The Functions of Serine 687 Phosphorylation of Human DNA Polymerase η in UV Damage Tolerance*§

Xiaoxia Dai§, Changjun You§, and Yinsheng Wang§‡

DNA polymerase η (polη) is a Y-family translesion synthesis polymerase that plays a key role in the cellular tolerance toward UV irradiation-induced DNA damage. Here, we identified, for the first time, the phosphorylation of serine 687 (Ser687), which is located in the highly conserved nuclear localization signal (NLS) region of human polη and is mediated by cyclin-dependent kinase 2 (CDK2). We also showed that this phosphorylation is stimulated in human cells upon UV light exposure and results in diminished interaction of polη with proliferating cell nuclear antigen (PCNA). Furthermore, we demonstrated that the phosphorylation of Ser687 in polη confers cellular protection from UV irradiation and increases the efficiency in replication across a site-specifically incorporated cyclobutane pyrimidine dimer in human cells. Based on these results, we proposed a mechanistic model where Ser687 phosphorylation functions in the reverse polymerase switching step of translesion synthesis: The phosphorylation brings negative charges to the NLS of polη, which facilitates its departure from PCNA, thereby resetting the replication fork for highly accurate and processive DNA replication. Thus, our study, together with previous findings, supported that the posttranslational modifications of NLS of polη played a dual role in polymerase switching, where Lys682 deubiquitination promotes the recruitment of polη to PCNA immediately prior to lesion bypass and Ser687 phosphorylation stimulates its departure from the replication fork immediately after lesion bypass. * Molecular & Cellular Proteomics 15: 10.1074/mcp.M115.052167, 1913–1920, 2016.

Living cells are constantly exposed to various DNA-damaging agents, such as UV irradiation, chemical carcinogens, and endogenous reactive oxygen species (1). The resulting DNA damage can block the progression of replication forks and/or induce mutations, which result in cell transformation, senescence, or apoptosis (2). To overcome replication blockages, cells are equipped with multiple DNA damage surveillance and repair systems, including translesion synthesis (TLS) (3). A polymerase switching model has been proposed for TLS, where the replicative DNA polymerase δ or ε is switched out for specialized TLS polymerases, a process thought to be mediated by the monoubiquitination of proliferating cell nuclear antigen (PCNA) (4). Replicative DNA polymerases are highly accurate in DNA synthesis, whereas DNA synthesis mediated by TLS polymerases is often error-prone (5, 6). Thus, timely removal of TLS polymerases from the replication fork and restoration of replicative polymerases after TLS are essential for avoiding undesirable mutagenesis during genome replication (7).

DNA polymerase η is a Y-family polymerase that plays a critical role in the TLS across UV-induced cyclobutane pyrimidine dimers (CPDs) (8–10). Deficiency in the gene encoding polη results in xeroderma pigmentosum variant syndrome in humans (11, 12). Xeroderma pigmentosum variant patients manifest high sensitivity to sunlight irradiation, and they are prone to developing skin cancer (11, 12). During TLS, PCNA becomes monoubiquitinated, which recruits polη to the replication fork through polη’s ubiquitin-binding zinc finger (UBZ) and PCNA-interacting peptide (PIP) domains (13–16). A recent study has extended the PCNA-interacting region of polη to amino acid residues 682–713, and PCNA-interacting region of polη can be monoubiquitinated, which masks its PCNA-interacting region and inhibits its interaction with PCNA (17). After UV irradiation, the monoubiquitination of polη is down-regulated to expose the PCNA-interacting surface, thereby stimulating polη’s interaction with PCNA and facilitating TLS (17). In addition, previous studies showed that polη can be phosphorylated at serine 587, threonine 617, and serine 601 (Ser601), which are required for normal survival and efficient recovery from UV damage (18, 19). It is therefore important to identify other possible posttranslational modification(s) of hu-

From the §Department of Chemistry, University of California, Riverside, California 92521–0403
Received May 22, 2015, and in revised form, September 21, 2015
Published, MCP Papers in Press, March 17, 2016, DOI 10.1074/mcp.M115.052167

Author contributions: X.D., C.Y., and Y.W. designed the research; X.D. and C.Y. performed the research; X.D., C.Y., and Y.W. analyzed data; and X.D. and Y.W. wrote the paper.

Molecular & Cellular Proteomics 15.6
man pol\(\eta\) for further understanding the regulatory mechanisms of this important TLS polymerase.

**MATERIALS AND METHODS**

**Cell Culture**—The HEK293T human embryonic kidney epithelial cells (ATCC) and XP30R human skin fibroblasts (20, 21) were cultured at 37 °C in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (Invitrogen) and 100 IU/ml penicillin (ATCC) in an incubator containing 5% CO\(_2\).

