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Evidence for the Involvement of Human DNA Polymerase N in the Repair of DNA Interstrand Cross-Links†

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ABSTRACT: Human DNA polymerase N (PolN) is an A-family nuclear DNA polymerase whose function is unknown. This study examines the possible role of PolN in DNA repair in human cells treated with PolN-targeted siRNA. HeLa cells with siRNA-mediated knockdown of PolN were more sensitive than control cells to DNA cross-linking agent mitomycin C (MMC) but were not hypersensitive to UV irradiation. The MMC hypersensitivity of PolN knockdown cells was rescued by the overexpression of DNA polymerase-proficient PolN but not by DNA polymerase-deficient PolN. Furthermore, in vitro experiments showed that purified PolN conducts low-efficiency nonmutagenic bypass of a psoralen DNA interstrand cross-link (ICL), whose structure resembles an intermediate in the proposed pathway of ICL repair. These results suggest that PolN might play a role in translesion DNA synthesis during ICL repair in human cells.

Escherichia coli DNA polymerase I (polI) is a high-fidelity DNA repair polymerase that conducts gap filling DNA synthesis during nucleotide excision repair (NER), base excision repair (BER), and DNA interstrand cross-link (ICL) repair. *E. coli* PolI is the prototypical member of A-family DNA polymerases (*1*). *Drosophila melanogaster* Mus308 is an A-family nuclear DNA polymerase, the mutants of which are hypersensitive to DNA cross-linking agents but not to other DNA-damaging agents (*2*−*4*). This suggests that Mus308 may play a role in ICL repair in *Drosophila*. Two nuclear A-family DNA polymerases, DNA polymerase N (PolN) and DNA polymerase Q (PolQ), were recently discovered (*5*, *6*). It has been proposed that PolN and PolQ are mammalian orthologs of Mus308 and that they participate in ICL repair in mammalian cells. However, additional studies are needed to confirm the precise role(s) of PolN and PolQ in DNA repair in mammalian cells.

ICLs are generated endogenously as bifunctional products of lipid peroxidation and by exogenous exposure to DNA-damaging agents, some of which are commonly used as cancer chemotherapeutic drugs (*7*, *8*). Because ICLs covalently link the two complementary strands of duplex DNA, they prevent progression of the DNA replication fork and block RNA transcription. This property makes ICLs highly toxic to proliferating cells. The molecular mechanism of human ICL repair is poorly understood (*7*−*10*). The current model of mammalian ICL repair (*7*−*10*) suggests that when a DNA replication fork stalls at an ICL, it is recognized by an endonuclease that generates a double-strand break (DSB) in the vicinity of the ICL (*11*−*14*). Subsequently, XPF-ERCC1 unhooks the ICL, resulting in a gap across from the unhooked ICL (*14*−*17*), which cannot be sealed by replicative DNA polymerase δ or ε. Gaps generated during ICL repair are thought to be repaired by translesion DNA synthesis (TLS) or by homologous recombination using the homologous chromosome (the sister chromatid is not available). Once the gap is sealed, the DSB is repaired and the collapsed replication fork is coordinately restored by homology-directed DSB repair (*13*, *18*). After DSB repair, the unhooked ICL is removed by NER (*18*). A recent biochemical study demonstrated that a triple-stranded structure with an unhooked ICL can occur during ICL repair in human cells, suggesting that a TLS polymerase is involved in ICL repair in human cells (*18*). However, the specific TLS DNA polymerases that play roles in ICL repair in human and other mammalian cells have not yet been identified.

Human PolN is a recently identified, low-fidelity A-family DNA polymerase that may facilitate TLS in human cells (*19*). Recent studies show that PolN bypasses thymine glycol DNA lesions in vitro (*19*). Nevertheless, the biological role of human PolN is not yet known. This study examined the biological function of PolN in human cells treated with PolN-targeted siRNA. The cellular sensitivity of the PolN-knockdown cells to various types of DNA-damaging agents, including DNA cross-linking agents, was examined. Using an unhooked psoralen ICL, which is a structure that mimics an intermediate in the proposed pathway of ICL repair, we also characterized a potential TLS activity of PolN in vitro.

EXPERIMENTAL PROCEDURES

Cloning of Human PolN and Construction of PolN Expression Plasmids. PolN was amplified from human cDNA in two fragments. For the N-terminus, PCR was performed using the primers 5′-GGCGCCATGGATCCGATGGAAAATTATGAGGCA and 5′-TGCTGACGTCTTCTCCATCTCCTC. For the

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C-terminus, PCR was performed using the primers 5′-GAGGAGATGGAGAAGACGTCAGCA and 5′-CAGCGTAAGCTTCTACAGACAAAATGAAGGCG. These primers introduced a *Bam*HI site at the 5′ end and a *Hin*dIII site at the 3′ end of the full-length gene. The PCR products were cloned into the pCR4-TOPO vector (Invitrogen) and sequenced. The two fragments were ligated at the central *Aat*II site in PolN to produce the full-length gene. The PolN gene was inserted into pcDNA3.1 (Invitrogen) or pETDuet-1 (Novagen) to generate an N-terminal six-His-tagged protein for expression in mammalian cells or *E. coli*, respectively.

A construct with a C-terminal truncation of the proline-rich region (PolNΔP) was also generated for better expression and solubility in *E. coli*, as described by Takata et al. (*19*). This deletion was reported not to affect PolN polymerase activity (*5*). The PolNΔP gene was ligated into pETDuet-1 to add a His tag at the N-terminus and a FLAG tag at the C-terminus.

