Up-regulation of the Fidelity of Human DNA Polymerase λ by Its Non-enzymatic Proline-rich Domain* 

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DNA repair pathways are essential for maintaining genome stability. DNA polymerase β plays a critical role in base-excision repair in vivo. DNA polymerase λ, a recently identified X-family homolog of DNA polymerase β, is hypothesized to be a second polymerase involved in base-excision repair. The full-length DNA polymerase λ is comprised of three domains: a C-terminal DNA polymerase β-like domain, an N-terminal BRCA1 C-terminal domain, and a previously uncharacterized proline-rich domain. Strikingly, pre-steady-state kinetic analyses reveal that, although human DNA polymerase λ has almost identical fidelity to human DNA polymerase β, the C-terminal DNA polymerase β-like domain alone displays a dramatic, up to 100-fold loss in fidelity. We further demonstrate that the non-enzymatic proline-rich domain confers the increase in fidelity of DNA polymerase λ by significantly lowering incorporation rate constants of incorrect nucleotides. Our studies illustrate a novel mechanism, in which the DNA polymerase fidelity is controlled not by an accessory protein or a proofreading exonuclease domain but by an internal regulatory domain.

A growing number of polymerases have been identified recently and many are hypothesized to function in DNA repair pathways. One of these polymerases is DNA polymerase λ (polλ),3 a new member of the X-family DNA polymerases (1–3). This family of DNA polymerases is a subdivision of a larger superfamily of nucleotidyltransferases (4). Human DNA polymerase λ is encoded by a gene located in human chromosome 10 (2, 3). The N terminus of the full-length polλ (fpolλ) is composed of a nuclear localization signal motif, a breast cancer susceptibility protein BRCA1 C-terminal (BRCT) domain, and a proline-rich domain (see Fig. 1). BRCT domains are known to mediate protein-protein and protein-DNA interactions in DNA repair mechanisms and cell-cycle checkpoint regulation upon DNA damage (5). The proline-rich domain, which contains multiple serine, threonine, and proline residues, is found to functionally suppress the polymerase activity of polλ (6) and limit strand-displacement synthesis (7). The C terminus of polλ (tPolλ in Fig. 1) possesses both 5′-deoxyribose-5-phosphate lyase and DNA polymerase activities while sharing 33% sequence identity with DNA polymerase β (polβ) (1–3, 8). polβ, on the other hand, is also an X-family polymerase and is known to be involved in base excision repair (BER) pathways in vivo (9, 10). The x-ray crystal structures of tpolλ (11, 12) display a high degree of similarity with the corresponding subdomains of polβ (13, 14), including the deoxyribose-5-phosphate lyase (also known as the 8-kDa domain), fingers, palm, and thumb subdomains. A comparison of the crystal structures of tpolλ single-nucleotide gapped DNA, tpolλ single-nucleotide gapped DNA dTTP, and tpolλ nicked DNA pyrophosphatase suggests that no major protein domain movement occurs during catalysis (11). polλ, like polβ, lacks 3′→5′ exonuclease activity (1–3) and possesses low processivity when copying non-gapped DNA (8).

At present, the biological role of polλ is unknown. polλ has been suggested to play a role in DNA repair synthesis associated with meiosis (1), in “short-patch” BER (15, 16), in the proliferating cell nuclear antigen-dependent BER pathway (15, 17), and in the repair of double-stranded breaks through non-homologous end-joining pathways (15, 18, 19). All these potential physiological functions require polλ to possess gap-filling polymerase activity. The fidelity of polλ when filling medium to large gaps has been estimated to be in the range of 10−4 by both forward and reverse mutation assays (8, 17, 18). But the fidelity of the full-length polλ when filling single-nucleotide-gapped DNA, which is relevant to BER, has not been determined. Previously, we measured the base substitution fidelity for filling single-nucleotide-gapped DNA catalyzed by human tpolλ and found it to be in the range of 10−2–10−4, which is relatively low when compared with polβ (15). However, the presence of the N-terminal BRCT and proline-rich domains may have significant effects on the fidelity of polλ. Here, we use pre-steady-state kinetic methods to determine the base substitution fidelity of human fpolλ and its N-terminal domain truncation fragments (see Fig. 1), based on all possible dNTP incorporations into single-nucleotide gapped DNA. Any difference in the fidelity between the full-length and the truncated polλ fragments will reveal the role of the BRCT and proline-rich

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domains in polymerization efficiency and fidelity. Our kinetic results strongly support a role for pol\(\lambda\) as the second polymerase in BER.