**Construction of Vectors**—Three tandem repeats of the FLAG epitope tag (DYKDDDDK) were inserted into BamHI and EcoRI sites of pRK7 vector to produce the pHK7-3×-FLAG vector. Human POLH gene was amplified from HEK293T cells by reverse transcription-PCR to introduce a 5′ Xbal site and a 3′ BamHI site and subcloned into pRK7-3×-FLAG vector. The pEGFPC3-pol\(\eta\) vector was a gift from Prof. Alan R. Lehmann (22). GST-pol\(\eta\) vector was generated by cloning the C-terional portion of pol\(\eta\) encoding the last 112 amino acids into pGEX-4T-1 vector that were pretreated with BamHI and Xhol. The pol\(\eta\)-S687A, pol\(\eta\)-S687D, PID (L704A, FF708–709AA), and PID/UB2 (D652A, L704A, FF708–709AA) mutants were amplified by site-directed mutagenesis using primers containing the indicated mutations. The primers are listed in Supplemental Table S1.

**Preparation of FLAG-Tagged Pol\(\eta\) Protein and Mass Spectrometric Analysis**—HEK293T cells were grown to 70–80% confluency in a six-well plate and transfected with 1.5 μg FLAG-tagged pol\(\eta\) plasmid using Lipofectamine 2000 (Invitrogen). After a 48-h incubation, the protein was isolated and purified using anti-FLAG M2 beads (Sigma). The purified protein was digested with trypsin (Roche) at an enzyme/substrate ratio of 1:50 and subjected to LC-MS/MS analysis.

**LC-MS/MS Experiments**—LC/MS/MS experiments were performed as previously described (23). Briefly, the peptides were separated on an EASY-100 LC II and analyzed on an LTQ Orbitrap Velos mass spectrometer equipped with a nanoelectrospray ionization source (Thermo). The trapping column (150 μm x 50 mm) and separation column (75 μm x 120 mm) were packed with ReproSil-Pur C18-AQ resin (3 μm, Dr. Maisch HPLC GmbH, Germany). The peptide samples were first loaded onto the trapping column in CH\(_3\)CN/H\(_2\)O (2:98, v/v) at a flow rate of 4.0 μl/min and resolved on the separation column with a 120-min linear gradient of 2–40% acetonitrile in 0.1% formic acid and at a flow rate of 300 n/min. The LTQ-Orbitrap Velos mass spectrometer was operated in the positive-ion mode, and the spray voltage was 1.8 kV. The full-scan mass spectra (m/z 300–2000) were acquired with a resolution of 60,000 at m/z 400 after accumulation to a target value of 500,000 in the linear ion trap. MS/MS data were obtained in a data-dependent scan mode where one full MS scan was followed with 20 MS/MS scans. The LC-MS/MS data were employed for the identification of pol\(\eta\) and its posttranslational modifications, which was conducted using MaxQuant, version 1.2.0.18 against International Protein Index (IPI) database, version 3.68 with 87,061 entries to which contaminants and reverse sequences were added. The maximum number of misscleavages for trypsin was 2 per peptide. Cysteine carbamidomethylation was set as fixed modifications. Methionine oxidation, lysine ubiquitination, and serine/threonine phosphorylation were set as variable modifications. The tolerances in mass accuracy for MS and MS/MS were 25 ppm and 0.6 Da, respectively.

The required false positive discovery rate was set to 1% at both the accuracy for MS and MS/MS were 25 ppm and 0.6 Da, respectively.

**UV irradiation**—The wild-type or mutant pol\(\eta\) plasmids were transfected into HEK293T cells as described above. After a 48-h incubation, the medium was replaced with PBS, and the cells were treated with 30 J/m\(^2\) UV light (254 nm) and recovered for 6 h in DMEM medium in a CO\(_2\) incubator at 37 °C, unless otherwise noted.

**Preparation of Recombinant Proteins**—PCNA-His\(_2\) (24), and the GST-pol\(\eta\) proteins were obtained by inducing transformed Rosetta (DE3) pLysS Escherichia coli cells with 1 mM isopropyl-1-thio-β-D-galactopyranoside when the culture reached OD\(_{600}\) of ~0.6 and culturing at 37 °C for 4 h. Subsequently, the PCNA-His\(_2\) and GST-pol\(\eta\) proteins were purified by using Talam affinity resin (Clontech) and glutathione agarose (Pierce), respectively, following the manufacturers’ recommended procedures.

**In Vitro Protein Kinase Assay**—The assay was performed with purified CDK2/Cyclin A2 complex (Sigma) according to the manufacturer’s instructions. Briefly, 10 μg recombinant GST-pol\(\eta\) was incubated with 250 μM ATP and a serial dilution of CDK2/Cyclin A2 in kinase assay buffer, which contained 5 mM MOPS (pH 7.2), 2.5 mM glycerol 2-phosphate, 1 mM EGTA, 0.4 mM EDTA, 5 mM MgCl\(_2\) and 0.05 mM DTT. The reaction mixture (25 μl) was incubated at 30 °C for 15 min and boiled in 1× SDS loading buffer for 5 min to terminate the reaction. The resulting protein mixture was reduced, alkylated, and digested with trypsin, and the peptide mixture was subjected to LC-MS/MS analysis, as described above.

