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The Steric Gate Amino Acid Tyrosine 112 Is Required for Efficient Mismatched-Primer Extension by Human DNA Polymerase κ†

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ABSTRACT: Human DNA is continuously damaged by exogenous and endogenous genotoxic insults. To counteract DNA damage and ensure the completion of DNA replication, cells possess specialized DNA polymerases (Pols) that bypass a variety of DNA lesions. Human DNA polymerase κ (hPolκ) is a member of the Y-family of DNA Pols and a direct counterpart of DinB in *Escherichia coli*. hPolκ is characterized by its ability to bypass several DNA adducts [e.g., benzo[*a*]pyrene diolepoxide-N^2-deoxyguanine (BPDE-N^2-dG) and thymine glycol] and efficiently extend primers with mismatches at the termini. hPolκ is structurally distinct from *E. coli* DinB in that it possesses an ∼100-amino acid extension at the N-terminus. Here, we report that tyrosine 112 (Y112), the steric gate amino acid of hPolκ, which distinguishes dNTPs from rNTPs by sensing the 2′-hydroxy group of incoming nucleotides, plays a crucial role in extension reactions with mismatched primer termini. When Y112 was replaced with alanine, the amino acid change severely reduced the catalytic constant, i.e., k_{cat}, of the extending mismatched primers and lowered the efficiency, i.e., k_{cat}/K_m, of this process by ∼400-fold compared with that of the wild-type enzyme. In contrast, the amino acid replacement did not reduce the insertion efficiency of dCMP opposite BPDE-N^2-dG in template DNA, nor did it affect the ability of hPolκ to bind strongly to template-primer DNA with BPDE-N^2-dG/dCMP. We conclude that the steric gate of hPolκ is a major fidelity factor that regulates extension reactions from mismatched primer termini.

The human genome is continuously exposed to a variety of genotoxic agents such as polycyclic aromatic hydrocarbons, ultraviolet light, and reactive oxygen species (*1*). To counteract genotoxic insults, cells possess a number of defense strategies that enable them to complete chromosome replication and maintain the integrity of the genome. One of these strategies is translesion DNA synthesis (TLS)[1] accomplished by specialized DNA polymerases (Pols) (*2*, *3*). These Pols can bypass a variety of DNA lesions, which would otherwise block DNA replication, to ensure the continuity of chromosome replication. Human cells possess more than 14 Pols, and approximately half of them are involved in TLS across DNA lesions, repair of DNA damage, or both (*4*, *5*). In particular, Y-family Pols play major roles in the damage tolerance process by carrying out error-free TLS, although in some cases they mediate error-prone TLS, which can result in mutagenesis, carcinogenesis, and genetic diversity (*6*−*8*).

Of the four human Y-family Pols, i.e., Polκ, -η, and -ι and REV1, Polκ is unique in that its orthologues are present not only in Eukarya but also in bacteria and Archaea (*9*−*11*). The *Escherichia coli* orthologue is DinB or Pol IV, which bypasses several DNA lesions, such as N^2-guanine adducts of benzo[*a*]pyrene diolepoxide (BPDE-N^2-dG), in an error-free manner (*12*, *13*). It is also active in spontaneous mutagenesis in λ phage and in stationary-phase *E. coli* cells (*14*−*17*). Like DinB, hPolκ bypasses BPDE-N^2-dG in an error-free manner by incorporating dCMP opposite the lesion (*18*−*23*) and correctly mediates bypass DNA synthesis across other lesions, e.g., thymine glycol, at least in vitro (*24*). hPolκ is also distinct from other human Y-family Pols or even from *E. coli* DinB in that it efficiently extends

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[1]Abbreviations: Pol, DNA polymerase; hPolκ, human DNA polymerase κ; BPDE-N^2-dG, benzo[*a*]pyrene diolepoxide-N^2-deoxyguanine; Y112, tyrosine 112; TLS, translesion DNA synthesis; rNTP, ribonucleotide triphosphates; F12, phenylalanine 12; PCR, polymerase chain reaction; 8-OH-dG, 8-hydroxyguanine; O^6-MedG, O^6-methylguanine; O^4-MedT, O^4-methylthymine; MOE, molecular operating environment; K_d, equilibrium dissociation constant; HNE-dG, *trans*-4-hydroxy-2-nonenal-dG.

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primers with terminal mismatches (*25*). hPolκ extends mismatched primers with a frequency of $10^{-1}-10^{-2}$, which is more than 10 times greater than that seen with hPolη or *E. coli* DinB. Thus, hPolκ may play a role in TLS in vivo as an extender of mismatched primers generated by other Pols (*2*). Structural analysis of the catalytic core of hPolκ suggests that the N-terminus, approximately 75 amino acids that are absent in bacterial and archaeal counterparts (Figure 1 of the Supporting Information), forms an "N-clasp" domain that enhances the binding of hPolκ to DNA (*26*). Despite distinct biochemical and structural characteristics, the amino acids in the vicinity of the terminal base in primers are similar to those of other family members. Therefore, the exact mechanism by which hPolκ mediates efficient mismatch extension reactions remains unresolved.

