Role of the ubiquitin-binding domain of Pol\(\eta\) in Rad18-independent translesion DNA synthesis in human cell extracts

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ABSTRACT

In eukaryotic cells, the Rad6/Rad18-dependent monoubiquitination of the proliferating cell nuclear antigen (PCNA) plays an essential role in the switching between replication and translesion DNA synthesis (TLS). The DNA polymerase Pol\(\eta\) binds to PCNA via a consensus C-terminal PCNA-interacting protein (PIP) motif. It also specifically interacts with monoubiquitinated PCNA thanks to a recently identified ubiquitin-binding domain (UBZ). To investigate whether the TLS activity of Pol\(\eta\) is always coupled to PCNA monoubiquitination, we monitor the ability of cell-free extracts to perform DNA synthesis across different types of lesions. We observe that a \(\text{cis}-\text{syn}\) cyclobutane thymine dimer (TT-CPD), but not a \(\text{N}\)-2-acetylaminofluorene-guanine (G-AAF) adduct, is efficiently bypassed in extracts from Rad18-deficient cells, thus demonstrating the existence of a Pol\(\eta\)-dependent and Rad18-independent TLS pathway. In addition, by complementing Pol\(\eta\)-deficient cells with PIP and UBZ mutants, we show that each of these domains contributes to Pol\(\eta\) activity. The finding that the bypass of a CPD lesion \textit{in vitro} does not require Ub-PCNA but nevertheless depends on the UBZ domain of Pol\(\eta\), reveals that this domain may play a novel role in the TLS process that is not related to the monoubiquitination status of PCNA.

INTRODUCTION

The translesion DNA synthesis (TLS) pathway is responsible for the vast majority of point mutations and is thus considered as a major process leading to genetic instability and carcinogenesis. Understanding the molecular mechanisms underlying this damage tolerance pathway and its regulation is therefore of major importance for our understanding of human pathogenesis.

In eukaryotes, TLS is carried out by specialized, low stringency, DNA polymerases belonging to the Y family (Pol\(\eta\), Pol\(i\), Polk and Rev1) and the B family (Pol\(\zeta\)). \textit{In vivo} or \textit{in vitro} studies have shown that these DNA polymerases have various substrate specificities and that, in many cases, TLS requires the concerted action of several TLS polymerases (1,2). Remarkably, human Pol\(\eta\) has the unique property to replicate past \textit{cis}-syn cyclobutane thymine dimers, one of the major photoproducts induced by UV irradiation, with the same efficiency and accuracy than it does on undamaged DNA (3). The loss of Pol\(\eta\) activity underlies the high susceptibility to skin cancers of \textit{Xeroderma pigmentosum} variant (XP-V) patients (4,5).

The mechanism by which TLS DNA polymerases gain access to the lesion site and take over the replicative polymerase to incorporate nucleotides opposite the damaged base is the subject of intense research. Numerous studies have highlighted the central role of replication processivity clamps (\(\beta\)-clamp in prokaryotes and PCNA in eucaryotes) in the fine tuning between replication and TLS. Yeast Pol\(\eta\) and human TLS polymerases such as Pol\(\eta\), \(\xi\) and \(\kappa\) functionally interact with the interconnecting loop of PCNA via their PCNA-interacting protein (PIP)
motif (6–8). Mutational inactivation of the PIP domain of Polβ sensitizes yeast cells to UV irradiation (9), while homologous mutations confer only moderate UV sensitivity in human cells (10,11). This suggests alternative targeting process(es) for the human polymerase. Recently, Acharya et al. (12) have identified a functional secondary PIP domain within the human Polβ that may explain the above-mentioned modest sensitivity. Furthermore, treatment of yeast or human cells with agents that affect DNA replication promotes the monoubiquitination of PCNA at its K164 residue by the Rad6–Rad18 enzyme complex (13). Genetic studies in Saccharomyces cerevisiae showed an epistatic relationship between PCNA-K164R mutation (a non ubiquitinable form of PCNA) and deletion of the Polβ and Polζ TLS polymerases, demonstrating that TLS in this organism is largely dependent on the monoubiquitination of the replication processivity clamp (14). Vertebrate Y-family DNA polymerases preferentially interact with the monoubiquitinated form of PCNA (15,16) via Ubiquitin (Ub) binding domains designated UBF (Polβ and Polk) or UBM (Pol and Rev1) that are required for their relocalization in nuclear foci after UV irradiation (10,17–19). In addition, it has been observed that some mutations in the UBZ domain of human Polη have a much more drastic effect on UV cell survival than mutations in the C-terminal PIP domain (10). Consequently, it has been proposed that the binding of Y-family polymerases to the Ub moiety of PCNA is required for their access to the site of a stalled replication fork. Such a model highlighting a crucial role of PCNA ubiquitination in the regulation of TLS should however be tempered by the results on the extensive mutational analysis of the UBZ domain of Polη conducted by Acharya et al. (12,20), which suggest that the binding of the Ub moiety by Polη is not a necessary requirement for the ability of this polymerase to function in TLS of UV-induced DNA lesions.