To generate a polymerase-defective mutant of PolN, the Asp (GAC) was changed to an Ala (GCC) at amino acid 624 by site-directed mutagenesis (*5*). Asp at amino acid 624 that is in highly conserved polymerase motif 3 (also called motif A) was substituted with Ala to inactivate the DNA polymerase activity. Motif 3 forms a pocket for the incoming dNTP (*20*, *21*). A substitution of the corresponding Asp in *E. coli* DNA polymerase I, which functions to coordinate the metal-mediated catalysis reaction, leading to the incorporation of the incoming nucleotide, abrogates the polymerase activity (*20*, *21*). The D624A mutant form of PolN was shown to be inactive in polymerase activity (*5*). The PolN gene in pETDuet-1 was used for PCR with the primers 5′-CACCTTTCTAGCAGCAGCCTTTTCACAGATTGAATTGCGGATTCT and 5′-AGAATCCGCAATTCAATCTGTGAAAAGGCTGCTGCTAGAAAGGTG (underlined nucleotides generate the mutation). A siRNA-resistant form of PolN was generated by site-directed mutagenesis using the PolN gene in pcDNA3.1. Seven silent mutations were introduced into the siRNA target region using the primers 5′-CCATTACAGTTAAAGT***CAATAGTACGTACGGGAAC***TCCTCAAGAAATATTGTG and 5′-CACAATATTTCTTGAGGA***GTTCCCGTACGTACTATTG***ACTTTAACTGTAATGG (bold/italic letters are the siRNA target region, and underlined letters are mutated nucleotides).

siRNA Experiments. All the siRNA and transfection reagents used in this study were purchased from Dharmacon. Cells were seeded 1 day before transfection. The cells were transfected with siRNA (100 nM) in the presence of DharmaFECT1. One day after transfection, cells were plated or cell lysates were prepared. Protein was analyzed by Western blot, and RNA was analyzed by RT-PCR. For cotransfection, the PolN expression plasmid was added to the mixture of siRNA and DharmaFECT1. The efficiency of siRNA knockdown was monitored in 293 T-cells transiently expressing His-tagged PolN by Western blot with anti-His antibodies. Western blot data showed that two siRNAs suppressed the expression of His-PolN without suppressing the expression of the p70 subunit of RPA (data not shown). The siRNA knockdown of PolN in HeLa cells was analyzed by RT-PCR. RT-PCR of endogenous PolN and PolQ confirmed that PolN and PolQ are expressed in HeLa cells and that PolN-siRNA#1 and -#2 suppressed PolN but not PolQ. The siRNA#2 (5′-GAACAGCACAUAUGGAAAU-3′) was selected for further experiments, because silent mutations could be introduced into its target sequence to generate a siRNA-resistant PolN for rescue experiments.

RT-PCR. RNA was isolated with Trizol reagent. The cDNA was prepared using SuperScript II RT (Invitrogen). Random hexamers (100 ng) were annealed to 2.5 μg of total RNA, incubated at 65 °C for 5 min, and cooled on ice for 1 min. The primed RNA was added to 1× RT buffer, 5 nM $MgCl_2$, 10 mM DTT, 1 μL of RNaseOUT Inhibitor, and 50 units of SuperScript II RT. The reaction mixture was incubated at 25 °C for 10 min and at 42 °C for 50 min. The reaction was terminated at 70 °C for 15 min, and 1 μL of RNase H was added. For amplification of PolN, the primers were 5′-CCAAGCACCCAATTCAGATT and 5′-GCGTACACCACCTTCTTGGT. For amplification of PolQ, the primers were 5′-CGAACTATCTGGGTGACTGG and 5′-GCCCTTTTCACTAACCACAC (*22*). GAPDH primers were 5′-ACCACAGTCCATGCCATCAC and 5′-TCCACCACCCTGTTGCTGTA. The PCR mixture (20 μL) contained 1× PCR buffer, each primer at 0.25 μM, each dNTP at 0.2 mM, and 0.4 μL of Taq. The program for PCR was as follows: 94 °C for 2 min and 25 cycles of (1) 95 °C for 5 s, (2) 59 °C for 10 s, and (3) 72 °C for 1.5 min. PCR products were analyzed with an agarose gel.

Colony Forming Assay. One day after siRNA transfection, cells were seeded at a density of 750 cells/100 mm dish and treated with DNA-damaging agents on the following day. HeLa cells were treated with 0.01, 0.03, or 0.05 μg/mL MMC, and U2OS cells were treated with 0.05, 0.1, and 0.5 μg/mL MMC. Cells were incubated at 37 °C for 2 h and washed twice with PBS, and fresh medium was added. For cisplatin treatment, the cells were exposed to 0.01, 0.1, and 1 μM cisplatin for 2 h and washed twice with PBS, and fresh medium was added. For UV irradiation, the medium was removed, cells were exposed to 4 or 8 J/m^2, and fresh medium was added. For IR treatment, cells were exposed to 1 or 3 Gy. Treated cells were incubated at 37 °C for 7−10 days. Colonies were fixed with ethanol, stained with a Giemsa solution, and counted. The surviving fraction was calculated by dividing the number of colonies on treated plates by the number on untreated plates.

Purification of PolNΔP from E. coli. Full-length PolN was poorly expressed and had poor solubility and thus could not be purified in large amounts from *E. coli* for biochemical experiments (data not shown). However, PolNΔP, which lacks the C-terminal proline region, had a much higher solubility and a higher level of expression. Therefore, PolNΔP was purified from *E. coli* for biochemical characterization in vitro. Importantly, PolNΔP rescued the DNA repair defect in PolN-siRNA-treated U2OS cells as efficiently as PolN did, indicating that the C-terminal proline-rich region is dispensable for the DNA repair activity of PolN in human cells (Figure S3 of the Supporting Information).