In this study, we examined the role of tyrosine 112 (Y112), the steric gate amino acid of hPolκ, in TLS and extension reactions from mismatched primer termini. The steric gate amino acid is the residue that distinguishes dNTPs from rNTPs by sensing the 2′-hydroxy group of rNTPs (*27*, *28*). We have previously demonstrated that the steric gate of the Archaea orthologue of hPolκ, i.e., phenylalanine 12 (F12) of *Sulfolobus acidocaldarium* (*Sac*) Pol Y1 (also known as DBH), plays a role in fidelity during DNA synthesis by regulating the efficiency with which oxidized dNTPs are incorporated into DNA (*29*). Both F13, the steric gate amino acid of *E. coli* DinB, and F12 of DBH were also shown recently to play important roles in TLS across N^2-deoxyguanine−DNA adducts (*12*). Here, we report that the steric gate amino acid of hPolκ is critical for mismatch extension reactions. When Y112 is replaced with alanine (A), the catalytic efficiency of extension from mismatched DNA substrates decreases more than 400-fold. Unlike in the bacterial and archaeal orthologues, the steric gate is unimportant in regulating correct counterbase insertion opposite N^2-deoxyguanine−DNA adducts. We suggest that the steric gate amino acid might have evolved into a major fidelity factor regulating mismatch extension reactions when the ancestral *E. coli* DinB- and archaeal DBH-type proteins developed the extended N-terminal domain. We propose a possible mechanism by which hPolκ extends mismatched primer termini depending on the steric gate.

EXPERIMENTAL PROCEDURES

More detailed experimental protocols are available in the Supporting Information.

Construction of the hPolκ Overexpression Vector. The hPolκ gene (NCBI GenBank POLK entry accession number XM_003930.2) was amplified by polymerase chain reaction (PCR) from the Clontech human testis large insert cDNA library. The amplified fragments were digested with *Nco*I and *Bam*HI, and the resulting fragment was ligated into similarly digested vector pYG8582, which is the same as pET-16b (Novagen) but has the translational DB (downstream box) enhancer (*30*). The resulting plasmid pYG8583 carries the coding sequence for N-terminal 10-His-tagged full-length hPolκ. To construct a C-terminally truncated hPolκ expression vector, a synthetic linker was ligated between the *Xba*I and *Bam*HI sites of pYG8582. This construct was then digested with *Xba*I and *Avr*II, and after dephosphorylation of both ends, the digested plasmid was ligated to the *Xba*I fragment of pYG8583, which carried the N-terminal portion of the hPolκ coding sequence. The resulting construct, overexpressed C-terminally truncated 10-His-tagged hPolκ_{1-559}, was named pYG8591. We refer to it as hPolκ throughout this study.

Construction of Mutant hPolκ Overexpression Vectors. The Y112A mutant of hPolκ was made from pYG8591 by site-directed mutagenesis using PCR. An amino acid change of Y112 to valine (V) was introduced by the same method. Overexpression vectors for Y112A and Y112V were named pYG331 and pYG332, respectively.

Overexpression and Purification. To express the wild-type and mutant proteins, plasmids pYG8591, pYG331, and pYG332 were transformed into Rosetta competent cells (Novagen), and expression was induced by adding IPTG. The harvested cells were resuspended in BugBuster lysis buffer (Novagen), and soluble proteins were collected by centrifugation. hPolκ and the mutant proteins were purified by binding to BD TALON Superflow resin (BD Biosciences) and eluted in accordance with the manual provided by BD Biosciences. The eluted proteins were further purified by gel filtration, followed by ion exchange chromatography (HiTrap Heparin HP, GE Healthcare) using an FPLC system (AKTAexplorer 10S, GE Healthcare). The purified proteins were stored at −80 °C.

Primer Extension Assay. Standard polymerase reactions (10 μL) were performed in 40 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM DTT, 0.1 mg/mL BSA, 60 mM KCl, 2.5% glycerol, and 250 μM dNTPs. To this reaction mixture were added annealed oligonucleotides (100 nM), consisting of primers (14mers, 18mers, or 19mers) and template 5′-GC GCGCTTCTGGCCAATXGCAGAATTCCTAGGGAAG-3′ (36mer), where X represents lesions, i.e., BPDE-N^2-dG, 8-hydroxyguanine (8-OH-dG), O^6-methylguanine (O^6-MedG), O^4-methylthymine (O^4-MedT), or thymine glycol, at position 19. If no lesions were present, X represents dG. The 36mer templates containing the lesions were synthesized by Japan BioServices Co. (Saitama, Japan), except for BPDE-N^2-dG. The template DNA bearing BPDE-N^2-dG, i.e., (−)-*trans*-*anti*-benzo[*a*]pyrene diol epoxide adduct at N^2-dG, at position 19 of the 36mer DNA was synthesized as reported previously (*31*). All other oligonucleotides were synthesized by BEX Corp. (Tokyo, Japan) and double purified by high-performance liquid chromatography. The primers were labeled with Cy3 at the 5′-terminus; the exact sequences are shown in Table 1 of the Supporting Information and in the top part of Figures 1, 2, and 4. Wild-type hPolκ, Y112A, or Y112V was added at a concentration of 40, 10, or 5 nM, respectively, in the experiments shown in Figures 1, 2, and 4, followed by incubation for 15 min (Figures 1 and 2) or 20 min (Figure 4) at 37 °C. When ribonucleotide incorporation was assayed, rNTP instead of dNTP was included in the reaction mixtures at concentrations of 0, 50, 100, 250, or 500 μM. After the reactions were terminated, the products were resolved by electrophoresis on a 15% polyacrylamide gel and visualized with the Molecular Imager FX (Bio-Rad). The band intensities were quantified with Quantity One (Bio-Rad).