**Assay for Monitoring the In Vitro Interaction between pol\(\eta\) and PCNA**—The assay for monitoring the physical interaction between pol\(\eta\) and PCNA was performed as previously described (13). Briefly, wild-type or mutant GST-pol\(\eta\) protein (4 μg) was mixed with PCNA-His\(_2\) (4 μg) in a binding buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.01% Nonidet P-40, 10% glycerol) and incubated at 4 °C for 30 min followed by 10 min at 25 °C. Subsequently, 10 μl of glutathione agarose (Pierce) was added to bind GST-pol\(\eta\). The samples were further incubated with rocking at 4 °C for 1 h. The beads were then washed five times using the binding buffer, and the bound proteins were eluted using the binding buffer containing 10 mM reduced glutathione and analyzed using Western blotting. The antibody that specifically recognized human PCNA (Cell Signaling Technology) was used at 1:2,000,000 dilution.

**RNA Interference and Western blotting**—RNA interference was carried out as described previously (23). Briefly, HEK293T cells were seeded in six-well plates at 40–60% confluence level and transfected with 100 pmol siRNAs using Lipofectamine 2000 (Invitrogen). After a 48-h incubation, 1.5 μg pol\(\eta\) expression plasmids were cotransfected into the cells together with another siRNA of siRNA. The knockdown efficiency of CDK2 was evaluated at 48 h after transfection by Western blotting as described previously (25). The antibodies that specifically recognized human CDK2 (Cell Signaling Technology) and β-actin (Abcam) were used at 1:2,000 and 1:5,000 dilutions, respectively.

**Flow Cytometry-Based Assay for Monitoring the Levels of CPD Lesion**—The cellular levels of CPD lesion were monitored by flow cytometry according to previously described procedures (26, 27). Briefly, HEK293T cells were irradiated with 10 J/m\(^2\) UV light and cultured in DMEM containing 100 ng/ml nocodazole. At various time intervals following UV irradiation, the cells were harvested and incubated first with mouse anti-CPD antibody (Kamiya Biomedical Co., 1:1000 dilution) and then with Alexa Fluor 647 goat anti-mouse secondary antibody (Invitrogen, 1:2000 dilution). The cells were then analyzed by flow cytometry (BD Biosciences FACSaria I).

**Fluorescence Microscopy**—For assessing the nuclear localization of EGFP-pol\(\eta\) proteins, HEK293T cells transiently expressing EGFP-tagged wild-type pol\(\eta\), pol\(\eta\)-S687A, or pol\(\eta\)-S687D were exposed
with 30 J/m² UVC or mock-treated. At 6 h after UV treatment, the cells were harvested, fixed with 4% paraformaldehyde or ice-cold methanol, mounted in Vectashield Mounting Medium (Vector Laboratories) containing DAPI, and imaged using a Leica TCS SP2/UV confocal microscope (Leica Microsystems).

Selection of Stable Transfectants—XP30RO cells were transfected with EGFP-polH, EGFP-polH-S687A, or EGFP-polH-S687D plasmid as described above. After a 24-h incubation, the cells were cultured in DMEM containing 600 μg/ml G418 (Sigma). The cells were selected for 2 weeks, and the stable transfectants were isolated. The stable transfectants expressing wild-type or mutant polH were harvested for flow cytometry analysis (BD Biosciences FACSAria I), and the proteins were isolated for Western blotting analysis as described above. The antibodies that specifically recognized human polH (Bethyl Laboratories) and β-actin (Abcam) were used at 1:2,000 and 1:10,000 dilutions, respectively.

Clonogenic Survival Assay—XP30RO cells stably expressing wild-type or mutant EGFP-polH were plated in six-well plates in triplicate. On the next day, the cells were exposed to various doses of UV light, after which the cells were incubated in DMEM containing 0.4 mM caffeine to inhibit DNA repair. Cell colonies grown for 10–14 days were then fixed with 6% (v/v) glutaraldehyde and stained with 0.5% (w/v) crystal violet. Colonies containing at least 50 cells were subsequently counted.

Cell-Based Translesion Synthesis Assay—The construction of CPD-bearing double-stranded shuttle vector and its lesion-free control and competitor vectors was described recently (28). The strand-specific PCR-based competitive replication and adduct bypass assay was performed following previously published procedures (28, 29). Briefly, the CPD-bearing and the corresponding nonlesion control plasmids were premixed individually with the competitor genome for the cell-based TLS assay, with the molar ratios of competitor vector to control or lesion-bearing genome being 1:1 and 1:30, respectively. The XP30RO cells stably expressing wild-type or mutant EGFP-polH were transfected with 300 ng mixed plasmids by using Lipofectamine 2000 (Invitrogen). At 24 h after transfection, the progenies resulting PCR products were restriction-digested with NcoI and SfaNI and subsequently analyzed by PAGE and LC-MS/MS, as described elsewhere (28, 29).

