zeiss).

RESULTS

Promotion of PCNA mono-ubiquitination by PolH is dependent on PIP2 and PIP3, but independent of PIP1

The three human Y-family DNA polymerases (Polη, γ and κ) share a basic architecture. Each protein contains a catalytic domain in the N-terminal half and various motifs/domains involved in interactions with other proteins in the C-terminal half (see Figures 1A and 4A, D). Human Polη contains multiple PIP boxes and a single copy of UBZ, which are believed to be involved in the interaction with mUB-PCNA in DNA-damaged cells. Recently, Durando and co-workers reported that depletion of endogenous Polη decreases the levels of damage-induced mUB-PCNA, and ectopically expressed Polη promotes mono-ubiquitination of PCNA in cells in a manner that depends on PIP2 at the C-terminus (29). However, those authors did not examine the contribution of another PIP box, PIP1, which is located in an internal region (5) (Figure 1A). To determine whether PIP1 plays any role in the promotion of PCNA mono-ubiquitination, we introduced the pip1 or pip2 mutation into FLAG-tagged Polη (Figure 1A), expressed the mutant proteins in XP-V cells, and analysed the levels of mUB-PCNA in cells by western blotting. As shown in Figure 1B, the result indicated that the pip1 mutant and the wild type promoted mono-ubiquitination of PCNA to similar extents. Importantly, PCNA ubiquitination was observed in the presence or absence of UV irradiation, although it was more extensive when the cells were UV-irradiated. By contrast, the pip2 mutant lost most of the ability to promote mono-ubiquitination. Because the pip1 pip2 double mutant still exhibited weak activity, similar to that of the single pip2 mutant, the residual activity could be attributable to additional PIP box(es). To identify another PIP box in Polη, we employed yeast two-hybrid assays and found one additional PIP box (hereafter, referred to as PIP3), which overlaps with a REV1-interacting region (RIR) (11,40) (Figure 1A; Supplementary Figure S1). Although short peptides carrying the PIP2 sequence interact with PCNA strongly enough for detailed physicochemical and structural analyses (10), the PCNA-binding activity of short peptides carrying the PIP1 sequence has never been detected, even using very sensitive yeast two-hybrid assay (11). Similarly, the PCNA-binding activity of PIP3 is not detected using short fragments; however, the activity of PIP3 appears stronger than that of PIP1, because the pip3 mutation caused a much more drastic reduction in the positive signal in the yeast two-hybrid assay than the pip1 mutation (Supplementary Figure S1). Subsequently, we made a series of pip3 mutants and expressed them in XP-V cells. As expected, the residual activities of the pip2 single and pip1 pip2 double mutants were diminished further by the additional introduction of the pip3 mutation. The levels of mUB-PCNA in pip3 pip2 double and pip1 pip3 pip2 triple mutants were similar to that in the vector control. On the other hand, the levels of mUB-PCNA in pip3 single and pip1 pip3 double mutants exhibited marginal differences from those in the wild type only in UV-unirradiated samples. Because all of these mutants were similarly detected in the chromatin fraction, the defects in the mutants were not attributed to alteration in sub-cellular localization (Figure 1B, bottom panel). These results indicate that PIP2 and PIP3 play a major and minor role, respectively, whereas PIP1 has no or little role, in promoting mono-ubiquitination of PCNA in cells, suggesting that two functional types of PIP boxes play distinct roles in the regulation of Polη.

Additionally, we showed that the ubz mutants severely reduced the ability to promote PCNA mono-ubiquitination (Supplementary Figure S2). However, these mutants had a significantly reduced ability to accumulate in chromatin (26) (Supplementary Figure S2), indicating that the mutations have additional effects, as postulated previously (5). Because the defect in promoting PCNA mono-ubiquitination could be also attributed to additional effects, such as alteration of sub-cellular localization, it remains unclear whether UBZ has the potential to promote ubiquitination of PCNA in vivo.