Steady-State Kinetic Analyses. The constituents of the reaction mixtures were the same as those in the primer extension assay except that only one dNTP was included and the protein concentrations were 5 nM. The reactions were initiated by adding dNTP. Both the concentration of dNTP and the incubation time were varied depending on the DNA substrate (matched or mismatched primer termini with or without lesions in the template). The products were resolved by electrophoresis on a 15% polyacrylamide gel, and the band intensities were quantified as described in the primer extension assay. Less than 20% of the

primers were extended in the steady-state kinetic analyses, ensuring single-hit kinetics. For each DNA substrate, the rate of incorporation was plotted as a function of dNTP concentration, and the V_{max} and K_m values were determined by Enzyme Kinetics Module 1.1 of SigmaPlot 2001 software (SPSS Inc., Chicago, IL). k_{cat} was calculated by dividing V_{max} by the enzyme concentration. All values are means ± standard errors from three experiments.

DNA Binding BIAcore Assay. The DNA binding assay was performed using the BIAcore3000 instrument (Biacore). The oligonucleotides that were used were basically the same as those used in the primer extension assay, except that primers were biotinylated at their 5′-termini to enable immobilization. The equilibrium dissociation constants (K_d) were calculated from the kinetic traces using BIAevaluation version 4.0 (Biacore) and employing local fitting according to the "1:1 binding with drifting baseline" predefined model.

Molecular Modeling. To visualize the relative position between Y112 or A112 and mismatched primer termini, active sites of wild-type hPol*κ* and its derivative Y112A complexed with a G:T mismatch and dATP were modeled on the basis of the reported crystallographic structure [Protein Data Bank entry 2OH2 (*16*)] using the 2007.09 version of the Molecular Operating Environment (MOE) (Chemical Computing Group Inc., Montreal, QC). Sequences of the template and primer were replaced with those which were applied to the experiments presented here, i.e., 3′-TCCTTAAGACGGTAA-5′ and 5′-AGGAATTCTGCT-3′, where G and T represent the mismatched template and primer bases, respectively. The incoming dTTP in the original structure was replaced with dATP, and Y112 was replaced with alanine in the model of Y112A. After all replacements, the total potential energy of the complex was minimized under the conditions where all coordinate sets of hPol*κ* were fixed.

RESULTS

Y112 Is the Steric Gate Amino Acid of hPolκ. By aligning the amino acid sequences of various Y-family Pols, we postulated that Y112 of hPol*κ* was the steric gate amino acid. To explore this possibility, Y112 was substituted with A or V; the resulting proteins and the wild-type enzyme (Figure 1 of the Supporting Information) were subjected to primer extension assays with rNTPs and dNTPs. The truncated form of hPol*κ* consisting of the 559 N-terminal residues is far more stable than the full-length protein and was therefore used throughout the study. In the presence of four rNTPs, the Y112A and Y112V proteins efficiently incorporated rNTPs and extended the primer, while the wild-type enzyme did not (Figure 1). In the presence of dNTPs, all three proteins incorporated dNTPs and extended the primers. These results clearly indicate that Y112 functions as the steric gate of hPol*κ*.

Translesion Activity of hPolκ Y112A and Y112V. To examine whether the steric gate residue of hPol*κ* is required for TLS across lesions, we performed in vitro primer extension assays using Y112A, Y112V, and the wild-type enzyme (Figure 2). The DNA lesions we analyzed were BPDE-N^2-dG, 8-OH-dG, O^6-MedG, O^4-MedT, and thymine glycol. hPol*κ* more efficiently bypasses the (−)-*trans-anti*-BPDE-N^2-dG adduct than the (+)-*trans-anti*-BPDE-N^2-dG adduct (*32*). To examine the effects of the amino acid substitution of Y112 on the bypass activities carefully, we employed the (−)-*trans-anti*-BPDE-N^2-dG

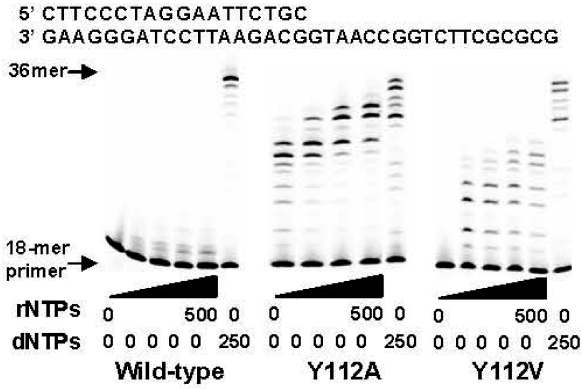

FIGURE 1: Incorporation of rNTPs by wild-type and mutant hPol*κ* proteins was analyzed by a primer extension assay using Cy3-labeled 18mer primers annealed to 36mer templates (i.e., 18/36G in Table 1 of the Supporting Information). The primer-template DNA (0.1 μM) was incubated with 40 nM wild type, Y112A, or Y112V in the presence of rNTPs for 15 min at 37 °C. Concentrations of rNTP were increased up to 500 μM. dNTP at a concentration of 250 μM was added as the control. The samples were resolved by 15% denaturing polyacrylamide gel electrophoresis and analyzed as described in Experimental Procedures.