**RESULTS**

**Serine 687 (Ser687) is Phosphorylated in Human PolH—** We employed a combined mass spectrometric and cell biological approach to identify and functionally characterize novel post-translational modification(s) of polH in human cells. To this end, we expressed FLAG-tagged human polH in HEK293T cells and purified the FLAG-tagged protein using anti-FLAG M2 beads. The purified protein was then digested with trypsin and the resulting peptides subjected to LC-MS/MS analysis. Database search of the LC-MS/MS results led to the identification of a number of peptides derived from polH with a sequence coverage of 93%. Our LC-MS/MS analysis also gave rise to the identification of the phosphorylation of serine residues 601 and 687 as well as the ubiquitination of lysine 682 (Lys682, Fig. 1A, Supplemental Fig. S1, and Supplemental Table S2). In this vein, Lys682 ubiquitination and Ser682 phosphorylation displayed a dose-dependent increase at 6 h following UV irradiation. Error bar represents the S.E. (n = 3). **, p < 0.01; ***, p < 0.001. The p values were calculated using unpaired two-tailed Student’s t test.
Phosphorylation were identified previously and found to be involved in UV-induced DNA damage response (17, 18). In addition, Ser<sup>687</sup>, similar as Lys<sup>682</sup>, resides in the highly conserved nuclear localization signal (NLS) region of human pol<sub>η</sub> (Fig. 1A).

Fig. 1A depicts the MS/MS for the [M+2H]<sup>2+</sup> ion of the peptide<sub>682-687</sub>NPK<sub>683</sub>PLACTNK<sub>694</sub>, with Ser<sup>687</sup> being phosphorylated. The Ser<sup>687</sup> phosphorylation was unambiguously identified from fragment ions found in the MS/MS, with the consideration of the mass shift introduced by phosphorylation, i.e. 80.0 Da. The existence of the b<sub>4</sub>-Pho, y<sub>8</sub>-Pho, y<sub>9</sub>-Pho, and y<sub>10</sub>-Pho ions and the absence of the b<sub>3</sub>-Pho and y<sub>7</sub>-Pho ions (“Pho” designates a phosphoric acid) in the MS/MS supported the phosphorylation of Ser<sup>687</sup> (Fig. 1A).

Trypsin cleaves the amide bonds on the C-terminal sides of lysine and arginine. Thus, the phosphorylated peptide<sub>682-687</sub>NPK<sub>683</sub>PLACTNK<sub>694</sub> contains a miscleavage site. We failed to observe the phosphorylated peptide without miscleavage, though the corresponding unmodified peptide, <sub>682</sub>SPLACTNK<sub>694</sub>, could be readily detected (MS/MS shown in Supplemental Fig. S2). Previous studies showed that phosphorylation can produce a greater degree of miscleavage by trypsin near the phosphorylation site compared with the unphosphorylated peptide (34). Thus, the miscleavage of the amide bond on the C-terminal side of Lys<sup>686</sup> provided another line of evidence to support the phosphorylation of Ser<sup>687</sup> in pol<sub>η</sub>.

Ser<sup>687</sup> Phosphorylation Involves Cyclin-Dependent Kinase 2 (CDK2)—CDK2 plays a major role in the S phase of cell cycle when DNA replication and repair occur (35, 36). To explore whether CDK2 is involved in Ser<sup>687</sup> phosphorylation in pol<sub>η</sub>, we first assessed the ability of CDK2 to phosphorylate directly Ser<sup>687</sup> in pol<sub>η</sub> with an in vitro kinase assay (Fig. 1C, Supplemental Fig. S3). Our results demonstrated that the recombinant CDK2/Cyclin A2 complex could indeed directly phosphorylate Ser<sup>687</sup> in recombinant human pol<sub>η</sub> (Fig. 1C, Supplemental Fig. S3).

We next asked whether CDK2 is involved in this phosphorylation in cells. Toward this end, we knocked down the expression of CDK2 in HEK293T cells by using siRNA and transfected the cells with the plasmid for expressing FLAG-tagged pol<sub>η</sub>. Upon efficient CDK2 knockdown (Fig. 1D), we observed a significant reduction in the level of Ser<sup>687</sup> phosphorylation in pol<sub>η</sub> relative to pol<sub>η</sub> isolated from cells treated with nontargeting control siRNA (Fig. 1D). In this vein, it is worth noting that CDK2 depletion was previously shown not to alter cell cycle progression (37, 38). Thus, the reduced phosphorylation of Ser<sup>687</sup> in pol<sub>η</sub> upon CDK2 depletion is unlikely attributed to cell cycle perturbation.

Ser<sup>687</sup> Phosphorylation Is Stimulated by UV Irradiation—Considering that pol<sub>η</sub>’s major functional role resides in its capability in bypassing UV-induced CPD lesions, we asked whether the level of Ser<sup>687</sup> phosphorylation is modulated by UV irradiation. To this end, we exposed HEK293T cells expressing FLAG-tagged pol<sub>η</sub> with UV irradiation, immunoprecipitated pol<sub>η</sub> by using anti-FLAG M2 beads, digested it with trypsin, and subjected the resulting peptide mixture to LC-MS/MS analysis in the selected-ion monitoring mode. We then estimated the relative level of phosphorylation based on the relative abundances of fragment ions formed from the unmodified and phosphorylated peptides (see Materials and Methods). The results showed that the phosphorylation level of Ser<sup>687</sup> increased with the dose of the UV irradiation (Fig. 1E). Along this line, our results also revealed an elevated phosphorylation of Ser<sup>687</sup> and a diminished ubiquitination of Lys<sup>682</sup> after UV irradiation, which are in keeping with previous findings (17, 18), thereby validating our method in quantifying the posttranslational modifications of human pol<sub>η</sub> (Supplemental Figs. S1C and S1D).