**MATERIALS AND METHODS**

Cloning, Expression, and Purification of fpol\(\lambda\), dpol\(\lambda\), and tpol\(\lambda\)—Preparation of tpol\(\lambda\) fused to a C-terminal His\(_8\) tag (38.2 kDa) was described previously (15). The human genes encoding fpol\(\lambda\) and dpol\(\lambda\) (Fig. 1) were PCR-amplified and separately inserted into the NdeI/XhoI sites of pET28b and pET24b to construct pET28b-fpol\(\lambda\) and pET24b-dpol\(\lambda\). A stop codon was engineered before the XhoI site of pET28b-fpol\(\lambda\). The constructed plasmids were individually transformed into Escherichia coli strain BL21 CodonPlus (DE3)-RIL competent cells (Stratagene) to express fpol\(\lambda\) fused to both an N-terminal and a C-terminal hexahistidine tag, and dpol\(\lambda\) fused to a C-terminal hexahistidine tag. Both fpol\(\lambda\) (65.6 kDa) and dpol\(\lambda\) (50.1 kDa) were overexpressed in these transformed E. coli cells as was tpol\(\lambda\) (15). fpol\(\lambda\) was purified through a nickel-nitrilotriacetic acid column (Qiagen), a heparin-Sepharose Fast Flow column, a DEAE-Sepharose column, and a Mono-S 10/10 column (GE Healthcare). tpol\(\lambda\) was purified through a nickel-nitrilotriacetic acid column (Qiagen), a heparin-Sepharose Fast Flow column, and a HiTrap Q column (GE Healthcare). The His\(_8\) tags of purified fpol\(\lambda\) and dpol\(\lambda\) were detected by Western blot analysis using anti-hexahistidine tag antibody (data not shown). The concentrations of the purified fpol\(\lambda\) and dpol\(\lambda\) were measured spectrophotometrically at 280 nm using the calculated extinction coefficients of 61,615 and 48,204 M\(^{-1}\) cm\(^{-1}\) respectively.

Synthetic Oligodeoxyribonucleotides—The oligodeoxyribonucleotides in Fig. 2 were purchased from Integrated DNA Technologies and purified by denaturing polyacrylamide gel electrophoresis (18% acrylamide, 8 M urea). Their concentrations were determined by UV absorbance at 260 nm with calculated extinction coefficients. The 21-mer was 5’-end-labeled by using OptiKinase (U. S. Biochemical Corp.) and \([\gamma-\text{P}]\text{ATP}\) (PerkinElmer Life Sciences), and subsequently purified using a Biospin column (Bio-Rad Laboratories). Each single-nucleotide-gapped DNA substrate was prepared by heating a mixture of 21-mer, 19-mer, and 41-mer in a 1:1.25:1.15 molar ratio, respectively, for 8 min at 95 °C, then cooling the mixture slowly to room temperature over 3 h as described previously (15).

**RESULTS**

Fidelity of Full-length Human pol\(\lambda\)—Previously, we established a minimal kinetic mechanism (Scheme 1) for the single-nucleotide gap-filling activity of tpol\(\lambda\) (15). Scheme 1 shows that an incoming deoxyribonucleotide binds to the E-DNA binary complex to establish a rapid equilibrium prior to nucleotide incorporation. We expect the full-length pol\(\lambda\) to have similar polymerase activity as tpol\(\lambda\) and thus follow the same minimal mechanism shown in Scheme 1. This mechanism allows us to measure the apparent affinity of dNTP (\(K_d\)) for the
Fidelity of DNA polλ Measured by Transient Kinetics

5’-GGCAGCGTTCAATGCTGCTC-3’
3’-GGTGCGGATGGTATGGTGAC-5’