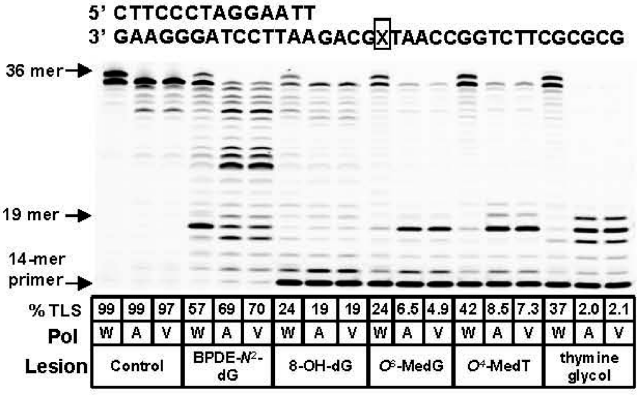

FIGURE 2: Effect of substituting A or V for Y112 on TLS across several lesions in DNA. The Cy3-labeled 14mer primer was annealed to each of six different 36mer templates containing an unmodified dG, dT, or lesions at position 19 (indicated as a boxed X). Reactions were allowed to proceed for 15 min at 37 °C with 100 nM primer-template and 10 nM hPol*κ* protein. W, A, and V represent wild-type hPol*κ*, Y112A, and Y112V, respectively. % TLS indicates the percentage of the amount of primers opposite and beyond the lesion relative to the total amount of the primer.

adduct in this study, although (+)-*trans-anti*-BPDE-N^2-dG is the major adduct of benzo[*a*]pyrene (*32*). Both Y112A and Y112V exhibited primer extension activities across BPDE-N^2-dG in DNA comparable to those of the wild-type enzyme. Rather, the mutant proteins displayed substantially reduced activities in inserting a base opposite other types of lesions except for 8-OH-dG compared to the wild-type enzyme. In particular, Y112A and Y112V exhibited a severely reduced ability to deal with thymine glycol in DNA. To gain insight into the roles of the steric gate amino acid in TLS in a quantitative manner, we conducted steady-state kinetic analyses of insertion and extension steps across BPDE-N^2-dG and thymine glycol in DNA (Table 1). The Y112A incorporation efficiency, i.e., k_{cat}/K_m, for dCMP opposite template BPDE-N^2-dG was 8-fold greater than that of the wild-type enzyme. In contrast, the amino acid change decreased the efficiency of incorporation of dCMP opposite undamaged dG by 90%. In the extension step, replacement of Y112 with

Table 1: Steady-State Kinetic Parameters for Incorporation and Extension across BPDE-N^2-dG and Thymine Glycol in DNA by the Wild Type and Y112A[a]

		Incorporation						
		k_{cat} (min^{-1})		K_m (μM)		k_{cat}/K_m (μM^{-1} min^{-1})		
template	dNTP	WT[b]	Y112A	WT	Y112A	WT	Y112A	Y112A/WT
G	dCTP	7.9 ± 2.0	8.2 ± 1.5	6.7 ± 2.6	60 ± 22	1.2	1.4×10^{-1}	1/8.6
BPG[c]		0.5 ± 0.088	1.6 ± 0.30	1900 ± 560	750 ± 340	2.7×10^{-4}	2.1×10^{-3}	7.8/1
T	dATP	13 ± 2.2	13 ± 2.0	3.4 ± 1.4	46 ± 15	3.8	2.9×10^{-1}	1/13
ThG1[d]		5.6 ± 0.97	0.20 ± 0.060	190 ± 74	2300 ± 1100	2.9×10^{-2}	8.4×10^{-5}	1/350
		Extension						
		k_{cat} (min^{-1})		K_m (μM)		k_{cat}/K_m (μM^{-1} min^{-1})		
base pair at th 3′-primer termini (primer-template)		WT[b]	Y112A	WT	Y112A	WT	Y112A	Y112A/WT
G/C		18 ± 1.1	15 ± 0.73	3.5 ± 0.40	65 ± 6.1	5.2	2.3×10^{-1}	1/23
BPG/C		11 ± 0.69	9.3 ± 1.7	44 ± 7.7	910 ± 380	0.25	1.0×10^{-2}	1/24
T/A		15 ± 0.72	18 ± 1.4	1.4 ± 0.16	17 ± 3.1	11	1.1	1/11
ThGl/A		4.4 ± 0.18	0.2 ± 0.026	240 ± 21	810 ± 240	1.8×10^{-2}	2.5×10^{-4}	1/75

[a] Incorporation reactions were analyzed by determining the steady-state kinetic parameters for incorporation of dCMP opposite template guanine (G) or BPDE-N^2-dG (BPG) and for incorporation of dAMP opposite template thymine (T) or thymine glycol (ThGl). Exact sequences of primer-template DNA, i.e., 18/36G or 18/36T, are described in Table 1 of the Supporting Information. The primer-template (100 nM) and the proteins (5 nM) were incubated at 37 °C. Extension reactions were analyzed by determining the steady-state kinetic parameters for incorporation of dAMP opposite template thymine (T) adjacent to G/C, BPG/C, T/A, or TG/A at the primer-template termini. Exact sequences of primer-template DNA, i.e., 19C/36G or 19A/36T, are described in Table 1 of the Supporting Information. The primer-template (100 nM) and the proteins (5 nM) were incubated at 37 °C. [b] WT, wild-type hPolκ. [c] BPG, BPDE-N^2-dG. [d] ThGl, thymine glycol.