Reconstitution of Polη-dependent mono-ubiquitination of PCNA in vitro

To study the molecular mechanisms underlying the promotion of PCNA mono-ubiquitination, we sought to develop in vitro experimental conditions for recapitulating the in vivo situation using purified enzymes (Supplementary Figure S3A) (21,34). Because Polη interacts with both RAD18 and PCNA (8,15,41), we first checked the possibility that such protein–protein interactions could themselves promote PCNA ubiquitination. However, we observed no ubiquitination of PCNA under such conditions (Figure 2A, lane 1). When poly(dA)-oligo(dT) was added into the reaction mixture, PCNA ubiquitination occurred and it was induced the ability to promote PCNA mono-ubiquitination (Figure 2A, lane 1). When poly(dA)-oligo(dT) was added into the reaction mixture, PCNA ubiquitination occurred and it was induced the ability to promote PCNA mono-ubiquitination (Figure 2A, lane 1). When poly(dA)-oligo(dT) was added into the reaction mixture, PCNA ubiquitination occurred and it was induced the ability to promote PCNA mono-ubiquitination (Figure 2A, lane 1). When poly(dA)-oligo(dT) was added into the reaction mixture, PCNA ubiquitination occurred and it was induced the ability to promote PCNA mono-ubiquitination (Figure 2A, lane 1).
Effects of pip and ubz mutations on the promotion of in vitro PCNA ubiquitination

Given that Polη promotes PCNA ubiquitination in vitro through direct interaction with PCNA on DNA, we next asked whether one or all of the PIP boxes are required for promotion of ubiquitination. To address this question, the pip mutants of Polη used for the in vivo experiments described above (Figure 1; Supplementary Figure S3A) were examined for the ability to promote ubiquitination in vitro. The results demonstrated that in vitro promotion of ubiquitination is dependent on PIP3 and PIP2, but independent of PIP1 (Figure 2B, C), in good agreement with the in vivo observations (Figure 1B). Together, these results support the interpretation that the in vivo accumulation of mUb-PCNA is a consequence of directly promoting de novo ubiquitination by Polη.
In addition, we demonstrated that the ubz mutant promoted mono-ubiquitination as efficiently as the wild type (Supplementary Figure S4B), indicating that the UBZ function is dispensable for promotion of ubiquitination in our in vitro system.

Different roles of the three PIP boxes of Polθ in stimulation of DNA synthesis

Next, to study how PCNA stimulates the polymerase activity of Polθ, we employed the primer extension assay using M13 mp18 ssDNA as a template in the presence of RPA and RFC. As shown in Figure 3A, we clearly detected stimulation of Polθ polymerase activity by PCNA. When the pip mutants were examined, the stimulation was slightly reduced in each of the single pip mutants relative to the wild type (Figure 3B), indicating that all of the PIP boxes contribute to stimulation to some extent. The activities of the pip1 pip2 and pip3 pip2 were further reduced. Surprisingly, the activity of the pip1 pip3 double mutant was inhibited by addition of PCNA, despite the fact that it still contains a PIP2 domain (Figure 3B). Similar inhibition was also observed with the pip1 pip3 pip2 triple mutant (Figure 3B). Based on these results, we conclude that PCNA binding to Pip1 or Pip3, both located in the adjacent region of the Polθ catalytic domain, is critical for the stimulation of DNA polymerase activity, and that even if PCNA binds to Pip2 at the C-terminus, PCNA does not stimulate Polθ polymerase activity in vitro unless Pip1 or Pip3 is present. These results suggest that the function of Pip2 in stimulation of DNA synthesis is largely Pip1- and Pip3-dependent.