FIGURE 2. Single-nucleotide-gapped DNA substrates D-1, D-6, D-7, and D-8. Primer 21-mer was 5’-32P-labeled. The downstream primer 19-mer was 5’-phosphorylated (circled ‘P’). “X” represents the unpaired base of template 41-mer.

fpolλ-DNA binary complex via the dNTP concentration dependence of the observed single-turnover rate constant \( k_{obs} \). Our results show that fpolλ with a dissociation rate constant \( k_f \) that is ~2-to 3-fold slower than the maximum nucleotide incorporation rate constant \( k_p \) (15), rendering the burst phase (amplitude = \( \frac{k_p}{(k_p + k_f)} \)) small. Thus, the experiments were performed with fpolλ in molar excess over DNA (or under single-turnover conditions) to avoid complications from the steady-state reaction phase (20). A preincubated solution of 5’-32P-labeled D-1 (Fig. 2) and 4-fold fpolλ was reacted with increasing concentrations of correct dTTP in buffer L. The DNA product 22-mer and remaining primer 21-mer at different time intervals were separated and quantitated. The product concentration was plotted against reaction time intervals. These data were subsequently fit to Equation 1, to yield a single-turnover rate constant at each concentration of dTTP (Fig. 3A). The single-turnover rates were then plotted against dTTP concentrations (Fig. 3B). These data were subsequently fit to Equation 2 (see “Materials and Methods”), to yield a \( k_p \) of 3.9 ± 0.2 s\(^{-1}\) for the maximum dTTP incorporation rate constant, and a \( K_d \) of 2.6 ± 0.4 \( \mu M \) for dTTP binding. The substrate specificity \( \left( \frac{k_p}{K_d} \right) \) of dTTP incorporation into D-1 was calculated to be \( 1.5 \times 10^6 \) M\(^{-1}\) s\(^{-1}\) (Table 1).

Similar pre-steady-state kinetic analyses were used to determine the kinetic parameters (Table 1) for the incorporation of each of the remaining three correct nucleotides (dCTP into D-6, dATP into D-7, and dGTP into D-8), and for the incorporation of each of the 12 possible misincorporations into D-1, D-6, D-7, and D-8 (Fig. 2). Notably, the ground-state-binding affinity of all nucleotides to the fpolλ-DNA (Table 1) and the tpolλ-DNA (15) binary complexes were similar, but the maximum incorporation rate constants, particularly for incorrect nucleotides, with fpolλ (Table 1) were slower than the corresponding parameters for tpolλ measured previously (15). The substrate specificity \( \left( \frac{k_p}{K_d} \right) \) of all four correct nucleotides was slightly lower for fpolλ when compared with tpolλ (Fig. 4A) with a nucleotide incorporation efficiency ratio, \( \left( k_p/K_d \right)_{tpolλ}/ \left( k_p/K_d \right)_{fpolλ} \), less than 2 (Table 1). In contrast, fpolλ incorporated incorrect nucleotides with much lower substrate specificities than tpolλ (Table 1 and Fig. 4B). The nucleotide incorporation efficiency ratio, \( \left( k_p/K_d \right)_{tpolλ}/ \left( k_p/K_d \right)_{fpolλ} \), was calculated to be in the range of 10–100 for all twelve incorrect base pairs (Table 1). The variation in this ratio was due to the sequence dependence of polymerization catalyzed by DNA polymerases. Nevertheless, the large ratios suggest that fpolλ achieves significantly higher fidelity than tpolλ by lowering the incorporation efficiency of mismatched nucleotides. To confirm this possibility, we calculated the fidelity of fpolλ, which was sequence-dependent and in the range of \( 10^{-1} \) to \( 10^{-3} \) (Table 1). In comparison, the fidelity of tpolλ with the same single-nucleotide-gapped DNA is also sequence-dependent and in the range of \( 10^{-2} \) to \( 10^{-4} \) (15). Therefore, the full-length polλ is 10-to 100-fold more faithful than the C-terminal fragment tpolλ in filling single-nucleotide-gapped DNA. To ensure the fidelity difference between fpolλ and tpolλ was not due to experimental errors, we repeated the above fidelity and kinetic measure-
Kinetic parameters of nucleotide incorporation into single-nucleotide-gapped DNA catalyzed by the full-length pol at 37 °C