Ser<sup>687</sup> Phosphorylation Does Not Alter the Nuclear Localization of Human Pol<sub>η</sub>—As stated above, Ser<sup>687</sup> is situated in the highly conserved NLS region of pol<sub>η</sub>. In the viewpoint that phosphorylation at or near the NLS may regulate the protein’s nuclear import (39), we next investigated whether Ser<sup>687</sup> phosphorylation affects pol<sub>η</sub>’s subcellular localization. Our fluorescence microscopy result showed that, similar as its wild-type counterpart, both the phosphorylation-deficient (i.e. EGFP-pol<sub>η</sub>-S687A) and phosphomimetic (i.e. EGFP-pol<sub>η</sub>-S687D) mutants were exclusively nuclear in both untreated and UV-treated HEK293T cells (Fig. 2 and Fig. S4). Thus, we conclude that Ser<sup>687</sup> phosphorylation does not alter the nuclear import of human pol<sub>η</sub>, suggesting that Ser<sup>687</sup> phosphorylation likely occurs after pol<sub>η</sub>’s nuclear import.

Increase in Ser<sup>687</sup> Phosphorylation Is Correlated with a Decrease in the Cellular Levels of CPD Lesion and Impedes Pol<sub>η</sub>’s Interaction with PCNA—We examined the levels of Ser<sup>687</sup> phosphorylation of pol<sub>η</sub> in HEK293T cells at various time intervals following UV irradiation. It turned out that Ser<sup>687</sup> phosphorylation exhibited a progressive increase after UV irradiation (Fig. 3A and Supplemental Fig. S5), which is accompanied with a concomitant reduction in CPD level, as revealed by a flow cytometry-based method (26) (Fig. 3B and Supplemental Fig. S6).

Previous studies demonstrated that interaction with PCNA plays an indispensable role in the recruitment of pol<sub>η</sub> to UV-induced CPD site to carry out TLS, and the dissociation of pol<sub>η</sub> from PCNA after TLS is essential for maintaining high-fidelity DNA replication (4–7). We next asked whether Ser<sup>687</sup> phosphorylation in human pol<sub>η</sub> is modulated by its interaction with PCNA. To this end, we generated a pol<sub>η</sub> variant with mutations in both the PIP and UBZ domains, which is defective in PCNA interaction (16, 20), and measured the level of Ser<sup>687</sup> phosphorylation in the mutant protein following UV irradiation. Our result showed that the mutation of PIP and UBZ domains of pol<sub>η</sub> did not affect appreciably the level of Ser<sup>687</sup> phosphorylation (Fig. 3C), suggesting that interaction with PCNA is not essential for pol<sub>η</sub>’s phosphorylation at Ser<sup>687</sup>.
The Role of Ser^687 Phosphorylation of Pol^h in Its Interaction with PCNA—Considering that Ser^687 resides in the PCNA-interacting region of pol^h that is essential for pol^h-PCNA interaction during UV damage response (17), we investigated the potential role of Ser^687 phosphorylation in the interaction between these two proteins. To this end, we performed GST pull-down experiments using GST fusion proteins of the C-terminal portion of pol^h (amino acids 602–713, GST-pol^hC) and recombinant PCNA. It turned out that, similar to what was reported previously (17), wild-type GST-pol^hC could effectively capture PCNA, whereas mutation of PIP box in pol^h compromised its binding to PCNA (Fig. 3D). Interestingly, mutation of Ser^687 to an alanine did not influence the binding; however, mutation of Ser^687 to an aspartic acid diminished significantly the pol^h-PCNA interaction (Fig. 3D). It is of note that wild-type GST-pol^hC purified from E. coli cells was not phosphorylated at Ser^687 (Fig. 1C); thus, recombinant wild-type pol^h and the S687A mutant should carry the same charges in the NLS, whereas the S687D mutant introduced a permanent negative charge to the NLS. Our observation of the compromised interaction between the phosphomimetic mutant of pol^h and PCNA is consistent with the previous finding that the positively charged lysines in the NLS are necessary for the binding of pol^h to PCNA (17).