Functional roles of PIP boxes of Polθ and Polκ

The findings described above regarding the PIP boxes of Polθ prompted us to investigate the PIP boxes of two other Y-family DNA polymerases, Polκ and Polκ. Polκ has a PIP box at the C-terminus, which is required for formation of nuclear foci in cells with DNA damage (42), and has the potential to promote mono-ubiquitination of PCNA in vitro when Polκ is ectopically expressed (29). More recently, another PIP box was found adjacent to the catalytic domain (Figure 4A; Supplementary Figure S5); therefore, the internal PIP box was named Pip1, and the C-terminal one was renamed Pip2, following the example of Polθ (Figure 4A) (11). Unlike the internal Pip1 and Pip3 boxes in Polθ, the PCNA-binding activity of the internal Pip1 box in Polκ was detected in short fragments by the yeast two-hybrid assay, implying that the activity is equivalent to that of the C-terminal Pip2 in Polκ (Supplementary Figure S5). When the wild-type Polκ protein was introduced into the in vitro PCNA ubiquitination reaction, a large amount of ubiquitinated PCNA was observed (the leftmost panel in Figure 4B), as in the case of Polθ. To study the roles of the respective PIP boxes, we examined the pip1, pip2 and double mutants (Figure 4A; Supplementary Figure S3B). As shown in Figure 4B, the pip1 and pip2 mutants exhibited reduced levels of PCNA ubiquitination: the pip2 mutant retained a relatively higher level, whereas the double mutant lost the activity. These results indicate that each of the two PIP boxes functions independently and have similar affinity for PCNA, and that both are required to promote the maximum level of ubiquitination (Figure 4B). Next, we examined effects of PCNA on DNA polymerase activity of the wild-type and mutant Polκ proteins. As shown in Figure 4C, the pip2 mutant was stimulated as efficiently as the wild type by PCNA, but pip1 and the double mutants failed to be stimulated by PCNA, indicating that the internal Pip1 box is responsible for stimulation by PCNA but the C-terminal Pip2 is not. Therefore, we conclude that the multiple PIP boxes of Polκ serve different functions: Pip1 stimulates DNA synthesis by PCNA, and Pip1 and Pip2 promote PCNA ubiquitination.

Next, we examined Pol( Figure 4D; Supplementary Figure S3C). In contrast to Polθ and Polκ, Pol did not promote PCNA ubiquitination in vitro (Figure 4E). On the other hand, PCNA stimulated DNA synthesis of Pol in vitro in a Pip1-dependent manner (Figure 4F), in line with previous reports that Pol has only one functional PIP box (for stimulation of DNA synthesis) immediately adjacent to the catalytic domain (7,10,12).

Subsequently, we examined the levels of mUb-PCNA in cells with ectopic expression of Polθ or Polκ, with or without UV irradiation (Figure 5). When GFP-Polθ was expressed in Polθ-deficient (XP-V) or proficient cells, promotion of PCNA mono-ubiquitination was observed in both types of cells (Figure 5A, B). The promoting effect of ectopic expression of Polκ is weaker than that of Polθ reported previously (29,43). The difference in the extent to which PCNA mono-ubiquitination was promoted by these enzymes could be attributed to differences in the expression system and/or cell types used in these experiments. Indeed, a difference is evident between the two types of cells used in our study (Figure 5A, B), indicating that it may not be appropriate to compare and draw conclusions from differences in mUb-PCNA levels between different cell lines. By contrast to Polθ and Polκ, ectopic expression of FLAG-Polθ in both cells did not increase the levels of mUb-PCNA (Figure 5C, D). These results are consistent with the in vitro properties described above (Figure 4B, E), implying that Polθ, but not Polκ, can promote de novo mono-ubiquitination of PCNA in vivo.

Interactions of Polθ, τ and κ with mUb-PCNA

PCNA is mono-ubiquitinated in DNA-damaged cells. Consequently, Y-family DNA polymerases with UBD(s), as well as PIP box(es), could interact with modified PCNA in preference to unmodified PCNA. To test this in vitro, we compared the stimulatory effects of mUb-PCNA and unmodified PCNA on DNA synthesis in vitro, using a primer extension assay (Figure 6; Supplementary Figure S3D). As shown in Figure 6A, mUb-PCNA stimulated DNA synthesis by Polθ more effectively than unmodified PCNA under our assay conditions. As expected, no additional stimulation was observed with the ubz mutant of Polθ (Figure 6B). DNA synthesis by either the pip1 pip2 or pip3 pip2 double mutants was stimulated by mUb-PCNA, although it was only marginally stimulated by unmodified PCNA (Figure 6C, D). The activity of the pip1 pip3 double mutant was slightly higher in the presence of mUb-PCNA than in the absence of PCNA (Figure 6E). The pip1 pip3 pip2 triple mutant exhibited a negative effect by the addition of mUb-
Figure 3. DNA polymerase assays of Polη in a reconstituted system in vitro. (A) DNA replication reactions using singly primed M13 mp18 ssDNA were reconstituted with the indicated factors. The reaction products were resolved in 10% polyacrylamide gels containing 7 M urea, and visualized using a PhosphorImager. (B) Analysis of pip mutants of Polη. Indicated mutants were subjected to replication assays shown in (A) in the presence or absence of PCNA.