Besides its role in facilitating the access of TLS polymerases to the lesion site, monoubiquitination of PCNA may also increase the kinetic of TLS by specialized DNA polymerases. Indeed, it has been shown that monoubiquitinated PCNA (Ub-PCNA) is more effective in facilitating the access of TLS polymerases to the lesion site, monoubiquitination of PCNA (Ub-PCNA) is more effective in promoting yeast Polα activity across an abasic site in vitro (21). However, such a kinetic activation by Ub-PCNA was not confirmed in another study (22). Finally, TLS in vertebrate cells appears to be only partially dependent on Rad18 activity since UV-mutagenesis is reduced only 2-fold, in Rad18KO (knock out) mouse cells (23). Furthermore, defective ubiquitination of PCNA in the chicken cell line DT40 is not epistatic to Polk and Rev1 mutants for UV sensitivity (24,25) indicating that at least some Y-family polymerases may be recruited in a Rad18-independent manner.

Taken together these data illustrate many aspects on the regulation of TLS by the post-translational status of PCNA that remain unclear and much debated. In an effort to elucidate these mechanisms and to investigate whether a Rad18-independent TLS pathway might operate in mammalian cells, we used Rad18-deficient cells and stable XPV fibroblast cell lines (XP30RO) complemented with Polη polymerases mutated in either their PIP or UBZ motifs. Protein extracts from these cell lines were prepared in order to analyze their ability to perform TLS across different DNA lesions. We could thus monitor the activity of Polη in the presence or in the absence of Rad18 and PCNA ubiquitination, and the activity of Polη variants that are differentially affected in their binding capacity to PCNA and/or to ubiquitin. We show that while Rad18 is required for the bypass of a N-2-acetylaminofluorene-guanine (G-AAF) lesion in cell-free extracts, it is dispensable for the bypass of a TT-CPD lesion. This implies that, under the experimental conditions tested here, the Polη-dependent bypass of CPD is independent of Ub-PCNA. However, we also demonstrate that both the PIP and UBZ domains of Polη facilitate the TLS reaction across both lesions. This result indicates that, in addition to its documented role in Ub-PCNA binding, the UBZ domain of Polη may play a novel role in the TLS process that is not related to the ubiquitination status of PCNA.

**MATERIAL AND METHODS**

**Plasmids and cell lines**

Cells were grown at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and gentamicin (0.5 mg/ml).

The human colon carcinoma cell line HCT116-Rad18KO cell line (26) was cultured in the presence of G418 (300 μg/ml) and puromycin (0.3 μg/ml). MRC5-V1 cells are SV40-transformed normal human lung fibroblasts (27). The XP30RO cell line (SV40-transformed X. pigmentation variant human fibroblasts) has a homozygous deletion in the Polη gene resulting in a truncated protein of only 42 amino acids (4). XP30RO cell lines expressing wild type (wt) or mutated forms of Polη were generated by transfection with a pcDNA3.1/zeo(–) plasmid harboring the corresponding Polη sequence (kindly provided by P. Kannouche); transfected cells were thereafter selected with zeocin. Mutations in the coding sequence of Polη were generated by site-directed mutagenesis. The sequences of the primers used for the generation of the D652A mutation are: 5′-CCAGAAACAATGGCATATCTTTTGCA-3′ and 5′-CATATCCCATACCCCGGTATACAGGGA-3′. The ΔPIP deletion, generated by introducing a stop codon at position 705, consists in a deletion of the last nine amino acids of the Polη amino acid sequence (QTLESFFKPLTH—QT). The sequences of the primers used for mutagenesis are: 5′-GAATCATTTTTAAGCCATTAACA-3′ and 5′-ACAAATTGTTGATGCCCTACAGGCT-3′.