A decreased the k_{cat}/K_m of dAMP incorporation opposite template dT adjacent to either BPDE-N^2-dG or dG by 95%. Therefore, the overall TLS across BPDE-N^2-dG was only moderately compromised by the amino acid change. For TLS across thymine glycol, however, the amino acid change severely reduced both the insertion and extension efficiency. The insertion and extension efficiencies of Y112A were only ~0.3 and ~1%, respectively, compared to that of the wild-type enzyme. From these results, we concluded that the steric gate amino acid of hPolκ is nonessential for TLS at least across (−)-*trans*-*anti*-BPDE-N^2-dG in DNA. Instead, Y112 may play roles in incorporation steps opposite lesions other than N^2-dG adducts, such as thymine glycol, and in extension steps from primer termini.

Strong Binding of hPolκ to Primer-Template DNA with BPDE-N²-dG/dCMP. Although the amino acid replacement of Y112 with A or V did not severely reduce TLS efficiency across BPDE-N^2-dG in DNA, it might still modulate binding to primer-template DNA. To test this possibility, we examined the physical interactions between hPolκ and primer-template DNA with or without lesions using surface plasmon resonance and calculated the equilibrium dissociation constants (K_d) (Figure 3 and Table 2 of the Supporting Information). Wild-type hPolκ bound to primer-template DNA with BPDE-N^2-dG ~3-fold more strongly than it bound to DNA without the lesion. Strong binding depended on the length of the primer and on the dNMP opposite the lesion. Specifically, strong binding was observed when the length of the primer was the same as the length of the template between the 3′-end and the lesion, i.e., a 19mer primer. Alternatively, the length of the primer could be one or two base pairs longer than the length of template, i.e., a 20mer or 21mer primer. No strong binding was observed when the primer was shorter than 19 bp or longer than 21 bp. Additionally, for strong binding, the dNMP opposite the lesion had to be correct (dCMP); strong binding was not observed when the 19mer primers had dGMP,

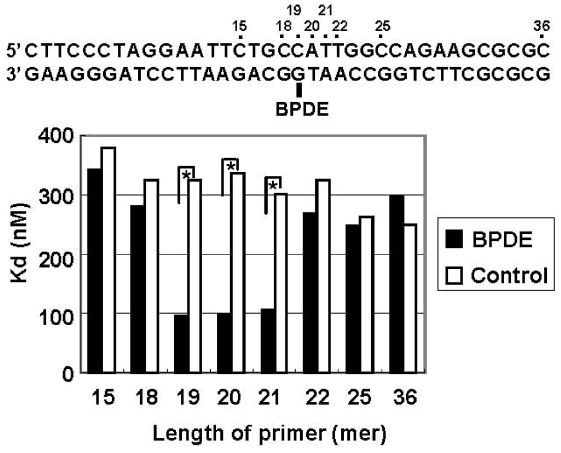

FIGURE 3: Affinity of hPolκ for primer-template DNA with a BPDE-N^2-dG adduct. Primers of different lengths, i.e., 15mers, 18mers, 19mers, 20mers, 21mers, 22mers, 25mers, or 36mers, were annealed with template DNA (36mer) with BPDE-N^2-dG at position 19. As a control, template DNA with an undamaged dG at position 19 was annealed to the various primers. The affinity between protein and DNA was measured with a BIAcore 3000, and the K_d was calculated with BIAevaluation version 4.0. The heights of bars represent the average K_d values of three experiments. An asterisk indicates $P < 0.001$ (*t*-test).

dTMP, or dAMP at the 3′-terminus. No strong binding was observed in primer-template DNA with other lesions, such as thymine glycol, or in primer-template DNA with mismatched termini (data not shown). Importantly, replacing the steric gate amino acid with A or V did not enhance or weaken binding to primer-template with BPDE-N^2-dG lesions (Figure 2 and Table 3 of the Supporting Information), nor did they modulate the binding affinity for control DNA. These results suggest that the steric gate amino acid may play roles in TLS other than modulating the ability of hPolκ to bind to damaged or undamaged DNA.