Figure 4. Analysis of Polκ, Polξ and their pip mutants in vitro. (A, D) Schematic structures of human Polκ (A) and Polξ (D), as shown in Figure 1A. (B, E) PCNA ubiquitination assays of His-Polκ (B) and Polξ (E), as shown in Figure 2. (C, F) DNA polymerase assays of His-Polκ (C) and Polξ (F), as shown in Figure 3.
PCNA (Figure 6F). These results suggested that UBZ function requires at least one PIP.

Next, we examined the stimulation of DNA synthesis by Pol and Polk by mUB-PCNA. Additional stimulation, albeit marginal, by mUB-PCNA was reproducibly observed for Polk (Figure 6G) as well as its pip1 mutant (Figure 6H). Similarly, mUB-PCNA stimulated Pol and its pip mutants to a slightly greater extent than unmodified PCNA (Figure 6I, J). The relatively lower contributions of ubiquitin moieties to Pol and Polk activity than to Polh activity could be attributed to the weaker affinity of Polk and Polk for mUB-PCNA than Polh (Supplementary Figure S6).

Cellular functions of the motifs of Polh

After UV irradiation, Polh forms nuclear foci that co-localize with PCNA (6,15,25–27,39,44). To determine the roles of each of Polh’s three PIP boxes in foci formation, wild type and pip mutants bearing a FLAG-tag, all of which were used in the experiments shown in Figure 1, were transiently expressed in XP-V cells. After UV irradiation, localization of Polh and PCNA was visualized using anti-Polh and anti-PCNA antibodies, respectively. As shown in Figure 7A, Polh foci co-localized with PCNA were observed in all of the samples except for the pip2 mutant. Because all of the proteins, including the pip2 mutant, could be detected with similar efficiency by western blotting (Figure 1B) and immunostaining (Supplementary Figure S7), we conclude that Pip2, but not Pip1 or Pip3, plays a crucial role in foci formation along with PCNA.

Next, we examined the abilities of the Polh mutants to complement the UV sensitivity of XP-V cells (Figure 7B). Although the pip and ubz mutants of Polh have been analysed previously using such assays, the levels of complementation were inconsistent among studies (5–6,25–27,30). Complementation of the UV sensitivity of XP-V cells differs among clones stably expressing a particular mutant Polh, but is not correlated with the expression levels of Polh (26). To avoid such complexities due to differences among clones, we used for our survival assays a mixture of the cells that were transfected with pIRESnneo2 carrying wild-type or mutant Polh and selected by G418. As shown in Figure 7B, the results indicated that the single and double pip mutants could complement the UV sensitivity of XP-V cells as efficiently as the wild type. By contrast, the pip1 pip3 pip2 triple mutant exhibited clearly reduced complementation activity (Figure 7B).

In contrast to such subtle phenotypes of individual single and double pip mutants, the ubz single mutant exhibited a severe defect (Supplementary Figure S8) (6,26). However, because the ubz mutant accumulated poorly in the chromatin fraction (see Supplementary Figure S2) (26), we hypothesized that this defect could be attributed to secondary effects due to the ubz mutation. To investigate this possibility, we made use of a deletion mutant carrying the 1–511 region of Polh (PolhΔC) (2–3,32) (Figure 1A). Because PolhΔC lacks the nuclear localization signal (NLS) as well as Pip2 and UBZ, we introduced an artificial NLS at the C-terminus. Because of the lack of Pip2, PolhΔC was expected to have a lower ability to promote PCNA ubiquitination and fail to form foci in co-localization with PCNA. Those properties were confirmed in vivo and in vitro (Supplementary Figure S9). Nevertheless, PolhΔC retained the ability to accumulate in the chromatin fraction (Supplementary Figure S9D), in contrast to the ubz mutants of full-length Polh (Supplementary Figure S2), supporting the idea that some ubz mutations provoke secondary effects (5,30). More importantly, PolhΔC could complement the UV sensitivity of XP-V cells much better than the ubz mutant (Supplementary Figure S8). As shown in Figure 7B, the pip1 and pip3 derivatives of PolhΔC exhibited reduced complementation activity, and the pip1 pip3 derivative of PolhΔC exhibited a severer defect than the pip1 pip3 pip2 triple mutant of full-length Polh. The defects were not attributable to alterations in the sub-cellular localization (Supplementary Figure S9D). Together, these results suggest that Pip1, Pip3, Pip2 and UBZ exert additive and redundant effects that protect cells from the lethal effects of UV irradiation.