PolNΔP in the pETDuet-1 vector was transformed into *E. coli* Rosetta 2 (DE3) pLysS. A single colony was grown at 37 °C in 20 mL of LB medium with 34 μg/mL chloramphenicol and 100 μg/mL ampicillin. The culture was transferred to 2 L of LB medium with 34 μg/mL chloramphenicol and 100 μg/mL ampicillin and grown at 37 °C until the OD_{600} reached 0.5. The culture was cooled on ice for 30 min and then induced with 1 mM IPTG at 16 °C for 16 h. Cells were harvested by centrifugation at 5000*g* for 15 min and washed with PBS, and the pellet was frozen. The pellet was thawed on ice and resuspended in 10 volumes of lysis buffer [50 mM Tris-HCl (pH 7.5), 10% glycerol, 5 mM EDTA, 0.1% Triton X, 0.1 mg/mL BSA, and EDTA-free protease inhibitors from Roche Applied Science]. The resuspended pellet was sonicated on ice (20 cycles of 10 s with a 20 s pause) and

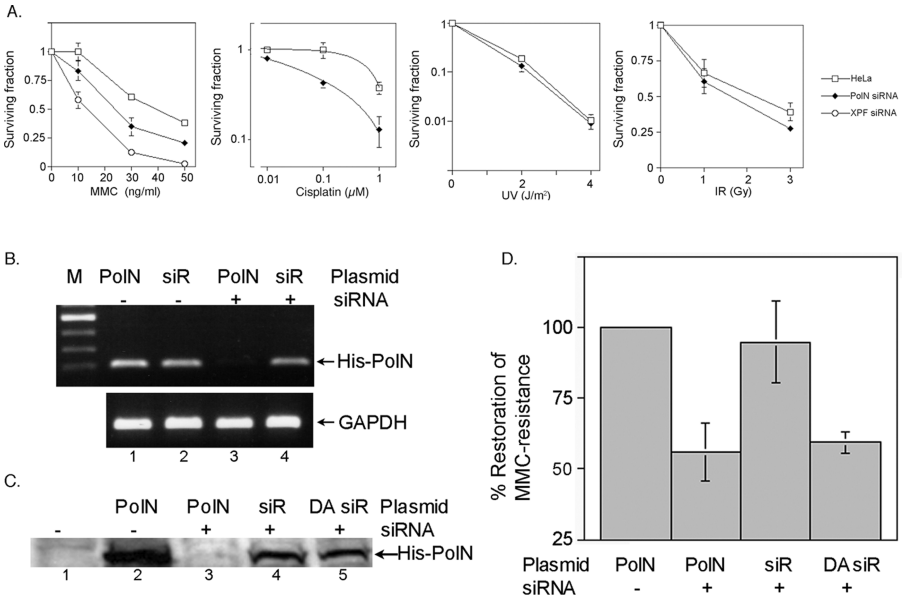

FIGURE 1: DNA damage sensitivity of HeLa cells treated with PolN-siRNA. (A) Suppression of endogenous PolN in HeLa cells results in cellular sensitivity, specifically to DNA cross-linking agents. HeLa cells were incubated with PolN-siRNA or XPF-siRNA for 24 h and then treated with the indicated concentration of MMC or cisplatin for 2 h or irradiated with the indicated dose of UV (254 nm) or IR. Cells were incubated for 10 days, and the cell number was measured with a colony forming assay. The bars in the graph represent the standard deviation from six independent experiments. (B−D) Rescue experiments using siRNA-resistant PolN. (B) An RNAi-resistant PolN was generated by introduction of seven silent point mutations at the target sequences of PolN-siRNA in pcDNA-His-PolN (siRPolN). PolN-siRNA was cotransfected into HeLa cells with either wild-type pcDNA-His-PolN (PolN, lanes 1 and 3) or siRPolN (siR, lanes 2 and 4). After 24 h, the expression level of His-PolN was determined by RT-PCR (lanes 1−4). GAPDH was used as a control. (C) The siRPolN or RNAi-resistant polymerase defective PolN mutant (siRPolN-DA) was cotransfected with PolN-siRNA#2. After 24 h, the expression level of His-PolN was determined by a Western blot with anti-His antibodies. (D) DNA polymerase activity of PolN is required for MMC resistance. Cellular sensitivity to MMC (50 ng/mL) was measured as described for panel A. The graph shows MMC resistance normalized to the control cells (defined as "100% MMC resistance"). The expression of a siRNA-resistant PolN (siR) restored the MMC resistance to the level of the control (third column), while the expression of a siRNA-resistant, polymerase-deficient PolN (DA siR) failed to fully restore MMC resistance (fourth column). Data shown are averages ± the standard deviation (error bars) based on data from the three independent experiments.

incubated with 0.25% polyethyleneimine for 15 min at 4 °C with rocking. The lysate was clarified by centrifugation at 13000 rpm for 30 min. The supernatant was incubated with 2 mM ATP and 10 mM $MgSO_4$ for 10 min at 37 °C. NaCl was added to a final concentration of 0.1 M, and the lysate was applied to a 6 mL DEAE column. The flow-through, containing PolNΔP, was collected and was added to a 50 mL phosphocellulose 11 column. The column was washed with 10 column volumes of a low-salt buffer [50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 10% glycerol], and PolNΔP was eluted with a high-salt buffer [50 mM Tris-HCl (pH 7.5), 1 M NaCl, 10% glycerol, and protease inhibitors]. Peak fractions, as determined by silver staining, were combined (45 mL) and incubated with 1 mL of TALON resin for 3 h at 4 °C with rocking. The resin was washed with 200 column volumes of high-salt buffer containing 10 mM imidazole. Proteins were eluted with an imidazole gradient (from 10 to 100 mM) and run on a 10% SDS−PAGE gel to determine purity. Immunoblots were performed with an anti-His polyclonal antibody (H-15, Santa Cruz) and anti-FLAG M2 monoclonal antibody (Sigma).