Ser^687 Phosphorylation Confers Cellular Tolerance toward UV DNA Damage and Promotes TLS across CPD Lesion—We next examined the role of Ser^687 phosphorylation in UV damage tolerance and in TLS across UV-induced CPD lesions. To this end, we first generated a set of stable cell lines by transfecting wild-type EGFP-pol^h and the corresponding S687A and S687D mutant plasmids into pol^h-deficient XP30RO fibroblasts and confirmed that the expression levels of the wild-type and mutant pol^h were similar in the transfected cells (Supplemental Fig. S7). We next assessed, by using clonogenic survival assay, the sensitivities of these cells toward UV light exposure. Our results showed that the cellular sensitivity toward UV light emanating from deficiency in pol^h could be restored to a similar extent by wild-type pol^h and pol^h-S687D whereas pol^h-S687A only partially rescued the cells from pol^h deficiency (Fig. 4A). This result suggests that the Ser^687 phosphorylation of pol^h conferred cellular resistance toward the cytotoxic effects of UV irradiation. In this regard, the moderate survival defect introduced by deficient Ser^687 phosphorylation of pol^h is in keeping with previous observations for the corresponding deficiencies in Ser^601 phosphorylation or Lys^682 deubiquitination (17, 18), suggesting that the functions of pol^h in cellular tolerance toward UV light exposure are regulated through multiple mechanisms, including Ser^601/Ser^687 phosphorylation, Lys^682 ubiquitination and perhaps some other as yet unidentified mechanism(s).
We also employed a cell-based replication assay to directly quantify how the efficiency of TLS across a site-specifically inserted CPD lesion in human cells is affected by Ser687 phosphorylation (28, 29). Our results revealed that the extent of TLS across a CPD lesion was markedly higher in cells stably expressing wild-type pol\textsubscript{H9257} than in the parental XP30RO cells (Fig. 4B and Supplemental Fig. S8). In agreement with our cell survival data, the cells stably expressing pol\textsubscript{H9257}-S687A mutant showed a moderate and significant decrease in TLS efficiency across a CPD lesion relative to the cells expressing the wild-type pol\textsubscript{H9257} or its phosphomimetic mutant (S687D Fig. 4B and Supplemental Fig. S8). These results support that the phosphorylation of pol\textsubscript{H9257} at Ser687 promotes DNA replication past UV-induced CPD lesion in human cells.

**Fig. 3.** The level of Ser\textsuperscript{687} phosphorylation is negatively correlated with the level of unrepaired CPD, and the relationship between Ser\textsuperscript{687} phosphorylation in pol\textsubscript{H9257} and its interaction with PCNA. (A) The level of Ser\textsuperscript{687} phosphorylation in pol\textsubscript{H9257} without UV treatment or at 3, 6, 12, and 24 h following UV irradiation (30 J/m\textsuperscript{2}). (B) Flow cytometry results showing the level of CPD at various time intervals following UV irradiation (10 J/m\textsuperscript{2}). (C) The level of Ser\textsuperscript{687} phosphorylation in wild-type pol\textsubscript{H9257} and the PIP/UBZ domain mutant of pol\textsubscript{H9257} after UV irradiation (30 J/m\textsuperscript{2}). (D) GST pull-down of PCNA by wild-type and various forms of pol\textsubscript{H9257} with mutations in the C terminus. Bottom panels show the quantification data. Error bar represents the S.E. (n = 3). *, p < 0.05; ***, p < 0.001. The p values were calculated using unpaired two-tailed Student’s t test.
human cells, perhaps through facilitating pol\(\gamma\)’s departure from PCNA after TLS across the UV-induced CPD lesion (vide infra).

**DISCUSSION**

Genetic deficiency in pol\(\gamma\) causes xeroderma pigmentosum variant syndrome in humans, which is manifested by sunlight sensitivity and elevated susceptibility to develop sunlight-induced skin cancer (11, 12). The C terminus of pol\(\gamma\) plays a critical role in protein–protein interactions and in intracellular localization of pol\(\gamma\) in response to UV damage. There are three important regulatory motifs in the C-terminal region of pol\(\gamma\), including the NLS, UBZ, and PIP domains (13–17). In this vein, PCNA is known to be monoubiquitinated in response to UV light exposure, and this ubiquitination stimulates its interaction with pol\(\gamma\) through the UBZ domain of the latter protein (16). This, together with the binding between PCNA and pol\(\gamma\) through the latter’s PIP and NLS domains, enables the recruitment of pol\(\gamma\) to stalled replication forks to perform TLS (10, 14, 22).

In this study, we found that the phosphorylation of Ser\(^{687}\) in pol\(\gamma\), which resides in the NLS of pol\(\gamma\), was stimulated upon UV irradiation and this phosphorylation could diminish pol\(\gamma\)’s interaction with PCNA (Fig. 1 and Fig. 3). This observation is in line with the previous finding that the positively charged amino acids in the NLS of pol\(\gamma\) are required for the interaction between pol\(\gamma\) and PCNA (17). We also observed a time-dependent elevation in the level of Ser\(^{687}\) phosphorylation following UV light exposure, and this increase is accompanied with a concomitant drop of the level of CPD lesion in cells (Fig. 3). On the basis of these findings, in conjunction with the importance of Ser\(^{687}\) phosphorylation in the cellular tolerance toward UV damage and in promoting TLS across a site-specifically incorporated CPD lesion (Fig. 4), we propose a tentative mechanistic model for the role of this phosphorylation in the reverse polymerase switching step of TLS: Ser\(^{687}\) phosphorylation introduces negative charges to NLS of pol\(\gamma\). The increased negative charge to the NLS diminishes pol\(\gamma\)’s binding affinity with PCNA and promotes its departure from the replication fork, thereby resetting the replication fork for highly accurate and processive replication of the downstream undamaged template. This is reminiscent of deubiquitination of Lys\(^{682}\) in the NLS of pol\(\gamma\) during the initial stage of TLS, where the removal of the ubiquitination mark unshields the UBZ domain of pol\(\gamma\) and renders it available for binding with monoubiquitinated PCNA (17, 28). Therefore, our study, along with the previous studies (17, 28), supports that the posttranslational modifications of the NLS of pol\(\gamma\) functions in both its recruitment to, and departure from replication fork, which involve Lys\(^{682}\) deubiquitination and Ser\(^{687}\) phosphorylation, respectively.