| dNTP | $K_d$ (μM) | $k_p$ (s⁻¹) | $k_p/K_d$ (M⁻¹s⁻¹) | Fidelity | Efficiency |
|------|----------------|----------------|---------------------|----------|------------|
| Template A (D-1) | | | | | |
| dTTP | 2.6 ± 0.4 | 3.9 ± 0.2 | 1.5 × 10⁶ | 1 | 1.9 |
| dATP | 1.86 ± 0.09 | 0.000034 ± 0.000001 | 1.8 × 10⁴ | 1.2 × 10⁻⁵ | 16 |
| dCTP | 10.4 ± 0.3 | 0.0052 ± 0.0009 | 5.0 × 10² | 3.3 × 10⁻⁴ | 22 |
| dGTP | 3.2 ± 0.5 | 0.00040 ± 0.00002 | 1.3 × 10¹ | 8.7 × 10⁻³ | 20 |

| Template G (D-6) | | | | | |
| dCTP | 0.9 ± 0.1 | 1.57 ± 0.04 | 1.8 × 10⁶ | 1 | 1.5 |
| dATP | 3 ± 1 | 0.00010 ± 0.000004 | 3.3 × 10³ | 1.8 × 10⁻⁵ | 20 |
| dGTP | 2.5 ± 0.3 | 0.00070 ± 0.00001 | 2.8 × 10² | 1.6 × 10⁻⁴ | 23 |

| Template T (D-7) | | | | | |
| dATP | 0.9 ± 0.3 | 1.5 ± 0.1 | 1.6 × 10⁶ | 1 | 1.4 |
| dTTP | 8.4 ± 0.6 | 0.0002 ± 0.00001 | 2.4 × 10⁴ | 1.5 × 10⁻⁵ | 88 |
| dCTP | 5.4 ± 0.2 | 0.004 ± 0.002 | 7.0 × 10³ | 4.4 × 10⁻⁴ | 11 |
| dGTP | 4 ± 1 | 0.0020 ± 0.00001 | 1.4 × 10⁴ | 8.8 × 10⁻⁴ | 51 |

| Template C (D-8) | | | | | |
| dATP | 2.1 ± 0.3 | 2.5 ± 0.1 | 1.2 × 10⁶ | 1 | 1.8 |
| dTTP | 1.5 ± 1.0 | 0.0003 ± 0.00003 | 1.7 × 10⁴ | 1.4 × 10⁻⁴ | 19 |
| dGTP | 4.7 ± 0.3 | 0.002 ± 0.002 | 3.8 × 10⁴ | 3.2 × 10⁻⁴ | 17 |
| dCTP | 3.4 ± 0.1 | 0.00047 ± 0.00001 | 1.4 × 10⁵ | 1.2 × 10⁻⁴ | 10 |

*Calculated as $(k_p/K_d)_{molar}/((k_p/K_d)_{measured}$ + $(k_p/K_d)_{molar}$.

*Calculated as $(k_p/K_d)_{molar}/((k_p/K_d)_{pol}$). The $k_p/K_d$ values for tpol are from Table 2 of Ref. 15.