Substrate Preparation (23). Primer oligonucleotides (100 pmol) were labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (1 mCi) and purified from a 10% sequencing gel. A 12-mer oligonucleotide (5′-GAAGCTACGAGC-3′) with a psoralen furan-side mono adduct at the T was a generous gift from J. E. Hearst. This 12-mer (100 pmol) was phosphorylated with cold ATP by T4 polynucleotide kinase and annealed to 100 pmol of a 74-mer oligonucleotide (5′-CCTGCTGCAGCCCAAGCTTGGCGCTCGCTCGTAGCTTCTCAGGGTGGCCAGCTGGCGCAGATCTGGCTCGAGGA-3′) (underlined portion complementary to the 12-mer). The partially duplexed DNA was exposed to UVA radiation (366 nm) for 10 min to convert the monoadduct to an ICL. The cross-linked substrate was purified from a 10% sequencing gel and annealed to a 5′-^{32}P-labeled 36-mer primer (5′-TCCTCGAGCCAGATCTGCGCCAGCTGGCCACCCTGA-3′) to generate a nicked substrate. A 5′-^{32}P-labeled 22-mer primer (5′-TCCTCGAGCCAGATCTGCGCCA-3′) was used as a primer for the gapped substrate. The substrate was then purified from a 6% native gel. Under these conditions, the cross-linked substrate is well-separated from the non-cross-linked substrate. Typically, less than 0.3% of the substrate was non-cross-linked (*23*). For the undamaged substrate, a ^{32}P-labeled 22-mer primer was annealed to the 74-mer template or a ^{32}P-labeled 18-mer primer was annealed to a 31-mer template.

In Vitro DNA Polymerase Assay. DNA polymerase assays for PolNΔP or DNA polymerase η (PolH, purchased from Enzymax) were performed as follows. Reaction mixtures [17 mM Tris-HCl (pH 7.5), 3.3% glycerol, 17 mM NaCl, 10 mM $MgCl_2$, 5 fmol of the substrate, and each dNTP at 77 μM in 30 μL] were incubated for 1 min at 37 °C before the addition of polymerase. After addition of the indicated amount of PolNΔP or PolH, the mixtures were incubated at 37 °C for the indicated time. The amount of PolNΔP or PolH used was determined to give similar primer-extension efficiency on an undamaged template (Figure S4 of the Supporting Information). The reactions were terminated by phenol/chloroform extraction. The reaction products were isolated by ethanol precipitation and analyzed on an 8%

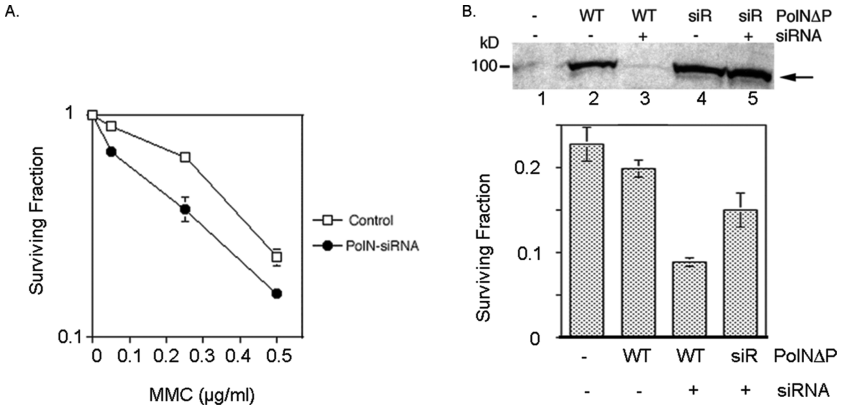

FIGURE 2: PolN-depleted U2OS cells are sensitive to MMC. (A) U2OS cells were transfected with PolN-siRNA. After 24 h, cells were plated for a colony forming assay as described in the legend of Figure 1A. PolN-depleted cells had increased sensitivity to MMC compared to mock-treated U2OS cells. Error bars represent the standard deviation of three independent experiments. (B) U2OS cells stably expressing siRPolNΔP are resistant to PolN-siRNA and restore resistance to MMC. U2OS cells stably expressing WT PolNΔP or siRPolNΔP were generated by selection with G418. U2OS cells stably expressing PolNΔP or siRPolNΔP were transfected with the PolN-siRNA and treated with MMC. A colony forming assay was used to determine survival. The top panel shows Western blot analysis with the anti-His antibody. PolNΔP and siRPolNΔP were stably expressed in U2OS cells (lanes 2 and 4). With the addition of siRNA PolN, PolNΔP expression was suppressed by more than 95%, while siRPolNΔP was resistant to the siRNA treatment (lanes 3 and 5). The bottom panel shows the sensitivity of PolN-depleted cells to MMC. Mock-treated U2OS cells are sensitive to MMC (0.5 μg/mL) (first column). It was noted that overexpression of PolNΔP did not increase resistance to MMC (second column). By the treatment with siRNA, PolNΔP-expressing U2OS cells became more sensitive to MMC (third column), while siRPolNΔP cells restored the MMC resistance similar to that of mock-treated cells (fourth column). Error bars represent the standard deviation of three independent experiments.

sequencing gel. The dried gel was exposed to a PhosphorImage screen; an image was obtained by scanning the screen with the Typhoon 9410 instrument (GE Healthcare), and the products were quantified with ImageQuant. The amount of 74-nucleotide full-length product was used to calculate the percent bypass activity. The sum of products 37−42 nucleotides in length was used to calculate the percent strand displacement activity. The 42-nucleotide product is caused by inhibition of polymerase activity one nucleotide before the ICL.