**Rad18 gene silencing in MRC5 cells**

siRNA design, cloning in pEBV vectors carrying a hygromycin B resistance cassette and establishment of stable knockdown or control cells were carried out as previously described (28). The RNAi sequence for Rad18 (NM_020165) stretches nucleotides 1459–1477. Control cells were obtained by stable transfection with the pBD650 vector that carries an inefficient shRNA (28).
The Rad18 knockdown and control cell lines were designated as MRC5-Rad18\textsuperscript{KD} and MRC5-CT, respectively. The transformed MRC5 cell lines were cultured in presence of hygromycin B (150\textmu g/ml, Roche).

**Cell survival analysis**

Cells were plated at 4000 (XP30RO) or 2500 (MRC5) cells per 10 cm diameter dish 24 h before UV irradiation (254 nm). After 7 days of culture in the presence of caffeine (1 mM), growing clones were fixed with 4% paraformaldehyde and stained with crystal violet in 10% ethanol. Clones containing more than 50 cells were counted. Experiments were performed at least four times. Colony formation was normalized as a percentage of the non-irradiated control.

**Immunostaining**

Cells grown on coverslips were UV-irradiated (15 J/m\textsuperscript{2}) and cultured for 4 h. The cells were fixed with methanol/acetone (1/1, v/v) for 30 min at 4°C and washed three times with phosphate-buffered saline (PBS) supplemented with Tween-20 (0.1%; v/v). Cells were stained by overnight incubation (4°C) with a monoclonal anti-Rad18 antibody (Rad18 Ab57447, Abcam, 1:500 dilution) washed three times with PBS supplemented with Tween-20 (0.1%; v/v), and incubated for at least 2 h at room temperature with Alexa Fluor 488 goat anti-mouse IgG (1:2000, Molecular Probes). Observations were made with a Leica TCSD4 confocal microscope equipped with an argon/krypton laser and suitable barrier filters.

**Immunoblotting**

Cell-free extracts (100\textmu g) were loaded onto SDS/polyacrylamide gels. After electrophoresis, separated proteins were transferred onto a PVDF membrane (Biorad) and probed with antibodies against Pol\eta (C17, sc-5938), Pol\delta (A9, sc-17776) and PCNA (PC10, sc-56) from Santa Cruz Biotechnology. Anti-HA antibody (HA.11) was purchased from Covance.

** Primer extension analysis and in vitro PCNA monoubiquitination assay**

Construction of single-stranded plasmids containing either a CPD lesion (pUC3G1-AAF.ss) or a single unique G-AAF adduct (pUC3G1-AAF-ss) has been extensively described (29). Primer extension analysis and standard ubiquitination assays were performed as previously described (30,31). Briefly, the reaction mixture (6.25\textmu l) containing 10 fmol of primed DNA and the whole-cell extract was incubated at 37°C in 50 mM HEPES-KOH (pH 7.8), 7 mM MgCl\textsubscript{2}, 1 mM DTT, 4 mM ATP, 500\textmu M of dNTPs, 40\textmu M creatine phosphate and 100\textmu g/ml creatine kinase. The reaction was stopped by adding an equal volume of proteinase K-SDS (4 mg/ml, 2%) and incubated for 30 min at 37°C. Purified replication products were further digested with EcoRI and PvuII restriction enzymes and analyzed by electrophoresis on a polyacrylamide–7 M urea denaturing gel. Quantification of the levels of TLS was determined by phospho-imager analysis.