Taken together, we identified Ser\(^{687}\) as a novel phosphorylation site of human pol\(\gamma\) by mass spectrometric analysis. We found that the phosphorylation of Ser\(^{687}\) in pol\(\gamma\) can be stimulated by UV irradiation, and this phosphorylation may function in the reverse polymerase switching step of TLS, which promotes cellular tolerance against UV irradiation. These findings reveal novel knowledge into the functional regulation of pol\(\gamma\) in UV damage tolerance at the posttranslational level.

**Acknowledgments**—We would like to thank Profs. Alan R. Lehmann, Zhongsheng You, and James E. Cleaver for providing plasmids and cell lines that were used in the present study.

* This work was supported by the National Institutes of Health (R01 DK082779). X. D. was supported by an NRSA Institutional Training grant (T32 ES018827). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

† To whom correspondence should be addressed: Tel.: (951)827-2700; E-mail: Yinsheng.Wang@ucr.edu.

**REFERENCES**

1. Lindahl, T. (1993) Instability and decay of the primary structure of DNA. *Nature* **362**, 709–715

2. Maslov, A. Y., and Vlij, J. (2009) Genome instability, cancer and aging. *Biochim. Biophys. Acta** **1790**, 963–969

3. Friedberg, E. C., Aguilera, A., Gellert, M., Hanawalt, P. C., Hays, J. B., Lehmann, A. R., Lindahl, T., Lowndes, N., Sarasin, A., and Wood, R. D. (2006) DNA repair: From molecular mechanism to human disease. *DNA Repair* **5**, 986–996

4. Hoege, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G., and Jentsch, S. (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419**, 135–141
18. Goehler, T., Sabbioneda, S., Green, C. M., and Lehmann, A. R. (2011) Regulation of DNA polymerase exchange between Polη and Polδ by monoubiquitination of PCNA and the movement of DNA polymerase holoenzyme. Proc. Natl. Acad. Sci. U.S.A. 105, 5361–5366.

8. Ohmori, H., Friedberg, E. C., Fuchs, R. P., Goodman, M. F., Hanaoka, F., Hinkle, D., Kunkel, T. A., Lawrence, C. W., Livneh, Z., Nohmi, T., Prakash, L., Prakash, S., Todd, T., Walker, G. C., Wang, Z., and Woodgate, R. (2001) The Y-family of DNA polymerases. Mol. Cell 8, 7–8.

9. Masutani, C., Kusumoto, R., Iwai, S., and Hanaoka, F. (1999) The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase η. Nature 399, 700–704.

13. Haracska, L., Johnson, R. E., Unk, I., Phillips, B., Hurwitz, J., Prakash, L., and Prakash, S. (2001) Physical and functional interactions of human DNA polymerase η with PCNA. Mol. Cell. Biol. 21, 7199–7206.

14. Kannouche, P. L., and Lehmann, A. R. (2004) Ubiquitination of PCNA and the polymerase switch in human cells. Cell Cycle 3, 1011–1013.

15. Maga, G., and Hubacher, U. (2003) Proliferating cell nuclear antigen (PCNA): A dancer with many partners. J. Cell Sci. 116, 3051–3060.

16. Bienko, M., Green, C. M., Crosetto, N., Rudolf, F., Zapart, G., Coull, B., Kannouche, P., Wider, G., Peter, M., Lehmann, A. R., Hofmann, K., and Dikic, I. (2005) Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis. Science 310, 1821–1824.

17. Bienko, M., Green, C. M., Sabbioneda, S., Crosetto, N., Matic, I., Hibbert, R. G., Begovic, T., Niimi, A., Mann, M., Lehmann, A. R., Hofmann, K., and Dikic, I. (2010) Regulation of translesion synthesis DNA polymerase η by monoubiquitination. Mol. Cell 37, 396–407.

18. Göhler, T., Sabbioneda, S., Green, C. M., and Lehmann, A. R. (2011) ATR-mediated phosphorylation of DNA polymerase η is needed for efficient recovery from UV damage. J. Cell Biol. 192, 219–227.

19. Chen, Y. W., Cleaver, J. E., Hatahet, Z., Honkanen, R. E., Chang, J. Y., Yen, Y., and Chou, K. M. (2008) Human DNA polymerase η activity and translocation is regulated by phosphorylation. Proc. Natl. Acad. Sci. U.S.A. 105, 16578–16583.

20. Despras, E., Delrieu, N., Garandeau, C., Ahmed-Seghir, S., and Kannouche, P. L. (2012) Regulation of the specialized DNA polymerase η revisiting the biological relevance of its PCNA- and ubiquitin-binding motifs. Environ. Mol. Mutagen. 53, 752–765.