Fidelity of dpol—To identify which of the one or more N-terminal domains up-regulates the fidelity of the full-length polα, we engineered and purified a fragment, dpolα (residues 132–575, Fig. 1), that lacks the BRCT domain. With two diagnostic substrates D-6 and D-7, the kinetic parameters and fidelity of dpolα were determined in the same manner as described above and are listed in Table 2. dpolα incorporated nucleotides into D-6 and D-7 with a fidelity in the range of 10⁻⁴–10⁻⁵, which was very similar to the fidelity of fpolα (Table 1). The nucleotide incorporation efficiencies ratios, $(k_p/K_d)_{dpolα}/(k_p/K_d)_{polα}$, were calculated to be in the range of 0.5–2.1 (Table 2 and Fig. 5). The average ratio for all eight possible nucleotide incorporations into both D-6 and D-7 was calculated to be 1.2. These ratios indicate that the substrate specificity of nucleotides with dpolα, $(k_p/K_d)_{dpolα}$, whether slightly larger or smaller, was within 2-fold of the substrate specificity of corresponding nucleotides with fpolα, $(k_p/K_d)_{polα}$. Because 2-fold differences are considered kinetically insignificant (20), we concluded that dpolα and fpolα have very similar, if not identical, gap-filling efficiency. Thus, the deletion of the BRCT domain in dpolα did not affect the nucleotide incorporation efficiency and the fidelity of fpolα. Similar polymerization efficiency and fidelity of dpolα and fpolα also indirectly confirmed that the N-terminal hexahistidine tag only present in the recombinant fpolα did not affect the fidelity of fpolα. Comparison of the fidelity of fpolα (10⁻⁴–10⁻⁵, Table 1), dpolα (10⁻⁴–10⁻⁵, Table 2), and fpolα (10⁻²–10⁻⁵) (15) reveals an intriguing conclusion: the non-enzymatic proline-rich domain alone significantly enhanced the fidelity of human polα.
**Fidelity of DNA polλ Measured by Transient Kinetics**

### TABLE 2

Kinetic parameters of nucleotide incorporation into single-nucleotide-gapped D-6 and D-7 catalyzed by dpolλ at 37 °C

| dNTP   | $K_{d}$ (μM) | $k_{p}$ (s$^{-1}$) | $k_{p}/K_{d}$ (10$^{-6}$ s$^{-1}$) | Fidelity* | Efficiency |
|--------|--------------|--------------------|---------------------------------|-----------|------------|
| **Template G (D-6)** |
| dCTP   | 6.0 ± 0.4    | 1.8 ± 0.2          | 1.8 × 10$^6$                    | 1         | 0.89       |
| dATP   | 3.2 ± 0.9    | 0.000010 ± 0.00002 | 3.3 × 10$^4$                    | 2.1 × 10$^{-6}$ | 1.0       |
| dTTP   | 1.8 ± 0.8    | 0.000030 ± 0.000007 | 1.7 × 10$^2$                   | 1.1 × 10$^{-4}$ | 0.61       |
| dGTP   | 3.3 ± 0.5    | 0.000022 ± 0.000002 | 6.7 × 10$^4$                   | 4.2 × 10$^{-5}$ | 1.3       |

* Calculated as $(k_{p}/K_{d})_{incorrect}/((k_{p}/K_{d})_{correct} + (k_{p}/K_{d})_{incorrect})$.

+ Calculated as $(k_{p}/K_{d})_{dpolλ}/(k_{p}/K_{d})_{dpolλ}$.

**Template T (D-7)**

| dATP   | 1.0 ± 0.4    | 1.8 ± 0.2          | 1.8 × 10$^6$                    | 1         | 1.1       |
| dTTP   | 11 ± 6       | 0.000046 ± 0.000005 | 4.2 × 10$^4$                   | 2.3 × 10$^{-5}$ | 1.7       |
| dCTP   | 4 ± 2        | 0.006 ± 0.0001     | 1.5 × 10$^3$                   | 8.3 × 10$^{-4}$ | 2.1       |
| dGTP   | 5 ± 1        | 0.0087 ± 0.0001    | 1.7 × 10$^3$                   | 9.4 × 10$^{-4}$ | 1.2       |

**FIGURE 5.** Both dpolλ (Table 2) and fpolλ (Table 1) incorporate nucleotides with similar efficiency with an average ratio of $(k_{p}/K_{d})_{dpolλ}/(k_{p}/K_{d})_{dpolλ}$ (Table 2) equal to 1.2 (indicated by the line) for all nucleotide incorporations into D-6 and D-7.