**RESULTS**

Rad18 is dispensable for the in vitro bypass of a CPD lesion while it is required for the bypass of an AAF adduct

In order to investigate whether Pol\eta functions were strictly dependent on Ub-PCNA and whether this may depend upon the nature of the DNA lesion, we compared the ability of extracts from Rad18-proficient (wild type) or Rad18-deficient (Rad18\textsuperscript{KD}) HCT116 cells to synthesize DNA opposite either a CPD or an AAF lesion. HCT116-Rad18\textsuperscript{KD} cells were significantly more sensitive than wild-type HCT116 to DNA-damaging agents such as cisplatin and methyl methanesulfonate but only moderately sensitive to UV (26). Strikingly, the difference between wild-type and Rad18-deficient cells was only detectable at high UV doses (Figure 1A). By using single-stranded mono-modified plasmids, we have already shown that normal cell extracts are able to bypass a blocking lesion such as a G-AAF adduct (30). In this assay, the replicative machinery that extends the radiolabeled primer (located 91 nucleotides away from the adduct site), is blocked one nucleotide before the lesion (L-1; Figure 1C). Pol\eta carries out the incorporation of a few nucleotides opposite and beyond the lesion site, and is then probably replaced by the replicative polymerase, as judged by the comparison of the extension profiles obtained with normal and XPV cell extracts (Figure 1C), or with XPV extracts complemented with Pol\eta (Supplementary Figure S1A). Therefore, our in vitro assay may mimic the successive steps promoting the access of TLS polymerases to damaged DNA. Indeed, we have clearly established that PCNA is required for the TLS reaction (Supplementary Figure S1B). Furthermore PCNA is monoubiquitinated during such primer extension reactions (31). This post-translational modification of PCNA depends on both relatively long tracts of DNA synthesis and upon the presence of replication hurdles such as hairpin structures or a single G-AAF adduct or a TT-CPD lesion (31 and Supplementary Figure S2). The ability of cell-free extracts to perform both PCNA monoubiquitination and DNA synthesis across a lesion gives us the unique opportunity to investigate whether the TLS activity of Pol\eta is coupled to this modification.

During primer extension, Ub-PCNA formation is easily detectable in HCT116 cell-free extracts, while it is abolished in HCT116-Rad18\textsuperscript{KD} cell-free extracts (Figure 1B). This demonstrates that under our conditions, the monoubiquitination reaction observed in cell-free extract is totally Rad18-dependent. As shown in Figure 1C, we observed that HCT116 cell-free extracts are able to bypass both CPD and AAF lesions, although higher efficiencies were observed for CPD. Extracts prepared from XP30RO cells which lack Pol\eta are largely defective in TLS past either AAF or CPD lesions, indicating that Pol\eta is involved in the bypass of both lesions. In Rad18-deficient cells, the bypass of the CPD lesion is as efficient as in...
wild-type cell extracts, while the bypass of the AAF adduct is totally abolished (Figure 1C). These results were further confirmed in a time course experiment showing that prolonged incubation of up to 60 min did not allow the bypass of an AAF adduct to occur in the absence of Rad18 (data not shown). Conversely, the kinetics of TLS through the CPD lesion in both HCT116 and HCT116-Rad18KO extracts are equivalent (Figure 1D). Together, these results indicate that Rad18 per se or the ubiquitination of PCNA is dispensable for the bypass of this lesion during primer extension in cell-free extracts.

We were concerned that the moderate UV sensitivity of HCT116-Rad18KO cells as compared to HCT116 cells might reflect a particular UV damage tolerance of this cell line that may interfere with the Polδ pathway. In order to confirm the results obtained with HCT116-Rad18KO cells, we thus have extended our analysis on TLS efficiency through CPD and AAF lesions by using a SV40-transformed MRC5 human fibroblast cell line in which Rad18 was stably silenced by means of long-term RNA interference. To assess the efficiency of Rad18 silencing, an MRC5 cell line ectopically expressing HA-tagged Rad18 protein was transfected with pEBV coding for shRNA against Rad18. After isolation of stable transformants upon hygromycin B selection, protein extracts were analyzed by immunoblotting with an anti-HA antibody. As shown in Figure 2A, expression of HA-Rad18 was drastically reduced in three independent cell clones expressing the shRNA against Rad18, while HA-Rad18 was readily detected as two bands, in extracts from either non-transfected cells (MRC5-HARad18-NT) or cells that express a non-related shRNA sequence (MRC5-HARad18-CT). This result demonstrates the effectiveness of the Rad18 shRNA sequence for silencing Rad18 expression. Therefore, this sequence was subsequently used to generate a MRC5-Rad18KD cell line in which endogenous Rad18 depletion was monitored by
immunostaining using an anti-Rad18 antibody (Figure 2B). The disappearance of the Rad18 signals in both non-irradiated and UV-irradiated MRC5-Rad18KD cells demonstrate the efficiency of Rad18 depletion in the selected clone (Figure 2B). Efficient silencing of Rad18 in this cell line was further confirmed by the absence of PCNA monoubiquitination after UV irradiation (data not shown). MRC5-Rad18KD cells also show increased sensitivity to UV as compared to MRC5 or MRC5-CT cells that express a non-related shRNA sequence (Figure 2C).