21. Bomgardner, R. D., Lupardus, P. J., Soni, D. V., Yee, M. C., Ford, J. M., and Cimprich, K. A. (2006) Opposing effects of the UV lesion repair protein XPA and UV bypass polymerase η on ATR checkpoint signaling. EMBO J. 25, 2605–2614.

22. Kannouche, P., Broughton, B. C., Volker, M., Hanaoka, F., Mullenders, L. H., and Lehmann, A. R. (2001) Domain structure, localization, and function of DNA polymerase η in xeroderma pigmentosum variant cells. Genes Dev. 15, 158–172.

23. Dai, X., Otake, K., You, C., Cai, Q., Wang, Z., Masumoto, H., and Wang, Y. (2013) Identification of novel α-N-methylation of CENP-B that regulates its binding to the centromeric DNA. J. Proteome Res. 12, 4167–4175.

24. Chen, X., Paudyal, S. C., Chin, R. I., and You, Z. (2013) PCNA promotes processive DNA end resection by Exo1. Nucleic Acids Res. 41, 9325–9338.

25. Zhang, F., Paramasivam, M., Cai, Q., Dai, X., Wang, P., Lin, K., Song, J., Seidman, M. M., and Wang, Y. (2014) Arsnelite binds to the RING finger domains of RNF20-RNF40 histone ε ubiquitin ligase and inhibits DNA double-strand break repair. J. Am. Chem. Soc. 136, 12884–12887.

26. Rouget, R., Auclair, Y., Ligonon, M., Affar el b., and Drobetsky, E. A. (2008) A sensitive flow cytometry-based nucleotide excision repair assay unexpectedly reveals that mitogen-activated protein kinase signaling does not regulate the removal of UV-induced DNA damage in human cells. J. Biol. Chem. 283, 5593–5611.

27. Cai, Q., Fu, L., Wang, Z., Gan, N., Dai, X., and Wang, Y. (2014) α-N-methylation of damaged DNA-binding protein 2 (DDB2) and its function in nucleotide excision repair. J. Biol. Chem. 289, 16046–16056.

28. Liang, Q., Dexheimer, T. S., Zhang, P., Rosenthal, A. S., Villamil, M. A., You, C., Zhang, Q., Chen, J., Ott, C. A., Sun, H., Lucci, D. K., Yuan, B., Simeonov, A., Jadhav, A., Xiao, H., Wang, Y., Maloney, D. J., and Zhuang, Z. (2014) A selective USP1-UAF1 inhibitor links deubiquitination to DNA damage responses. Nat. Chem. Biol. 10, 298–304.

29. You, C., Swanson, A. L., Dai, X., Yuan, B., Wang, J., and Wang, Y. (2013) Translesion synthesis of 8,5-cyclopurine-2'-deoxynucleosides by DNA polymerases η, ζ, and η. J. Biol. Chem. 288, 28548–28556.

30. Ziegler, K., Bui, T., Frisque, R. J., Grandinetti, A., and Nerurkar, V. R. (2004) A rapid in vitro polyomavirus DNA replication assay. J. Virol. Methods 122, 123–127.

31. Burns, J. A., Dreij, K., Cartularo, L., and Scicchitano, D. A. (2010) O6-methylguanine induces altered proteins at the level of transcription in human cells. Nucleic Acids Res. 38, 8178–8187.

32. Sanchez, J. A., Marek, D., and Wangh, L. J. (1992) The efficiency and timing of plasmid DNA replication in Xenopus eggs: Correlations to the extent of prior chromatin assembly. J. Cell Sci. 103, 907–918.

33. Taylor, E. R., and Morgan, I. M. (2003) A novel technique with enhanced detection and quantitation of HPV-16 E1- and E2-mediated DNA replication. Virol. 315, 103–109.

34. Previs, M. J., Van Buren, P., Begin, K. J., Vergoreaux, J. O., LeWinter, M. M., and Matthews, D. E. (2008) Quantification of protein phosphorylation by liquid chromatography-mass spectrometry. Anal. Chem. 80, 5864–5872.

35. Ishidate, T., Elewa, A., Kim, S., Mello, C. C., and Shirayama, M. (2014) Divide and differentiate: CDK/Cyclins and the art of development. Cell Cycle 13, 1384–1391.

36. Luo, R. A., and Poon, R. Y. (2003) Cyclin-dependent kinases and S phase control in mammalian cells. Cell Cycle 2, 316–324.

37. Tetsu, O., and McCormick, F. (2003) Proliferation of cancer cells despite CDK2 inhibition. Cancer Cell 3, 233–245.

38. Berthet, C., Aleem, E., Coppola, V., Tesserollo, L., and Kaidis, P. (2003) CDK2 knockout mice are viable. Curr. Biol. 13, 1775–1785.

39. Haneman, M. T., Kline, T. M., Milford, H. G., Harben, M. B., Hodel, A. E., Corbett, A. H. (2004) Regulation of nuclear import by phosphorylation adjacent to nucleosome localization signals. J. Biol. Chem. 279, 20613–20621.