RESULTS

siRNA-Mediated PolN Knockdown Causes Sensitivity to MMC. The biological role of PolN was investigated by knocking down the level of PolN expression in HeLa cells with siRNA. For this purpose, four siRNA were purchased from Dharmacon and screened for selective and efficient knockdown of PolN expression (Figure S1 of the Supporting Information). One of these siRNAs, PolN-siRNA#2, was selected for further study, and its effects on PolN expression were confirmed by RT-PCR (Figure 1B) and Western blot (Figure 1C). As a positive control, HeLa cells were treated with XPF-siRNA, which efficiently knocks down the level of expression of the NER gene XPF (Figure S2 of the Supporting Information). HeLa cells treated with or without PolN-siRNA or XPF-siRNA were exposed to MMC, cisplatin, or UV or γ irradiation. Survival was measured using a colony forming assay (Figure 1A). After exposure to MMC, the extent of survival of cells treated with PolN-siRNA was lower than that of wild-type HeLa cells but higher than that of cells treated with XPF-siRNA. Cells treated with PolN-siRNA were also sensitive to another DNA cross-linking agent, cisplatin, while they were not sensitive to UV irradiation and were moderately sensitive to γ irradiation at a high dose. Similar results were observed in U2OS cells treated with PolN-siRNA (Figure 2A). Suppression of endogenous PolN in the U2OS cells led to cellular MMC sensitivity. These results demonstrate that PolN is required for resistance to DNA cross-linking agents, MMC and cisplatin, in HeLa cells, the first cellular phenotype associated with the knockdown of PolN.

The DNA Polymerase Activity of PolN Is Required for MMC Resistance in Human Cells. To confirm the specificity of PolN-siRNA, PolN expression plasmids were constructed to express recombinant wild-type PolN or siRNA-resistant PolN (siRPolN) (see Materials and Methods). Furthermore, to examine whether the DNA polymerase activity of PolN is required for resistance to MMC, a plasmid expressing polymerase-deficient PolN (siRPolN-DA) was also constructed. This plasmid expresses PolN with a D624A mutation that abolishes PolN polymerase activity (Figure S3 of the Supporting Information) (*5*), and expression of PolN-DA from this plasmid is resistant to siRNA knockdown (Figure 1C, lane 5). Figure 1B−D shows that the expression of siRPolN rescued sensitivity of HeLa cells to MMC (Figure 1D, third column), but the expression of siRPolN-DA failed to rescue MMC sensitivity in PolN knockdown cells (Figure 1D, fourth column). A Western blot confirmed that siRPolN and siRPolN-DA are expressed at similar levels in these cells (Figure 1C, lanes 4 and 5). In addition, overexpression of siRPolNΔP, which is a variant form of PolN used for in vitro experiments in this study, restored wild-type resistance to MMC in the U2OS cells treated with PolN-siRNA (Figure 2B). These results suggest that the DNA polymerase activity of PolN is required for resistance to MMC in human cells.

In Vitro Bypass of an Unhooked Psoralen ICL by PolNΔP. The results presented above suggest that PolN-catalyzed DNA synthesis may contribute to repair of ICLs induced by MMC in HeLa cells (Figure 1). Previous studies suggest that ICLs are repaired during the S phase in mammalian cells (*11*, *13*, *14*), and that this reaction involves TLS (*18*). In vitro studies also showed that purified PolNΔP, which lacks the C-terminal proline-rich PolN region, acts as a TLS DNA polymerase on a DNA substrate containing thymine glycol (*19*). Importantly, the expression of PolNΔP rescued the MMC sensitivity in PolN knockdown cells as efficiently as full-length PolN, indicating that the C-terminal proline-rich region is dispensable for the DNA repair activity of