DISCUSSION

Cellular functions of the respective motifs of Polh are routinely monitored in two ways: co-localization with PCNA and complementation of UV sensitivity of XP-V cells. In this study, we demonstrated that these two phenotypes are mediated by different PIP boxes and distinct modes of interaction with PCNA. We also showed that the diverse functions of PIP boxes are conserved in Polh.
Figure 6. Interactions between Y-family DNA polymerases and mUb-PCNA in vitro. (A–J) Analysis of DNA synthesis by Polη (A–F), Polκ (G–H) and His-Polκ (I–J), as shown in Figure 3, in the absence or presence of PCNA (designated as PCNA or +) or mUb-PCNA (designated as uPCNA or u). Concentrations of polymerases increase in the order 0.25, 0.5, and 1 nM (A–F and I) or 1, 2, and 4 nM (G–H), or remain constant at 1 nM (J).

Functions of PIP boxes in ubiquitination of PCNA and foci formation

In this study, we found that Polη promotes mono-ubiquitination of PCNA in a manner dependent on PIP2 and to a lesser extent on PIP3, but independent of PIP1 in vivo (Figure 1B). These findings were perfectly correlated with the in vitro observations regarding promotion of PCNA mono-ubiquitination by purified proteins (Figure 2B, C). Together, the data strongly suggest that the intracellular accumulation of mUb-PCNA with ectopically expressed Polη is a consequence of direct promotion of de novo ubiquitination. Importantly, we found that DNA is an absolute requirement for Polη-dependent PCNA ubiquitination reactions in vitro (Figure 2A). We suggest that...
Figure 7. Cellular functions of the motifs of Polη (A) Co-localization of Polη with PCNA. XP-V cells were transiently transfected with plasmids encoding wild-type FLAG-Polη or the indicated mutants. After UV irradiation, FLAG-Polη and PCNA were visualized by immunostaining with anti-Polη and anti-PCNA antibodies, respectively. Nuclei were stained by Hoechst 33342. Scale bars represent 5 μm. Control experiments confirming expressions of FLAG-Polη were shown in Supplementary Figure S7. (B) UV sensitivities of XP-V cells stably expressing FLAG-Polη. Cells were irradiated with the indicated dose of UVC, incubated with 1 mM caffeine for 4 days, and their viabilities were measured. Error bars show SD from three independent experiments. (C) A model for a regulatory network for foci formation and the TLS function of Polη/Pol96/Pol97. Interaction of Polη/Pol96/Pol97 with PCNA, together with RAD6-(RAD18)2, leads to their accumulation by promoting mono-ubiquitination of PCNA around stalled 3′-OH ends. Interactions of Polη/Pol96/Pol97 with mUb-PCNA via PIPs and UBDs stimulate DNA synthesis at stalled 3′-OH ends. See text for details.

the mode of interaction between PIP3 or PIP2 and PCNA on DNA for the promotion of PCNA ubiquitination could act in such a way as an appropriate substrate for RAD6-(RAD18)2 catalysis, which was independent of the interaction between RAD18 and Polη in vitro. The partial involvement of the interaction in the promotion of PCNA ubiquitination in vivo (29) could be attributed to an additional function, such as recruitment of Polη to damage sites (15).

