Human DNA polymerase η activity and translocation is regulated by phosphorylation

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Contributed by James E. Cleaver, September 2, 2008 (sent for review May 4, 2007)

Human DNA polymerase η (pol η) can replicate across UV-induced pyrimidine dimers, and defects in the gene encoding pol η result in a syndrome called xeroderma pigmentosum variant (XP-V). XP-V patients are prone to the development of cancer in sun-exposed areas, and cells derived from XP-V patients demonstrate increased sensitivity to UV radiation and a higher mutation rate compared with wild-type cells. pol η has been shown to replicate across a wide spectrum of DNA lesions introduced by environmental or chemotherapeutic agents, or during nucleotide starvation, suggesting that the biological roles for pol η are not limited to repair of UV-damaged DNA. The high error rate of pol η requires that its intracellular activity be tightly regulated. Here, we show that the phosphorylation of pol η increased after UV irradiation, and that treatment with caffeine, siRNA against ATR, or an inhibitor of PKC (calphostin C), reduced the accumulation of pol η at stalled replication forks after UV irradiation or treatment with cisplatin and gemcitabine. Site-specific mutagenesis (S587A and T617A) of pol η at two putative PKC phosphorylation sites located in the protein-protein interaction domain prevented nuclear foci formation induced by UV irradiation or treatment with gemcitabine/cisplatin. In addition, XP-V cell lines stably expressing either the S587A or T617A mutant form of pol η were more sensitive to UV radiation and gemcitabine/cisplatin than control cells expressing wild-type pol η . These results suggest that phosphorylation is one mechanism by which the cellular activity of pol η is regulated.

xeroderma pigmentosum variant | lesion bypass

Xeroderma pigmentosum (XP) is an autosomal recessive condition characterized by premature skin aging, pigmentary changes, photosensitivity, and malignant tumor development. The manifestations associated with XP are because of a cellular hypersensitivity to UV radiation resulting from defects in any one of a number of genes encoding nucleotide excision repair (XP-A to -G) proteins (1, 2). Human DNA polymerase η (pol η) is an important enzyme that replicates across pyrimidine dimers introduced by UV radiation (3), and defects in the gene encoding pol η result in xeroderma pigmentosum variant (XP-V) syndrome (3, 4). Similar to patients with other forms of XP, patients with XP-V are highly sensitive to UV radiation and prone to the development of skin cancer (5). Furthermore, cells derived from XP-V patients exhibit a higher mutation rate (6) than the wild-type cells. In addition to pyrimidine dimers, pol η has been shown to replicate across 8-hydroxyurea-induced lesions, *O*⁶-methylguanine, and cisplatin cross-linked intrastrand GG sites (7). Recently, we have shown that pol η incorporates, extends, and bypasses chemotherapeutic nucleoside analogs AraC and gemcitabine (8). pol η is also involved in Ig hypermutation (9), strand invasion during homologous recombination (10), and replication during nucleotide starvation (11). These studies suggest that in addition to its roles in the protection of cells from DNA damage, pol η has other physiological roles.

Biochemical and cellular studies have shown that pol η has high fidelity while replicating across many different types of DNA lesions, inserting the correct complementary nucleotides, especially inserting adenines opposite thymidine dimers (12). In contrast, *in vitro* studies have shown that pol η replicates undamaged DNA with much lower fidelity (12). Therefore, to achieve a balance between genomic integrity and cell survival, the *in vivo* activity of pol η needs to be tightly regulated in the cell.

UV irradiation has no apparent impact on pol η expression at RNA or protein levels (13). However, confocal microscopy has shown that UV irradiation or treatment with certain DNA-damaging agents induces the translocation of pol η to stalled DNA replication forks, which is seen as formation of foci in the nucleus (13). These observations suggest that pol η is recruited to replication forks to facilitate bypass of DNA lesions that block replicative polymerases. pol η is also recruited to replication forks during nucleotide deprivation by hydroxyurea (11), suggesting that pol η translocation is responsive to DNA damage-independent inhibition of replication fork progression (14). This relocation of pol η appears to be critical for its cellular activity because complementation of XP-V cells with a mutant form of pol η that fails to relocate after UV irradiation does not restore wild-type phenotype (13).