**DISCUSSION**

DNA polymerases λ and β share high sequence homology (54%) and sequence identity (33%) (1–3, 8). polλ, like polβ, possesses the two key enzymatic activities (gap-filling polymerase and deoxyribonuclease-5-phosphate lyase) required by BER. The role of polλ in DNA repair is further supported by the following observations: (i) like polβ (21), polλ is expressed at high levels in the developing mouse testes, suggesting a possible function of polλ in DNA repair pathways associated with meiotic recombination (1); (ii) in an *in vitro* BER reconstitution assay, recombinant human polα and polβ can replace each other to efficiently repair uracil-containing DNA in the presence of human uracil-DNA glycosylase, human AP endonuclease, and human DNA ligase I (22); (iii) polλ is the only X-family DNA polymerase found in higher plants and is induced by DNA-damaging treatments (23); (iv) mouse embryonic fibroblast cell extract contains substantial amounts of active polλ, which contributes to uracil-initiated short-patch BER (16); (v) monoclonal antibodies against polλ strongly reduce *in vitro* BER in the polβ$^{-/-}$ cell extract (16); and (vi) polλ protects mouse fibroblasts against oxidative DNA damage and is recruited to oxidative DNA damage sites (24). Thus, polλ may compliment or support the function of polβ in BER *in vivo*. But this hypothesized role was initially weakened by the observed difference in single-nucleotide gap-filling fidelity between polβ and polλ. The fidelity of polλ (10$^{-2}$ to 10$^{-3}$) (15) is ~10- to 100-fold lower than the fidelity of rat polβ (recalculated as 10$^{-9}$ to 10$^{-5}$ using the definition of fidelity in Table 1), which was measured under similar single-turnover conditions with four single-nucleotide-gapped DNA 25–19/45-mer (primer-primer/template) substrates (25). If the full-length polλ had similar fidelity to polλ, it would make 10- to 100-fold more base substitution errors than polβ if it participated in the repair of single-base lesions. DNA base modifications/losses are known to account for a large portion of total cellular DNA damage.

Under single-turnover conditions, the deoxyribonucleotide incorporation fidelity of human fpolλ was measured in the presence of four different single-nucleotide-gapped DNA substrates shown in Fig. 2. Surprisingly, the fidelity of fpolλ was determined to be in the range of 10$^{-4}$ to 10$^{-5}$ (Table 1) and was indeed significantly higher than that of fpolλ (10$^{-2}$ to 10$^{-3}$) (15). This range for fpolλ was identical to the fidelity range determined for polβ (26). In addition, the efficiency for correct nucleotide incorporation into single-nucleotide-gapped DNA catalyzed by fpolλ (1.2 × 10$^6$–1.8 × 10$^6$ M$^{-1}$ s$^{-1}$, Table 1) was only slightly lower than polβ (4 × 10$^6$–6 × 10$^6$ M$^{-1}$ s$^{-1}$) (26). Similar polymerization efficiency and fidelity in combination with other biological evidence discussed above strongly support the hypothesis that polα and polβ both possess similar *in vivo* roles, such as functioning in BER. So far, the generation of knock-out mice through deletion of exons 5–7 of the polλ gene has not yet confirmed the involvement of polλ in BER or any other biological processes (27). The published mouse polλ knock-out experiments (27, 28) are likely complicated by the existence of polβ (28), which could fill in and compensate for the loss of functional polλ.