We previously noticed that induction of Ub-PCNA during primer extension is much more efficient than that after UV irradiation of cultured cells (31). While Ub-PCNA is easily observable in control cell extracts, it is barely detected in MRC5-Rad18KD cell extracts (Figure 2D). As shown previously with HCT116 cells, extracts prepared from both Rad18-proficient and -deficient MRC5 cells were able to carry out TLS through a CPD lesion with equal efficiency (Figure 2E). In contrast, MRC5-Rad18KD extracts show reduced TLS capacities opposite an AAF adduct (Figure 2E). Quantitative analysis of the data shows that TLS activity through an AAF-modified substrate in the MRC5-Rad18KD cell extracts drops to about 50% of that measured in control cell extracts. We consider that the remaining PCNA monoubiquitination observed in MRC5-Rad18 KD cell extracts (Figure 2D) may be responsible for the residual Polδ-dependent TLS activity through an AAF adduct in these assays.

Data from both HCT116-Rad18KO and MRC5-Rad18KD cell extracts demonstrate that Rad18 activity is dispensable for the bypass of a CPD lesion in vitro while it is required for the bypass of an AAF adduct. Altogether, our observations strongly suggest the existence of both Rad18-dependent and -independent Polη-mediated TLS pathways.

**UV survival of XP30RO cells expressing PIP and UBZ mutants of Polη**

The Polη-deficient XP30RO cell line was used to generate several clones stably expressing different mutants of Polη.
The ΔPIP mutation consists of a deletion of the last nine amino acids of the Polη sequence containing the PCNA binding site consensus sequence (6,32). The UBZ mutation consists of a D to A mutation at position 652. The solution structure of this domain shows a classical zinc-finger structure coupled with a carboxy-terminal, a helix that mediates the interaction with ubiquitin (33). The D652 residue, lying in the α-helix, is conserved in both yeast and vertebrate Polη and was shown, by co-immunoprecipitation assays and by yeast two-hybrid analysis (10; A.C., unpublished results) to be essential for Ub binding. The single D652A as well as the double mutant D652A-ΔPIP were constructed.

The UV sensitivity of stable transformants expressing mutated Polη was determined using a colony-forming assay (Figure 3). XP30RO cells expressing either wild-type Polη (WT) or the empty vector (V) were included as positive and negative controls, respectively. We found a strong positive correlation between the ability of ΔPIP Polη mutants to complement the UV sensitivity of XPV cells and the level of ectopic expression of the Polη protein in the different clones analyzed (Figure 3A and C). This result emphasizes the crucial importance of controlling the expression level of a protein in complementation assays. Indeed, while high levels of expression of the ΔPIP Polη allows an almost complete restoration of UV survival, the ΔPIP mutant clone expressing the lowest levels of Polη fails to efficiently complement the UV sensitivity of XPV cells.

On the other hand, the UBZ domain mutant, is only partially able to complement the UV sensitivity of XPV cells, whatever its expression level (Figure 3B and C). Finally, the UV survival of the transfectant expressing a high level of the double mutant D652A-ΔPIP Polη was significantly lower than either of the single mutants (Figure 3B). Altogether, these observations are consistent with a model in which both the PIP motif and the D652 residue within UBZ domain contribute to the in vivo function of Polη.