The molecular mechanisms that enable the recruitment of pol η to stalled replication forks are not yet clear. Kannouche *et al.* (15) reported that UV irradiation induces proliferating cell nuclear antigen (PCNA) ubiquitination and that pol η interacts exclusively with monoubiquitinated PCNA. However, a recent report revealed that nonubiquitinated and monoubiquitinated PCNA have similar affinities for pol η (16). In addition, ataxia-telangiectasia mutated Rad3-related (ATR) is activated by DNA replication stresses introduced by UV radiation, cisplatin, and methyl methanesulfonate (MMS) (17), and XP-V cells exhibit enhanced ATR signaling after UV irradiation (18). ATR proteins are kinases like PI3-kinase, which are known to play key roles in DNA damage-induced checkpoint control. Here, we explored the potential role of protein kinases in the regulation of pol η activity. The results presented suggest that phosphorylation controls the intracellular translocation of pol η to stalled DNA replication forks and indicates that both ATR and protein kinase C (PKC) are involved in the process.

Author contributions: Y.-w.C., J.E.C., Z.H., R.E.H., Y.Y., and K.-m.C. designed research; Y.-w.C., Z.H., J.-Y.C., Y.Y., and K.-m.C. performed research; J.E.C., Z.H., and K.-m.C. contributed new reagents/analytic tools; Y.-w.C., J.E.C., Z.H., R.E.H., J.-Y.C., Y.Y., and K.-m.C. analyzed data; and K.-m.C. wrote the paper.

The authors declare no conflict of interest.

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Fig. 5. Sensitivity of S587A and T617A pol η mutants to UV radiation and gemcitabine/cisplatin combination treatments. Cell survival curves of XP30RO cells stably transfected with wild-type-, S587A-, or T617A-EGFP-pol η expression plasmids. (A) UV radiation. (B) Gemcitabine/cisplatin combination treatments. (C) WT and S587A pol η expressing cells treated with caffeine (1 mM) and UV irradiation. (D) WT and S587A pol η cells treated with caffeine (1 mM) and gemcitabine/cisplatin.

lular localization in response to UV irradiation (13). As shown in Fig. 4*A*, sequence analysis of pol η revealed two putative PKC phosphorylation sites, S587 and T617, that lie within the protein-protein interaction domain. To evaluate the impact of phosphorylation on pol η , S587 and T617 were individually mutated in an EGFP-pol η expression vector. The plasmids that carried the S587A or T617A mutations were then independently transfected into XP30RO cells, and the stably transfected cells were selected with geneticin and sorted by using FACS. Then, the intracellular location of the mutant pol η proteins in response to UV irradiation and gemcitabine/cisplatin combination was examined. Like wild-type pol η in cells that have not encountered DNA damage treatments, the two mutant proteins were equally distributed in the nucleus (Fig. 3*A–C*). However, unlike wild-type pol η (Fig. 3*D* and *G*), both mutant proteins have significantly reduced nuclear foci formed in response to UV irradiation (Fig. 3*E* and *H*) or gemcitabine/cisplatin treatment (Fig. 3*F* and *I*). The foci formation efficiency of both mutant proteins was also summarized and presented as Fig. 3*J* and *K* and Table 1. These results suggest that amino acids S587 and T617 play a critical role in determining the intracellular location of pol η .

Because the intracellular movement of pol η is critical for its biological activity (13), the impact of the S587A and T617A mutations on cellular sensitivity to UV and gemcitabine/cisplatin treatments was evaluated. As shown in Fig. 5, the T617A pol η -expressing cells were moderately more sensitive to both UV irradiation (Fig. 5*A*) and gemcitabine/cisplatin combination treatments (Fig. 5*B*) than the cells transfected with wild-type pol η . In contrast, cells that expressed S587A mutant pol η were more sensitive to both UV irradiation and gemcitabine/cisplatin combination treatments, exhibiting sensitivity similar to that observed with the parental XP30RO cells (Fig. 5*A* and *B*). These results indicated that residue S587 of pol η is more important for its biological activity.