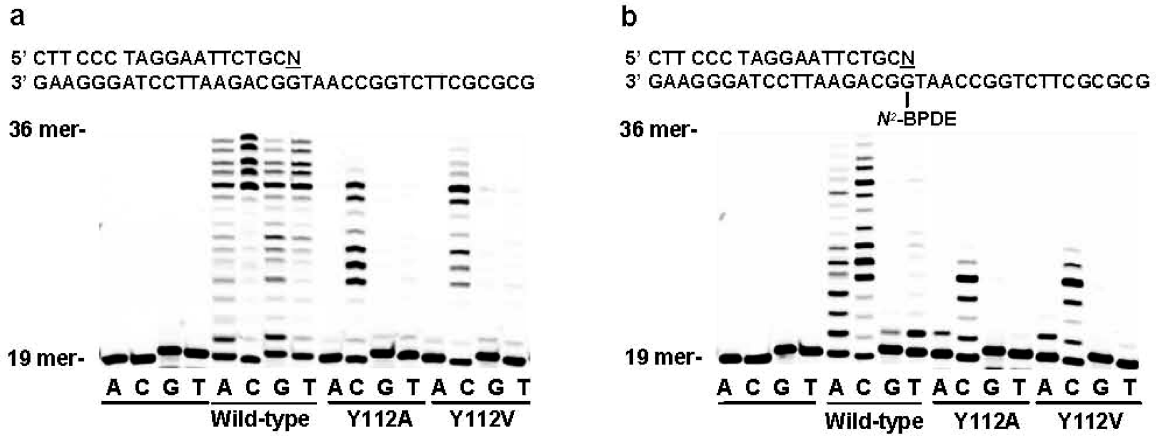

FIGURE 4: Extension from mismatched and matched DNA substrates by wild-type hPolκ, Y112A, and Y112V. Each of four 5′-Cy3-labeled primers (19mers) with either A, C, G, or T at the terminus (N) was annealed to a 36mer undamaged template (a) or a template with BPDE-N^2-dG at position 19 (b). Reactions were conducted for 20 min at 37 °C. The products were resolved by 15% polyacrylamide gel electrophoresis and visualized using a Molecular Imager FX (Bio-Rad) equipped with Quantity One software.

Table 2: Steady-State Kinetic Parameters for Mismatch Extension by WT and Y112A[a]

	k_{cat} (min^{-1})		K_m (μM)		k_{cat}/K_m (μM^{-1} min^{-1})		
base pair at 3′-primer termini (primer-template)	WT[b]	Y112A	WT	Y112A	WT	Y112A	Y112A/WT
C/G	18 ± 1.1	15 ± 0.73	3.5 ± 0.40	65 ± 6.1	5.2	2.3×10^{-1}	1/23
A/G	3.1 ± 0.26	0.11 ± 0.020	89 ± 16	1400 ± 450	3.5×10^{-2}	8.2×10^{-5}	1/420
G/G	4.3 ± 0.40	0.24 ± 0.024	73 ± 16	1700 ± 300	5.9×10^{-2}	1.4×10^{-4}	1/430
T/G	3.6 ± 0.24	0.34 ± 0.039	44 ± 8.3	1500 ± 320	8.2×10^{-2}	2.2×10^{-4}	1/360

[a] Steady-state kinetic parameters were determined for incorporation of dAMP opposite template thymine (T) adjacent to matched (C/G) or mismatched (A/G, G/G, or T/G) primer termini. Exact sequences of matched and mismatched primer-template DNA, i.e., 19C/36G, 19A/36G, 19G/36G, and 19T/36G, are described in Table 1 of the Supporting Information. Primer-template DNA (100 nM) and the proteins (5 nM) were incubated at 37 °C. [b] WT, wild-type hPolκ.

Crucial Role of Y112 in Extension Reactions from Mismatched Termini. Because hPolκ efficiently extends from primer-terminal mismatches, we next examined the possibility that the steric gate amino acid might play a role in mismatch extension reactions. Remarkably, the ability of both Y112A and Y112V to extend primers from mismatched termini was severely reduced compared with extension from matched termini (Figure 4). Reduced extension from mismatched termini was observed when the template DNA had either BPDE-N^2-dG or undamaged dG. To further analyze the effects of the amino acid changes on the mismatched extension reactions, we carried out steady-state kinetic analyses with an undamaged template (36mer) annealed to a 19mer primer with mismatched (A/G, G/G, and T/G) or matched (C/G) termini (Table 2). Y112A exhibited a severely reduced ability to extend primers from mismatched termini; the extension efficiency (k_{cat}/K_m) was 360−430 times lower than that of the wild-type enzyme. In contrast, the extension efficiency from matched termini was only 23-fold lower than that of the wild-type enzyme. Therefore, the amino acid change has an effect that is ~20 times greater on the ability of hPolκ to extend primers from mismatched termini, compared to the extension of primers with matched termini. The reduction in extension efficiency from mismatched termini was due to a reduction in the catalytic constant, i.e., k_{cat}. The k_{cat} values for mismatch extension were reduced 10−30-fold by the Y112A amino acid change, whereas the value for matched-primer extension was not affected by the amino acid change. In contrast, the K_m values were increased 20−30-fold by the Y112 to A change in both mismatched and matched primer termini. From these results, we conclude that the steric gate amino acid is crucial to the chemistry of extension reactions catalyzed by hPolκ from mismatched primer termini.

DISCUSSION

In this study, we report for the first time, to the best of our knowledge, that the steric gate residue of hPolκ plays a critical role in extension from mismatched DNA substrates. Mismatch extension reactions catalyzed by hPolκ were substantially compromised by the replacement of the Y112 amino acid with A or V (Figure 4). The steric gate residue appears to be critical to the chemistry of mismatch extension, i.e., the nucleophilic attack of the misaligned primer 3′-OH group upon the α-phosphate of dNTP, because the replacement of Y112 with A specifically reduced the k_{cat} values for mismatch extension reactions (Table 2). No reduction was observed in the k_{cat} values for matched-primer extension (18 ± 1.1 and 15 ± 0.73 min^{-1} in the wild-type and Y112A enzymes, respectively). Interestingly, amino acid replacement had similar distinct effects on the k_{cat} values for extension from the primer-template with thymine glycol/dAMP or BPDE-N^2-dG/dCMP at the termini (Table 1). The k_{cat} value for extension from primer-template DNA with thymine glycol/dAMP was reduced more than 20-fold by the amino acid replacement (4.4 ± 0.18 and 0.20 ± 0.026 min^{-1} in the wild-type and Y112A enzymes, respectively), while virtually no reduction was seen for extension from primer-template DNA with BPDE-N^2-dG/dCMP (11 ± 0.69 and 9.3 ± 1.7 min^{-1} in the wild-type and Y112A enzymes, respectively). Therefore, we propose that, at least partially, the termini of primer-template DNA with

thymine glycol/dAMP or BPDE-N^2-dG/dCMP lesions structurally resemble "mismatched" and "matched" termini, respectively, in the active site of hPolκ.