**Both PIP and UBZ domains of Polη contribute to CPD and AAF bypass in vitro**

In order to analyze the contribution of the PIP and/or UBZ domains to Polη’s TLS function, extracts from cells expressing the different mutants were prepared and assayed for in vitro bypass through either CPD or AAF lesions. With extracts from cell lines expressing the mutated Polη at moderate levels (lower than 10-fold above the MRC5 basal level; Figure 4A), we observed a decrease in the amount of TLS products in comparison with the wild-type control (Figure 4A). These results suggest that mutations in either of these two domains significantly reduce the bypass efficiency through both lesions, even if the PIP truncation appears to have a more pronounced effect than the D652A mutation on the bypass efficiency (compared at identical levels of Polη expression: lanes 2 with lanes 6 and lanes 3 with lanes 7).

Finally, cell extract from the D652A-ΔPIP Polη double mutant, although containing a high level of Polη cannot restore efficient TLS through G-AAF or TT-CPD lesions. Altogether, these data show that, in accordance with the in vivo studies, both regions of Polη participate to its function. Interestingly, despite the independence of the CPD bypass with regard to Rad18, the D652 residue...
within the UBZ domain contributes to the bypass of a CPD lesion in vitro. In turn, this points to a specific role of the D652 residue that does not involve interaction of Pol η with the ubiquitin moiety of the modified PCNA.

DISCUSSION

Bypass of TT-CPD, in contrast to G-AAF, is independent of Rad18 in cell-free extracts

Our results demonstrate that, in cell-free extracts, Rad18 is dispensable for the bypass of a CPD lesion while it is required for the bypass of an AAF adduct (Figures 1 and 2). While we cannot rule out a minor undetectable Rad18-independent monoubiquitination of PCNA (34–36), we infer from these data that CPD bypass in cell-free extracts is independent of Ub-PCNA. Our observation is consistent with a recent report showing that Pol η is able to gain access to replication complexes and to catalyze TLS through CPD dimers in the presence of the non-ubiquitinable K164R PCNA mutant during replication of double-stranded DNA in HeLa cell-free extracts (37). What could be the rationale for such a difference between the CPD lesion and the AAF adduct? One obvious difference is the facility by which each of these lesions is bypassed by Pol η. Steady-state kinetic analysis have shown that purified yeast and human Pol η can replicate through a T–T CPD dimer with the same kinetics and fidelity as through an undamaged T–T sequence (38,39), while the bypass kinetics of a G-AAF adduct by purified human Pol η is significantly lower (40). By altering the Pol η–PCNA interaction, monoubiquitination of the clamp may directly stimulate Pol η TLS activity through the AAF adduct, as previously shown in vitro for an abasic site (21). Ub-PCNA may also recruit additional co-factors that could facilitate the Pol η-dependent bypass of the AAF adduct. Alternatively, Rad18 per se may be required for this specific reaction since it has been shown that interaction of Rad18 with Pol η participates in the polymerase function in vivo (16).

Contributions of the PCNA- and ubiquitin-binding domains to the activity of Pol η in vivo

We examined the ability of PIP truncation and D652A Pol η mutants to complement the UV sensitivity of XP30RO cell line. Consistent with the predicted essential role of the PIP domain in PCNA binding, we found that expression of the ΔPIP Pol η mutant (even fivefold above the MRC5 basal level) results in a substantial sensitization of the cells as compared to those expressing exogenous WT Pol η. Surprisingly, this mutation can be compensated by high overexpression of the mutant polymerase, raising the possibility that the secondary PIP domain recently identified within the Pol η sequence (12) may substitute for the C-terminal one, although with a lower efficiency. The hypothesis that the binding of the Ub moiety on PCNA via the UBZ domain may replace the Pol η PIP–PCNA interaction seems unlikely since recent structural studies have shown that the PIP motif contributes more significantly to the interaction between Pol η and PCNA than the UBZ domain (33,41). Alternatively, the defect in the targeting of Pol η to the DNA template/primer junction by the C-terminal PIP domain might be compensated by interaction with other proteins, such as Rad18 or Rev1, that may recruit Pol η to the replication stalling sites (16,42).
In agreement with our data, it has already been shown that a mutation of the D652 residue resulted in UV sensitivity in yeast and human cells (12,20,43). When overproduced about 10-fold, both D652A and ΔPIP PolZ mutants partially complement the UV sensitivity of XPV cells to the same extent. While complementation by the ΔPIP PolZ mutant varies notably with the expression level within a 1- to 10-fold overexpression range, complementation with the D652A PolZ mutant does not change significantly with the level of overproduction (Figure 3C). We conclude from these results that the D652 residue of PolZ participates in a specific role that is not exchangeable with that of the PIP domain and that is essential for full activity of PolZ.