The contribution of individual Polη PIP boxes to co-localization with PCNA was correlated with the effect on promotion of PCNA ubiquitination. These effects could be largely attributed to PIP2 (Figures 1B, 2B, C and 7A). Polk also promotes mono-ubiquitination of PCNA (Figures 4B and 5A, B) (29) and co-localizes with PCNA (42). Polk failed to promote mono-ubiquitination of PCNA (Figures 4E and 5C, D) and failed to co-localize with PCNA by itself (45,46). These results suggest that a large part of the function of the PIP2 box in foci formation is the promotion of PCNA mono-ubiquitination, which is a prerequisite for co-localization of polη with PCNA in nuclear foci. The following observation supports this idea: first, accumulation is dependent on RAD18 and its catalytic activity (15,29). Second, accumulation of Polη is barely detectable in a human cell line in which the PCNAK164R mutant is expressed instead of endogenous PCNA (28), or in a PCNAK164R knock-in murine cell line (23). Therefore, we
suggest that one of the functions of PIP2 in foci formation is to promote mono-ubiquitination of PCNA. The resultant mUb-PCNA could stabilize Polη via interaction with UBZ, because mUb-PCNA has a higher affinity for Polη than unmodified PCNA (Supplementary Figure S6). Thus, even though UBZ is dispensable for the promotion of PCNA ubiquitination (at least in vitro), UBZ may still contribute to stable foci formation. This idea does not exclude another role for PIP2 in foci formation via direct interaction with PCNA. Indeed, among the three PIPs of Polη, only the PIP2 peptide has been shown to interact directly with PCNA in yeast two-hybrid and structural analyses (10,11). Furthermore, enhancement of the PIP2-PCNA interaction of Polκ by manipulations of its PIP2 sequence (10) improves foci formation (29,47). These results suggest that stable interactions with mUb-PCNA via both PIP and UBZ are required for detectable focus formation.

Interestingly, PIP3 and RIR1 share the same FF residues for binding to PCNA and REV1, respectively (40). Therefore, it is unlikely that PIP3 could act as a PIP box and RIR at the same time. However, Polη has two RIRs, RIR1 and RIR2 (40). So far, we have been unable to detect any defect in the polη-REV1 interaction in the rir1 single mutant in cells (39) or in yeast-two hybrid assays (40) (Supplemental Figure S1). We believe that the PIP3-PCNA interaction or pip3 mutation does not interfere with REV1-related function(s) because of the presence of RIR2.

Functions of PIP and UBZ in stimulating DNA synthesis

In this study, we demonstrated that all three PIP boxes of Polη play roles in stimulating DNA synthesis, although the mechanisms are different. We showed that PIP-less Polη has an intrinsic defect in accessing the PCNA-loaded 3′-end; the presence of Pip1 or Pip3, but not Pip2, compensates for this inhibition (Figure 3B). It is likely that both Pip1 and Pip3 stimulate DNA synthesis via direct interaction with PCNA, and that Pip2 facilitates the Pip1-PCNA and Pip3-PCNA interactions. Because Pip1 and Pip3 are proximal to the catalytic domain of Polη, the PCNA-Polη interaction via Pip1 or Pip3 may enable the active site of Polη to effectively bind the 3′-OH end of the primer-terminus, whereas the interaction via Pip2, located distant from the C-terminus, may be less efficient in this respect. This mechanism seems conserved in Polκ and Polδ, since each Pip1 box located adjacent to the catalytic domain can stimulate DNA synthesis. More recently, during the preparation of this manuscript, another group reported that Pip1 in Polκ is responsible for PCNA stimulation of in vitro DNA synthesis (48).

We demonstrated for the first time that mUb-PCNA stimulates DNA synthesis by human Polη, γ and κ (Figure 6A–H). We showed that the UBZ-function of Polη is largely Pip1- and Pip3-dependent and slightly Pip2-dependent (Figure 6A–F), suggesting that adequate stimulation by mUb-PCNA requires interaction with at least one Pip box. Alternatively, an increased local concentration of Polη around the primer end, achieved through Pip-PCNA interactions, indirectly promotes the interaction between UBZ and the ubiquitin moiety. Collectively, these results suggest that interactions between mUb-PCNA and Polη stimulate DNA synthesis via diverse mechanisms.