The first 18 N-terminal residues of hPolκ also play an important role in mismatch extension (*26*). hPolκ missing the first 18 amino acids has a severely reduced ability to extend mismatched primer termini, although it retains primer extension activity from matched termini. Because the first 18 amino acids contribute to the proficiency of DNA binding by hPolκ, it is surmised that the 18 amino acids and the subsequent N-clasp domain may contribute to the encirclement of DNA and may increase the time during which the mismatched primer 3′-OH group can acquire proper alignments for nucleophilic attack (*26*). Although the catalytic core of hPolκ_{19-526} in a ternary complex with DNA and an incoming nucleotide has been crystallized and the structure has been determined, it lacks the first 18 amino acids (*26*). Therefore, the exact position adopted by these N-terminal amino acids in the complete structure is unclear. We suggest that Y112 may play a critical role in mismatch extension reactions in a manner distinct from that of the action of the 18 N-terminal amino acids. This is because replacing Y112 with A did not reduce the affinity of hPolκ for primer-template DNA (Figure 2 and Table 3 of the Supporting Information), while removing the 18 N-terminal amino acids strongly affects the enzyme's ability to bind to DNA. In addition, the steric gate amino acid is close to primer termini and incoming dNTPs, whereas the 18 N-terminal amino acids may not be. Given the location of Y112, we speculate that Y112 may directly interact with a mismatched terminal base in the primer, thereby preventing the terminal base from moving from the position of the cognate Watson−Crick pairing terminal bases (Figure 5a). This may be a prerequisite for extension from mismatched primer termini, because the mismatched terminal bases, which are otherwise moved from the normal base pairing positions, should be in the proximal normal positions in the active site to acquire proper alignment. The presence of the extended N-terminal domain of hPolκ may enable the steric gate amino acid to interact with the terminal base in the primer, which thereby plays a decisive role in mismatch primer extension. Carlson et al. (*33*) reported that hPolκ forms productive complexes with mismatched primer termini but not with matched primer termini. Both the 18 N-terminal amino acids and Y112 may play roles in the formation of the productive complexes with the mismatched termini. In addition, the steric gate may guide incoming dNTPs to a particular position, where the chemistry between the primer 3′-OH group and α-phosphate of the dNTP can take place. Y112A may have a less constrained active site, which decreases the probability of achieving proper alignment for the phosphoryl transfer reactions, resulting in a decrease in k_{cat} values for mismatch primer extension (Figure 5b).

Immediately adjacent to the steric gate in hPolκ is the highly conserved amino acid F111. Replacing the corresponding amino acid with certain other amino acids in several B-family Pols such as yeast Polζ results in a decrease in the fidelity of DNA synthesis and an increase in the efficiency of mismatch extension (*34*, *35*). Although the exact mechanisms by which this conserved amino acid plays a role in the extension of mismatched termini are not known, amino acid substitutions may alter the geometry of the nascent base pair binding pocket and/or the chemistry of the reaction (*35*). To examine whether hPolκ F111 affects the efficiency of extension reactions from mismatched termini, we changed F111 to A and purified the protein. However, F111A displayed significantly reduced DNA synthesis activity (data not shown). Replacing both F111 and Y112 with A resulted in greatly reduced activity. Thus, we could not determine the efficiency of extension reactions from mismatched termini with F111A or F111A/Y112A. Interestingly, F34L of yeast Polη, which is located next to the steric gate amino acid F35, exhibits substantially reduced DNA synthesis activity (*34*). Therefore, we suggest that unlike B-family Pols, the conserved amino acids adjacent to the steric gate in Y-family Pols, i.e., F111 in hPolκ and F34 in yeast Polη, may primarily play roles in DNA synthesis activity.

Although Y112 was nonessential for TLS across BPDE-N^2-dG in DNA, replacing Y112 with A weakened the ability of hPolκ to bypass several other lesions such as thymine glycol (Figure 2 and Table 1). The efficiency (k_{cat}/K_m) of incorporation of dAMP opposite thymine glycol was ~350 times lower than that of the wild-type enzyme, while the efficiency of incorporation of dAMP opposite undamaged thymine was only 13 times lower than that of the wild-type enzyme. In addition, the k_{cat}/K_m for incorrect incorporation of dNMP opposite an undamaged base was 40−60 times lower in Y112A than in the wild-type enzyme (Table 4 of the Supporting Information), while the k_{cat}/K_m for correct incorporation of dCMP opposite template guanine was ~9 times lower than that of the wild-type enzyme. We propose therefore that replacing Y112 with A may alter the geometry of the nascent base pairing binding pocket, which in turn increases selectivity against both incorporation of dNTP