As described above, both ATR and PKC play an important role in recruiting pol η to stalled replication forks. Because S587 is a putative PKC phosphorylation site and has more profound impact on the cell sensitivity to both UV irradiation and

gemcitabine/cisplatin treatments than T617, we examined whether caffeine and the S587A mutation have additive effects on cellular sensitivity to DNA damage. As shown in Fig. 5 C and D, cells expressing S587A pol η were more sensitive to both UV and drug treatments in the presence of caffeine than in its absence. These results suggested that, in addition to the phosphorylation of residue S587, other mechanisms suppressed by caffeine, presumably other downstream targets of ATR, are also involved in regulating the sensitivity of cells to UV irradiation or gemcitabine/cisplatin combination treatments.

Discussion

The recruitment of pol η to transiently associate with stalled replication forks in response to certain types of DNA damage suggests that the intracellular movement of pol η is regulated by the DNA-damage response. To date, the mechanism(s) that regulates this controlled recruitment of pol η to DNA damage sites is not well understood. Here, we demonstrate that pol η phosphorylation occurs after UV irradiation and implicates phosphorylation in modulating the intracellular activity of pol η . Phosphorylation is known to regulate the activity of several DNA repair enzymes (36, 37). In particular, the PI3-kinase-like kinases ATM and ATR are two key DNA damage checkpoint proteins. Whereas numerous studies have reported that ATM is mainly responsible for DNA double strand break signaling, ATR has been implicated as a key G₁/S-phase-regulating component that coordinates cellular responses to DNA replication stress, such as UV-irradiation-induced DNA damage (38). On UV irradiation, replication protein A (RPA) has been shown to recruit ATR to stalled replication forks with help from ATR-interacting protein (ATRIP) (17). Then, the activated ATR independently recruits multiple proteins to the stalled replication fork. In this report, we show that the phosphorylation of pol η increased in response to UV irradiation. Moreover, by using two independent methods of inhibiting ATR activity (treatment with caffeine and ATR siRNA), we observed blocking of UV-induced pol η foci formation, suggesting that ATR is one of the kinases that mediates the recruitment of pol η to stalled replication forks.

In addition to the role played by ATR, our studies indicate that PKC also participates in the recruitment of pol η to the stalled replication fork. The first observation was that the PKC inhibitor calphostin C blocked the intracellular relocation of pol η after UV irradiation or gemcitabine/cisplatin treatment. PKC overexpression has been shown to protect cells from UV-induced apoptosis (39–41), and PKC is involved in many signaling cascades regulating cell proliferation, apoptosis, and differentiation. There are 11 different PKC isoforms (28) and several of them are involved in regulating DNA repair processes. For example, PKC ζ regulates ubiquitination, degradation, and intracellular levels of hMutS α proteins (37), and PKC δ is responsible for DNA damage induced phosphorylation of Rad9 (42). Our current results indicate that PKC may reduce UV-induced apoptosis by enhancing the cellular activity of pol η to resume DNA replication stalled at DNA damage sites. In support of this hypothesis, cells expressing pol η mutated specifically at putative PKC phosphorylation sites were found to be more sensitive to DNA damaging agents than cells expressing wild-type pol η . In addition, the different sensitivity of the S587A and T617A mutant-expressing cell lines to both UV radiation and gemcitabine/cisplatin treatments implies that amino acid residue S587 plays a more important role than T617 in regulating pol η translocation. Caffeine-treated S587A cells were also more sensitive to both UV radiation and gemcitabine/cisplatin treatments than wild-type cells, suggesting that the PKC-mediated and caffeine-induced responses may be complementary. Therefore, a reduction of intracellular relocation of pol η by caffeine would be expected to increase cellular sensitivity in response to UV. Nevertheless, other studies have shown that caffeine has minor effects on the cellular sensitivity of wild-type cells to UV radiation as compared with XP-V cells (25, 43–45). In addition, caffeine inhibits the phosphorylation of other targets in addition to ATR (i.e., protein kinases involved in cell cycle G₂/M checkpoint), which may also contribute to cell survival. Furthermore, the S587A mutant pol η did not form nuclear foci after UV irradiation or treatment with gemcitabine/cisplatin. The S587A-expressing cells and the parental XP30RO cells were also equally sensitive to both treatments. Together, the information implies that other downstream targets of ATR become more important for cell survival when pol η is either lost or inactive. For example, during DNA homologous recombination, pol η was shown to promote DNA chain extension during strand invasion on defined substrates. However, homologous recombination is enhanced in pol η -deficient cells (10, 46). Obviously, further studies are needed to fully illuminate the relationship between downstream signaling factors that regulate pol η activity.