Functions of PIP and UBZ in Polη in enhancing survival after UV irradiation

All three PIP boxes and UBZ of Polη serve additive and redundant functions in enhancing survival after UV irradiation (Figure 7B). The survival rate in vivo (Figure 7B) correlated well with the level of stimulation of DNA synthesis by these mutants by PCNA or mUb-PCNA in vitro (Figure 6A–H). However, there were two exceptions. One was the ubz point mutant that exhibited severe defects (Supplemental Figure S8), which might be attributed to a secondary effect related to accumulation on chromatin (Supplemental Figure S2). Indeed, the deletion mutation of UBZ and Pip2 in PolηΔC restored the sub-cellular localization (Supplementary Figure S9D) and increased the ability to complement the UV sensitivity of XP-V cells relative to the ubz point mutant (Supplementary Figure S8). This partial contribution of UBZ is consistent with reports of PCNA ubiquitination-independent TLS in PCNA-K64R knock-in murine cells (22,23). The other exception was the pip1 pip3 double mutant. The defect in complementation of UV sensitivity of XP-V cells by the pip1 pip3 mutant was marginal (Figure 7B), although its DNA synthesis was poorly stimulated by mUb-PCNA in vitro (Figure 6E). Because the mutant is proficient in the promotion of PCNA ubiquitination and foci formation (Figures 1B, 2B and 7A), the elevated local concentration of the mutant could compensate for the defect in stimulation, and this could explain the minor defect of the pip1 pip3 mutant in vivo. Overall, the data suggest that the level of stimulation of DNA synthesis by mUb-PCNA could directly affect the efficiency of TLS in vivo.

A model for the cellular functions of PIP and UBZ of Polη/γ/κ in the orchestration of TLS

Taking the results of previous reports together with the in vivo and in vitro data obtained in this study, we propose a model for cellular functions of the various motifs of Polη/γ/κ (Figure 7C). We suggest that interactions between Polη/κ and PCNA constitute a network that regulates promotion of mono-ubiquitination and accumulation of Polη/γ/κ. PCNA is concentrated on DNA in close proximity to replication forks (21,35,49). In the model, Polη/κ are recruited to these locations via interactions with Pip boxes together with RAD18 (15,41,43). The initial Pip-PCNA interactions are too transient and unstable to be detected as foci, but the interaction turns PCNA into an appropriate substrate for RAD6-(RAD18)2 catalysis, which promotes ubiquitination of PCNA. The resultant mUb-PCNA recruits additional Polη/γ/κ molecules via interaction with both PipPs and UBDs. Because Polη has a much higher affinity for mUb-PCNA than Polκ and Polδ (Supplementary Figure S6), it is likely that Polη is predominantly recruited. In the case of Polκ, the interactions are sufficiently stable for detectable foci formation, but in the case of Polκ they are relatively weak. By contrast, Pol binds mUb-PCNA too weakly to form stable foci by itself. Increased
local concentrations of Polη/κ further promote ubiquitination of PCNA. Consequently, Polη/κ and ubiquitinated PCNA robustly accumulate around the stalled primer ends until accumulation of Polη/κ is saturated. Any mutation that disrupts the regulatory network should attenuate the response in vivo via activities of de-ubiquitination enzymes. This model is compatible with the dynamic mobile properties of Polη/κ in cells (50).

Accumulation of Polη in replication foci plays a minor role in survival, as reflected by the observation that accumulation-defective Pip2 mutant could perfectly complement UV sensitivity of XP-V cells (Figure 7B) (27). In addition, PolηΔAC with a C-terminal deletion encompassing UBZ and Pip2 also exhibited considerable complementation activity (Figure 7B). These results suggest that Polη can perform TLS of UV lesions without accumulating in foci. The minor contribution of foci formation to cellular function is also true in other members of the Y family. Although accumulation of Polκ in replication foci is dependent on Polη (45,46), Polκ appears to be functional in TLS of UV lesions in Polη-knockout mice (51). Deficiency in the Polη-dependent co-localization of REV1 with UV lesions does not affect survival, but does modulate mutagenesis (39).

In this scenario, one of physiological functions of the accumulated mUb-PCNA could be to establish an order of recruitment for TLS polymerases, via dynamic interactions between mUb-PCNA and their PIPs and UBDs, depending on their respective affinities around the stalled primer ends (50). This possibility is compatible with the observation that Polκ can be induced by specific agents that produce DNA damage cognate for Polκ (52). Increasing the ratio of Polκ forces it to predominantly access specific DNA damage. We believe that this model provides an integrated picture of the cellular functions of various motifs of the Y-family DNA polymerases.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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