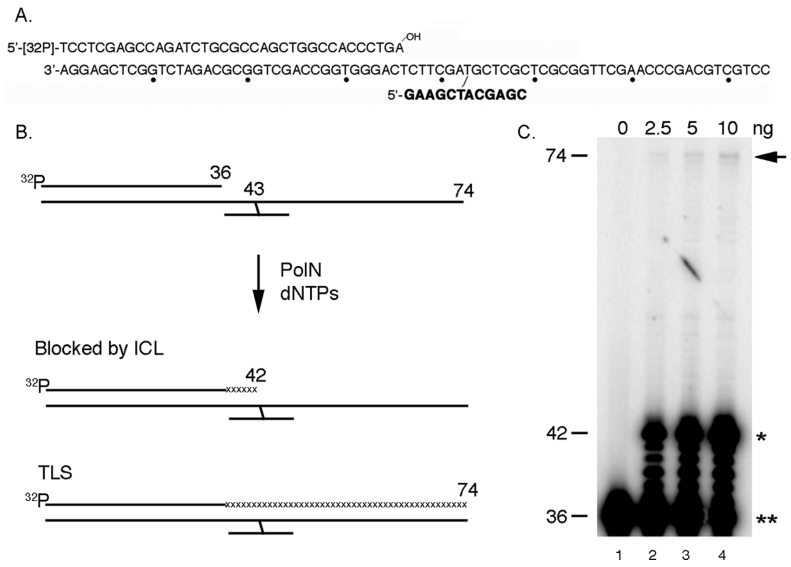

FIGURE 3: In vitro ICL bypass activity of PolNΔP. (A) Substrate DNA used in this study. A 12-mer with a single psoralen furan-side monoadduct (bold) was annealed to a 74-mer. The partially duplexed DNA was exposed to UVA light to generate an ICL. The 5′-^{32}P-labeled primers were annealed to the cross-linked template. Dots indicate every 10th nucleotide. (B) Scheme of the in vitro TLS assay. A ^{32}P-labeled 36-mer was annealed adjacent to the 12-mer cross-linked fragment, leaving a nick. The primer can only be extended by strand displacement activity. When in vitro chain elongation is inhibited at the site of a psoralen ICL, a 43-nucleotide fragment is produced. When the elongation is inhibited one nucleotide before an ICL, a 42-nucleotide fragment is generated. A full TLS reaction gives a 74-nucleotide fragment. (C) ICL bypass by PolNΔP. Purified PolNΔP (2.5, 5, or 10 ng) was incubated with a nicked substrate containing an unhooked ICL for 1 h. The extension of the primer of this template requires strand displacement activity. In lanes 2−4, the percent bypass was 0.03, 0.05, and 0.09%, respectively. The arrow indicates the fully bypassed product (74 nucleotides in length); the single asterisk represents the termination product at one nucleotide before the ICL (43 nucleotides in length), and the double asterisk shows the primer (36 nucleotides in length).

PolN in human cells (Figure 2). The ability of PolNΔP to function as a TLS polymerase was examined using a psoralen ICL. The substrate contains a 12-mer oligonucleotide attached to a 74-mer oligonucleotide via a psoralen ICL (see Materials and Methods), which resembles the "unhooked" intermediate in the proposed pathway of ICL repair (Figure 3A,B) (*23*). The 74-mer or 12-mer was hybridized to a complementary upstream 36-mer or 22-mer, to generate either a nicked or gapped DNA substrate, respectively (*23*).

The nicked DNA substrate was incubated in vitro with recombinant purified PolNΔP or polymerase-deficient PolNΔP-DA (Figures 3 and 4). Purified PolNΔP showed strong strand displacement activity 5′ to the ICL (Figure 3C, lanes 2−4; Figure 4, lanes 1−3); however, DNA synthesis by PolNΔP was strongly inhibited by the unhooked ICL and proceeded beyond the DNA lesion at 42 nucleotides with very low efficiency (Figure 3C, lanes 2−4; Figure 4, lanes 1−3). It is estimated that PolNΔP bypassed the DNA lesion ~0.1% of the input DNA substrate (Figure 4, lane 3). However, the low-level bypass activity detected in this assay is intrinsic to PolNΔP, because it is not detected in reactions with polymerase-deficient PolNΔP-DA (Figure 4, lanes 4−6). For comparison, a Y-family TLS polymerase, DNA polymerase η (PolH), was also incubated with the unhooked ICL in vitro. PolH is a well-characterized TLS polymerase implicated in the error-prone repair of ICLs in mammalian cells (*24*, *25*). This error-prone ICL repair pathway is mediated by nucleotide excision repair (NER). NER is proposed to initiate the unhooking of an ICL, generating a structure similar to that of the substrate used in this study. PolH is believed to bypass the unhooked ICL with the expense of induced mutations (*24*, *25*). Purified PolH exhibited very weak strand displacement activity and was unable to bypass the unhooked ICL in the nicked (Figure 5A, lanes 2−4) or gapped

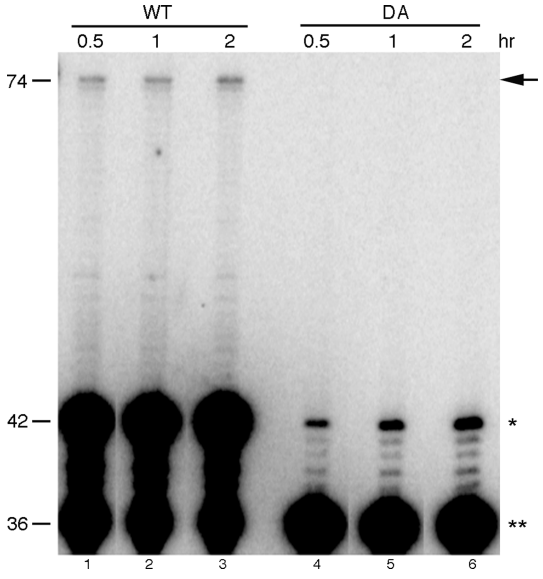

FIGURE 4: DNA polymerase activity-deficient PolN (DA) cannot support TLS of the unhooked ICL in vitro. Wild-type PolNΔP (5 ng) and a DNA polymerase activity-deficient variant DA (10 ng) were incubated at 37 °C with the nicked ICL substrate for the indicated times, and the products were analyzed on an 8% sequencing gel. Products longer than 36 nucleotides are due to strand displacement by PolNΔP. Bypass products are longer than 42 nucleotides, and the full-length product is 74 nucleotides in length. The arrow indicates the fully bypassed product, and the single and double asterisks represent the termination product at one nucleotide before the ICL (42 nucleotides in length) and the primer (36 nucleotides in length), respectively. The percent bypass was 0.05% (lane 1), 0.07% (lane 2), and 0.11% (lane 3). In lanes 1−3, the prcent strand displacement was 55, 64, and 82%, respectively.