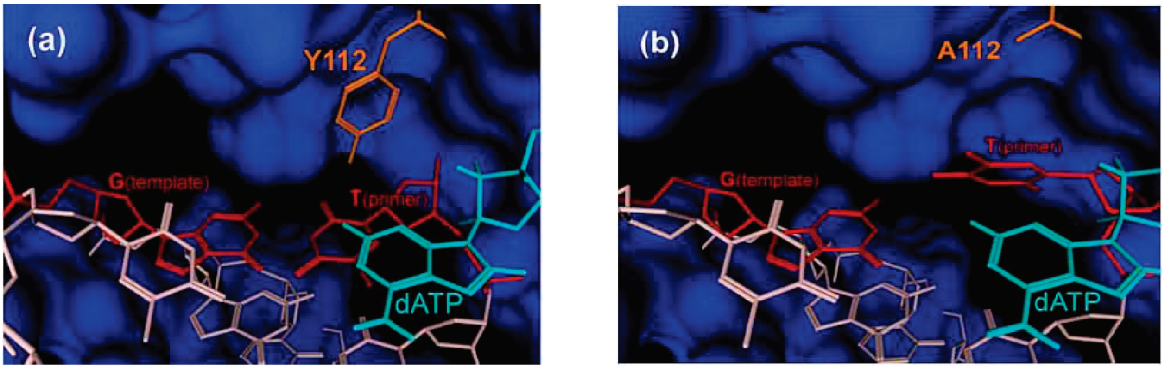

FIGURE 5: Images of the active site of wild-type hPolκ (a) and Y112A (b). The steric gate Y112 may interact with primer T, thereby locking T in a position close to template G (a). A112 has no interactions with primer T, and thus, primer T is positioned far from template G (b). The distance between A112 and primer T is more than 5 Å greater than that between Y112 and primer T. Y112 (a) and A112 (b) are highlighted in orange. A mismatched base pairing of template G with primer T (red) was modeled in the active site with incoming dATP (light blue). The remaining template and primer strands are colored light brown. The active site of hPolκ is displayed as a Connolly surface (blue).

opposite thymine glycol and incorrect incorporation of dNTP opposite an undamaged template base. In this respect, BPDE-N^2-dG is exceptional, because changing Y112 to A or V enhanced the efficiency of incorporation of dCMP opposite the lesion (Figure 2 and Table 1). Template guanine base modified with BPDE might better fit the less constrained active site in Y112A or Y112V than in the native one.

hPolκ strongly binds to primer-template DNA with BPDE-N^2-dG when the primer possesses dCMP opposite the lesion (Figure 3 and Table 2 of the Supporting Information). No such strong binding was observed with primer-template DNA with any of the other lesions that were examined in this study. Structural analysis of the hPolκ catalytic core suggests a model in which there is a cleft in the active site, through which the long chain of *trans*-4-hydroxy-2-nonenal-dG (HNE-dG) in the template extends into the solvent (*26*). By analogy with the position of the HNE-dG adduct, we postulate that the BPDE adduct also fits into the cleft, thereby reducing the level of obstruction of DNA synthesis mediated by hPolκ. Similar strong binding to primer-template DNA with a thymine dimer/dAMP base pair is observed with hPolη (*36*). Intriguingly, the strong binding by hPolη requires the presence of the correct nucleotide, i.e., dAMP, opposite the lesion. The strong binding disappears after Polη has inserted two nucleotides beyond the lesion, which is remarkably similar to the mode of binding of hPolκ to DNA with BPDE-N^2-dG. Because hPolκ and hPolη bypass BPDE-N^2-dG and thymine dimer, respectively, in an error-free manner, it is tempting to speculate that they are cognate lesions for these two Pols. Alternatively, structurally similar but endogenous DNA lesions such as steroid hormone DNA adducts (*37*) could be the cognate lesions for hPolκ. It has been proposed that the strong binding of hPolη may have implications for Pol switching and the restriction of error-prone Pols to damaged sites (*36*). Likewise, the strong binding of hPolκ may contribute to the mechanism of transient access to primer-template DNA with BPDE-N^2-dG by this intrinsically error-prone Pol.

In summary, we found that the steric gate amino acid Y112 was crucial to mismatch extension reactions catalyzed by hPolκ. Y112 appears to play an important role in the chemistry of mismatch extension. It may directly interact with the mismatched terminal base in the primer strand and prevent movement from the normal matched base pairing position in the active site (Figure 5). Unlike bacterial and archaeal orthologues, in which the steric gates are essential for TLS across N^2-dG adducts in DNA (*12*), hPolκ Y112 may be unnecessary in bypass reactions across BPDE-N^2-dG in DNA. *E. coli* DinB, which lacks the N-terminal clasp domain (Figure 1 of the Supporting Information), does not display high efficiency in mismatch extension, although it has a steric gate (*38*). We speculate, therefore, that the steric gate amino acid may have evolved into a major fidelity factor that regulates mismatch extension in hPolκ when the ancestral bacterial DinB and archaeal orthologue gained the extra N-terminal domain.

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SUPPORTING INFORMATION AVAILABLE

Detailed experimental protocols, sequences of primer-template DNA, affinity of hPolκ and the mutants for primer-template DNA with or without BPDE-N^2-dG, kinetic parameters for incorporation of dNTP opposite template G by hPolκ and Y112A, and purification of hPolκ and the mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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