The deletion mutation studies of pol η reported by Kannouche *et al.* (13) indicate that the region between amino acids 594 and 713 is sufficient for pol η to form foci in response to UV irradiation. Our data indicate that phosphorylation of T617 is important for the foci formation of pol η , which is consistent with the deletion studies. Although S587 is not within the region of amino acids 594–713, our mutation studies indicate that the phosphorylation on S587 is also important for the intracellular location of full-length pol η . It is possible that the deletion of amino acids 1–593 has an intrusive impact on the conformation of pol η and expose cryptic amino acids that normally are not accessible for other proteins in full-length pol η . In contrast, S587A is a site-directed point mutation, and the hydrogen on Ala is not likely to disturb the folding of pol η . However, if the hydroxyl on S587 is playing a conformational role, then phosphorylation on S587 could induce a structural change, possibly rearranging the protein–protein interaction domain of pol η . Such a change may impact binding to proteins like PCNA and Rad18 whose interaction with pol η has been shown to be important for the cellular response to UV irradiation (47, 48). Because the crystal structure of the full-length human pol η has

not been solved to date, further structural information will be necessary to understand how amino acids within pol η impact its conformation.

In summary, we present evidence implicating ATR and PKC in the regulation of nuclear localization of pol η after exposure to UV radiation or DNA-damaging agents commonly used for cancer chemotherapy. It is not yet clear if phosphorylation is direct or if these kinases are upstream regulators in a signaling network leading to pol η phosphorylation. The data add to the list of known ATR functions, which include regulation of cell cycle arrest and apoptosis in response to UV, hydroxyurea, and cisplatin through activating cell cycle regulatory proteins.

Materials and Methods

Cell Culture. SV40-transformed pol η -deficient XP30RO fibroblasts were derived from an XP-V patient as described, and the XP30RO cell line stably transfected with EGFP-pol η expression plasmids was generated as described in refs. 49 and 50.

Chemicals. Tissue culture media minimum essential media (MEM) and geneticin were purchased from Invitrogen. Calphostin C was purchased from Calbiochem. Cisplatin was obtained from Sigma–Aldrich.

Confocal Microscopy. Exponentially growing XP30RO cells, stably transfected with EGFP-pol η , were plated in chamber slides for 24 h before an addition of 2 mM caffeine or 100 nM Calphostin C for an additional 60 min. Then, cells were treated with either UV irradiation (11.3 J·m^{−2}) or gemcitabine (10 nM)/cisplatin (10 μ M) combination followed by 6 h of additional incubation, at the end of which the intracellular location of pol η was analyzed with a Leica laser scanning confocal system equipped with krypton/argon laser.