DNA substrate (Figure 5B, lanes 6−8). In contrast, PolH DNA polymerase activity was comparable to PolNΔP on an undamaged

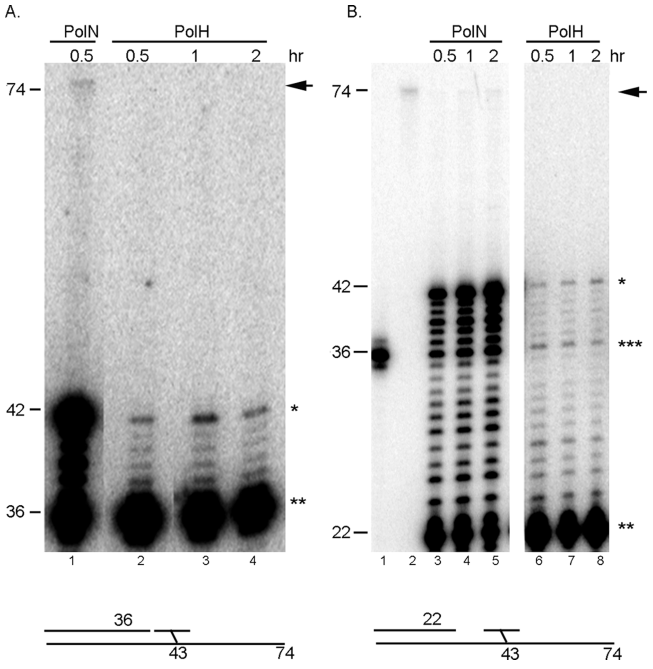

FIGURE 5: PolH is unable to bypass the unhooked ICL in vitro. We used 40 ng of PolH and 2.5 ng of PolN, which give a similar level of polymerase activity on undamaged DNA under the conditions used (Figure S4 of the Supporting Information). (A) ICL bypass activity with the nicked substrate. PolNΔP was incubated with the substrate for 30 min (lane 1). PolH was incubated with the substrate for the indicated time at 37 °C (lanes 2−4). The percent bypass was 0.06%, and 62% of the primer was extended in lane 1. No bypass activity was detected in lanes 2−5. The percentage of the extended primer by PolH was 8% (lane 2), 12% (lane 3), or 12% (lane 4). The arrow indicates the fully bypassed products (74 nucleotides in length), and the single and double asterisks represent the termination product at one nucleotide before the ICL (42 nucleotides in length) and the primer (36 nucleotides in length), respectively. (B) ICL bypass activity with the gapped substrate. The percent bypass was 0.03% in lane 5, and 53, 66, and 83% of the primer was extended in lanes 3−5, respectively. No bypass activity was detected in lanes 6−8. The percentage of the extended primer by PolH was 17% (lane 6), 22% (lane 7), or 26% (lane 8): lane 1, 36-mer marker; and lane 2, 74-mer marker. Products longer than 36 nucleotides (indicated by the triple asterisks) are due to strand displacement by PolNΔP. The arrow indicates the fully bypassed products, and the single and double asterisks represent the termination product one nucleotide before the ICL (42 nucleotides in length) and the primer (22 nucleotides in length), respectively.

DNA substrate (Figure S4 of the Supporting Information). These results indicate that PolN is capable of low-efficiency bypass of an unhooked ICL in vitro, which is consistent with a biochemical role for PolN polymerase activity in the repair of ICLs in human cells.

To examine the accuracy of bypass by PolNΔP, the DNA sequence of the 74-nucleotide bypass products was determined by PCR-based DNA sequencing (*23*). This method was employed previously to sequence bypass products of abasic lesions by Dpo4 DNA polymerase from *Sulfolobus solfataricus* (*26*, *27*). First, labeled 74-nucleotide fragments were purified from an 8% denaturing DNA sequencing gel and amplified for 10 cycles using primers that hybridize to the 5′ and 3′ ends of the DNA substrate. PCR products were cloned directly into the pCR vector using the TA cloning system, and 31 clones were selected for DNA sequencing. The results revealed that there were no point or frameshift mutations in the 31 clones (>97% accuracy). Regardless of the mechanism, PolNΔP conducts low-efficiency nonmutagenic bypass of an unhooked ICL in vitro.

DISCUSSION

This study provides genetic evidence of the involvement of PolN in resistance to MMC and repair of ICLs in human cells and biochemical evidence that PolN may act as a TLS polymerase during ICL repair. These conclusions are supported by the fact that siRNA-mediated knockdown of PolN reduces resistance to MMC in HeLa cells, and that wild-type resistance to MMC is restored by DNA polymerase-proficient PolN but not by DNA polymerase-deficient PolN. In vitro studies also demonstrate that purified PolNΔP has a weak but measurable ability to bypass an unhooked psoralen ICL and that ICL bypass is not mutagenic under the conditions tested.