Alternative models for the role of the PolZ UBZ domain in TLS through a CPD lesion

We also examined the effects of PolZ PIP truncation and D652A mutation on the ability of cell-free extracts to promote TLS through a CPD lesion and an AAF adduct. We observed that whatever the lesion, both mutations impair TLS efficiency in vitro. This result together with the UV survival analysis of XP30RO cells complemented with PIP and UBZ mutants, are in agreement with a model in which both regions contribute to PolZ function in TLS (10,12,20,43). The dependence of TLS on the PIP motif confirms the involvement of PCNA in the reaction since it has been shown that this motif mediates a functional interaction between PolZ and PCNA (6). Interestingly, while Rad18 is not required for the bypass through a CPD lesion, we observed that the D652 residue, which is involved in the interaction with ubiquitin, contributes to this reaction. This result suggests that, for the bypass of this specific lesion, the UBZ domain of PolZ may bind the ubiquitin moiety of a modified protein that is ubiquitinated in a Rad18-independent manner. Another possibility might be that the D652A mutation abrogates a novel, uncharacterized, function of the PolZ UBZ domain that is distinct from ubiquitin binding. In agreement with this latter interpretation, recent genetic data provide evidences that some mutations in the C2H2 motif of the UBZ domain of PolZ impair the binding to ubiquitin but have no perceptible effect on UV sensitivity and UV mutagenesis (12,20), suggesting that the binding of ubiquitin by PolZ via its UBZ domain is not a necessary prerequisite for PolZ to function in TLS.

CONCLUSION

Despite the recent advances in the knowledge of the TLS process, the mechanisms by which the TLS Pols gain access to the template–primer junction when the replicative machinery encounters a blocking lesion remain unclear. Several data indicate that PolZ contributes not only to gap filling after the replication apparatus has moved away from the damaged site, but also helps to maintain the continuous progression of the replication fork after DNA damaging treatment. Indeed, as compared to WT cells, XPV cells accumulate shorter replication products after UV irradiation and elongation of these intermediates was markedly retarded (25,44). We propose that, depending on the nature of the lesion (and its sequence context), different mechanisms control PolZ activity. Efficient bypass of lesions that do not require Rad18-dependent PCNA monoubiquitination, as a T–T CPD dimer, may occur ‘on the fly’ during processive DNA synthesis, without actual uncoupling of the replicative polymerases. If this first TLS trial is not successful, the uncoupling of leading and lagging strand synthesis provides the substrate for Rad6/Rad18-dependent PCNA monoubiquitination. Rad18 and Ub-PCNA may then facilitate the bypass reaction as exemplified here by the analysis of the AAF adduct TLS. We were concerned that our system may not be fully relevant to test this model, since the CPD lesion is located on a ssDNA template and is thus able to stimulate PCNA monoubiquitination in the absence of fork uncoupling (Supplementary Figure S2). However, as shown in Figures 1 and 2, this modification is clearly dispensable for PolZ to bypass a CPD lesion.

We have established that in cell-free extracts, TLS of both an AAF adduct and a CPD lesion requires the UBZ domain. We speculate that this domain plays a dual role in the TLS process. On one hand, it may mediate the binding to Ub-PCNA during the gap filling reaction. On the other hand, the D652 residue may participate in a distinct function, such as the interaction with another protein that is necessary for the coordination of the PolZ activity at the replication fork. In agreement with this model, Edmunds et al. (25) have recently shown that, in DT40 cells, PCNA monoubiquitination is dispensable for maintaining replication fork progression on damaged DNA while it is required post-replicatively. Interestingly, the data presented in this latter paper show that the UBM domains of Rev1 are required for its activity at the replication fork in the absence of Ub-PCNA. Thus, it is conceivable that the regulatory mechanism described in this study may apply to the UBD domain of Y-family polymerases in general.

SUPPLEMENTARY DATA

Supplementary data are available at NAR Online.

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