In Vivo Phosphorylation, Immunoprecipitation, and Western Blotting Analysis of pol η . XP30RO cells transfected with EGFP-pol η were seeded and grown on 15-cm Petri dishes at 37°C for 24 h before 100 μ Ci of ³²P-labeled orthophosphate was added for an additional 2 h followed by UV irradiation. pol η was immunoprecipitated 6 h after UV irradiation. The cells were washed with cold PBS, harvested, and lysed with 10 mM Tris-HCl (pH 7.4), 4 mM EDTA, 30 mM KCl, 1% Nonidet P-40, 500-fold dilution of protease inhibitor mixture from Sigma, 10 mM β -glycerol phosphate, 0.5 mM NaF, 1 mM orthovanadate, and 1 mM PMSF. The cell lysates were centrifuged at 17,000 $\times$ g for 15 min at 4°C, and the supernatant was collected. The amount of total protein in the supernatants was determined by using the Bradford protein assay (Bio-Rad) to ensure that equal amounts of total protein were used for immunoprecipitation. Identical dilutions of a monoclonal anti-pol η antibody (Santa Cruz Biotechnology) were added for 12 h at 4°C followed by the addition of anti-mouse IgG agarose. The immune complexes were precipitated by centrifugation and washed with cold PBS. SDS/PAGE gel loading buffer was added to the collected beads, and the samples were heated in boiling water for 5 min. The proteins in the immune complexes were separated on SDS/10% PAGE gels and then transferred onto nitrocellulose membranes. The blots were probed with an anti-pol η polyclonal antibody (Santa Cruz Biotechnology) and visualized by using SuperSignal West Dura (Pierce) Western blotting detection reagents. The signal was detected by using a Fuji LAS-1000 imaging system. The ³²P-labeled pol η was detected and quantified with a phosphorimager (Fuji FLA 5000).

Down-Regulation the Expression Level of ATR. To down-regulate the expression of ATR, specific siRNA targeting ATR (Stealth siRNA, Invitrogen, Co.) was used. The control siRNA (fluorescein-conjugated) was a nontargeting 20- to 25-nucleotide siRNA (Santa Cruz Biotechnology) that used siRNA as a transfection control and to monitor transfection efficiency. The siRNA was preincubated in α -MEM in the presence of Lipofectamine RNAiMax (Invitrogen) at 37°C for 20 min in six-well plates. Then, 1.25 $\times$ 10⁵ cells in MEM were plated and gently mixed with siRNA transfection complexes. After 72 h of incubation at 37°C, the cells were seeded in either chamber slides for 24 h before treatments for confocal microscopy study or preparation of whole-cell lysates for Western blotting analyses.

Western Blotting Analysis of the Expression Level of ATR. Cellular lysates from XP30RO-EGFP-pol η cells with or without ATR targeting siRNA transfection

treatment were collected at the end of confocal microscopy study described in Fig. 2G. The expression level of ATR was analyzed by using Western blotting. ATR protein was probed on the nitrocellulose membrane with an anti-ATR monoclonal antibody (Cell Signaling Technology). The expression of ATR protein was visualized by using SuperSignal West Dura (Pierce) Western blotting detection reagents and the signal was detected by using a Fuji LAS-1000 imaging system.

Cell Sensitivity Assays. The cell sensitivity of XP3ORO cell lines stably expressing wild-type or mutant pol η to UV irradiation or gemcitabine/cisplatin combination treatments was determined by using a cell number-based assay or clonogenic assay as described previously (8, 51).

Site-Directed Mutagenesis and Cell Transfection. Mutagenesis was performed by the PCR-based method using the QuikChange Site-directed Mutagenesis Kit (Stratagene) on pEGFP-N1 expression plasmid (BD Biosciences), containing wild-type pol η (50). The primers used to create each mutant form

are as follows: Pol η -S587A primer, 5'-ag cta gaa gaa gcc tct aaa gca ac-3'; pol η T617A primer, 5'-ctg gag gtg gct cag aaa gca-3'. The DNA sequences of the expression plasmids containing the wild type or the site-directed mutant *pol* η genes were confirmed by DNA sequencing at Howard Hughes Medical Institute/Keck Facility, Yale University. The plasmids with the different mutations were independently transfected into XP3RO cells by using electroporation, and stably transfected cells were selected with geneticin (600 μ g/ml). The EGFP-pol η -expressing cells were further analyzed and sorted with FACS to ensure that all mutants have similar EGFP expression.

ACKNOWLEDGMENTS. We are grateful to Drs. Roger Woodgate and Fumio Hanaoka for their consultations and Dr. S. de Feraudy for the FACS analysis of normal and XP cells. The work described here was supported, in part, by grants from the National Institutes of Health, National Cancer Institute Grants CA112446 (K.-M.C.) and CA60750 (R.E.H.), National Institute of Neurological Disorders and Stroke Grant NS052781 (J.E.C.), and the Comprehensive Cancer Center Support Grant CA82103 (J.E.C.).

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