HeLa cells treated with PolN-siRNA were moderately sensitive to MMC, but less sensitive to MMC than HeLa cells treated with XPF-siRNA (Figure 1). This may reflect the presence of residual PolN activity that escaped suppression by PolN-siRNA. Alternatively, other DNA polymerase(s) expressed in HeLa cells could promote ICL repair in the absence of PolN. In fact, other DNA polymerases have been identified, whose inactivation renders cells sensitive to DNA cross-linking agents. These include DNA polymerase θ (PolQ) and DNA polymerase ζ (PolZ). Like PolN, PolQ is a nuclear DNA polymerase with an A-family DNA polymerase domain (*6*, *28*, *29*). PolQ knockout mice have been generated, and PolQ(−/−) ES cells are moderately sensitive to MMC and IR (*30*); thus, PolQ knockout mouse cells have a phenotype similar to PolN knockdown human cells, described here for the first time. These results suggest that PolQ may have overlapping function(s) with PolN in ICL repair. PolZ is a TLS polymerase that has homology to the B-family DNA polymerase domain (*31*). PolZ knockout MEF cells are sensitive to a variety of DNA-damaging agents, including UV irradiation and DNA cross-linking agents (*32*). Significantly, PolZ is required for the mutagenic effects of these DNA-damaging agents (*32*). Systematic genetic studies are needed to investigate potential redundancy in the function of PolN, PolQ, and PolZ during ICL repair in human cells.

Interestingly, PolN orthologs have only been identified in vertebrate species thus far. Recently, a PolN knockout was generated in chicken DT40 cells (*33*). These PolN knockout cells, the only PolN-knockout cell line reported to date, lack a detectable phenotype. In contrast, orthologs of PolQ exist in lower eukaryotes, including *D. melanogaster* (*3*), *Caenorhabditis elegans* (*34*), and *Arabidopsis thaliana* (*35*). Mutation in *Drosophila* Mus308, a PolQ ortholog, results in severe cellular sensitivity to DNA cross-linking agents (*4*). However, PolQ knockout DT40 cells are not sensitive to MMC and display sensitivity to H_2O_2, a phenotype similar to that of DNA polymerase β knockout DT40 cells (*33*). In addition, genetic and biochemical studies demonstrated that chicken PolQ participates in BER of oxidative DNA damage, suggesting that the biological roles of PolQ and DNA polymerase β may overlap (*33*). These results clearly suggest complex and overlapping role(s) for nuclear A-family DNA polymerases in DNA repair. Nonetheless, the results presented here strongly suggest that human PolN acts as a repair DNA polymerase during ICL repair in HeLa cells.

Our in vitro experiments indicate that PolNΔP bypasses an unhooked psoralen ICL with very low efficiency (Figure 2). One explanation for this result is that a PolN accessory factor, which stimulates PolNΔP's bypass activity, is lacking from the in vitro reaction performed here. In fact, our preliminary data suggest the existence of such accessory factors in nuclear extracts from HeLa

cells (Figure S5 of the Supporting Information). The addition of the nuclear extract stimulated the PolNΔP-mediated TLS of the unhooked ICL, while there was no significant effect on the PolH-mediated TLS. Identification of these factors is currently underway. As a candidate approach, PCNA and RPA, which stimulate several other DNA polymerases (*36*, *37*), were tested for their ability to stimulate human PolNΔP. However, recombinant PCNA and RPA (purified from *E. coli*) did not stimulate ICL bypass by PolNΔP under the conditions used here (data not shown). Post-translational modifications of these factors (*38*, *39*) and/or other accessory factors might be required for efficient bypass of an unhooked ICL by PolNΔP. Alternatively, coordination with other DNA polymerase(s) could enhance the bypass activity of PolNΔP. In fact, it has been reported that the efficient bypass of some DNA lesions in vitro requires two DNA polymerases (*37*, *40*). Many Y-family DNA polymerases, including PolH and Rev1, insert a nucleotide opposite a DNA lesion with relatively high efficiency but extend the primer from the lesion with low efficiency (inserter DNA polymerases). In contrast, PolZ extends the primer from the DNA lesion with relatively higher efficiency (an extender DNA polymerase). Thus, a combination of a Y-family DNA polymerase and PolZ accomplishes bypass of DNA damage efficiently in vitro (*37*, *40*). This "inserter−extender" mechanism may also be relevant for efficient bypass of an unhooked ICL.

Displacement of short oligonucleotides 5′ or 3′ to an ICL might also be required for bypass of an unhooked ICL. Our results indicate that PolNΔP is capable of displacing a DNA oligonucleotide on the 5′ side of an ICL with high efficiency. Interestingly, PolQ has ssDNA-dependent ATPase activity (*6*), and it was also reported recently that PolQ is an extender (*41*). Thus, it is possible that more than two TLS polymerases might be required for bypass of an unhooked ICL, including PolN, PolQ, PolZ, and Rev1.

Interestingly, PolNΔP appears to conduct nonmutagenic bypass of the unhooked psoralen ICL in vitro. Although we cannot formally eliminate the possibility that the fully bypassed products were the results of polymerization of undamaged DNA that existed in the substrate preparation, this suggests that PolNΔP may only extend primers with dAMP incorporated opposite the unhooked ICL. It was reported that PolNΔP conducts efficient error-free bypass of thymine glycol in vitro, preferentially incorporating dAMP opposite the DNA lesion (*19*) and extending the primer. *E. coli* DNA polymerase I Klenow fragment, the prototype A-family DNA polymerase, also incorporates dAMP opposite a psoralen ICL (*23*) or thymine glycol (*42*, *43*). Thus, preferential incorporation of dAMP opposite these DNA lesions and efficient extension of the primer may be characteristic of A-family DNA polymerases. In future studies, it will be important to determine whether PolNΔP conducts nonmutagenic bypass of cisplatin ICLs, which are guanine-derived DNA lesions.

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SUPPORTING INFORMATION AVAILABLE

Supplemental Figures 1−5. This material is available free of charge via the Internet at http://pubs.acs.org.

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