The measured fidelity of fpolλ (Table 1) was 10- to 100-fold higher than the fidelity of polλ (10$^{-2}$ to 10$^{-3}$), estimated by employing the same single-turnover kinetic assay with identical DNA substrates (15). This indicated that the N-terminal domains of polλ (Fig. 1) significantly enhance the fidelity of polλ. Because fpolλ possesses slightly higher correct nucleotide incorporation efficiency than fpolλ (Table 1) and both enzymes bind to all correct and incorrect nucleotides with similarly high affinity (Table 1 and Ref. 15), it is unlikely that the absence of the N-terminal domains will cause any type of misfolding of
tpolα and thus contribute to its low fidelity. This assumption was validated based on the following structural evidence: (i) when the high resolution tpolα binary structure (2.1 Å) (12) and the full-length polβ ternary structure (2.2 Å) (14) are overlaid, the α carbon superimposed very well with a root mean square deviation of 1.4 Å for 113 C-α atoms (12), suggesting that tpol folds well and similarly to polβ in the absence of the two N-terminal domains of polα and (ii) no unfolded regions in tpolα are observed in its 1.95–2.3 Å binary (12) and ternary crystal structures (11). Interestingly, the average ratio of \( k_p/K_D \) for all eight possible nucleotide incorporation with D-6 and D-7 was 1.2 (Fig. 5), indicating that dpolα and fpolα almost have equal nucleotide incorporation efficiency. As expected, these two enzymes also have very similar fidelity (Tables 1 and 2). Thus, the deletion of the BRCT domain affected neither the single-nucleotide gap-filling efficiency nor the fidelity of polα, whereas differences in fidelity between dpolα and tpolα demonstrated that the non-enzymatic proline-rich domain dramatically enhanced the fidelity of polα by up to a 100-fold. This conclusion is consistent with the proposed function of the BRCT domain, which is generally known to mediate the protein-protein or protein-DNA interactions required for particular DNA repair pathways and cellular responsiveness to DNA damage (1–3, 8). Our conclusion is also supported by the observation of Shimazaki et al. (6) in which they found that deletion of the BRCT domain of human polα did not affect DNA synthesis with DNA substrates, including poly(dA)/oligo(dT) and activated DNA. The proline-rich domain has been proposed to merely couple polymerase action to the protein-protein and protein-DNA interactions required during DNA repair (1). Thus, the proline-rich domain is generally assumed to be dispensable for the polymerase activity of polα. However, the difference in fidelity between tpolα and dpolα observed here suggested that the flexible proline-rich domain actively regulates the polymerase fidelity. Enhancement of the fidelity of a polymerase by a non-enzymatic domain is both surprising and unprecedented. Therefore, the 11-kDa proline-rich domain must interact with the subdomains of tpolα and optimize the geometry of the polymerase active site to achieve higher fidelity. The fidelity modulation observed here differs dramatically from the fidelity enhancement contributed by the enzymatic activity of a 3′→5′ exonuclease domain of a replicative DNA polymerase, or by an accessory protein as in the case of the mitochondrial DNA polymerase complex (29). Amazingly, the enhancement of the polα fidelity by the proline-rich domain is as large as what has been contributed by the proofreading 3′→5′ exonuclease domain to a replicative DNA polymerase (29, 30). Our results further suggest that DNA polymerases, the vital enzymes that replicate and maintain genomic DNA, have evolved through different mechanisms to adjust their polymerization fidelity to best perform diverse physiological functions. Interestingly, the fidelity difference between tpolα and fpolα resulted mainly from stronger discrimination against mismatched nucleotides by fpolα. The average substrate specificity of mismisincorporations was 10- to 100-fold lower with fpolα than with tpolα (Fig. 4B). In comparison, fpolα incorporated correct dNTPs with an efficiency \( k_p/K_D \) of \((1.2–1.8) \times 10^5 \text{m}^{-1}\text{s}^{-1}\) (Table 1), which was only ~2-fold lower than the corresponding range of \((2.1–2.7) \times 10^6 \text{m}^{-1}\text{s}^{-1}\) with tpolα (Fig. 4A). The larger decrease in the incorporation efficiency of mismatched over matched dNTPs (Table 1 and Fig. 4) with fpolα led to the higher fidelity of polα. In comparison, the accessory subunit enhances the fidelity of mitochondrial DNA polymerase by 14-fold by increasing the incorporation efficiency of a correct A:T base pair more than a T:T mismatch (29). Furthermore, the decrease in the incorporation efficiency of both matched and mismatched dNTPs was due to slower incorporation rate constants \( k_p \) with fpolα than with tpolα, because the binding of all dNTPs \( K_D \) by these two enzymes was similarly tight (Table 1 and Ref. 15). This suggested fpolα was slower yet more faithful than tpolα in filling single-nucleotide-gapped DNA. This correlation goes against the general trend summarized from a survey of the A-, B-, X-, and Y-families by Beard et al. (31) that a more catalytically efficient polymerase has a lower base substitution rate. It is not clear what contributes to this intriguing correlation between the polymerase fidelity and nucleotide incorporation rate. We speculate that the presence of the proline-rich domain in both fpolα and dpolα may either somewhat tighten the polymerase active site to achieve better geometric selection (32, 33), or shield the active site of pol from solvent, which leads to greater desolvation of a nascent base pair and amplifies the free energy differences between matched and mismatched nucleotide incorporations (34). These possibilities can be evaluated by the active site structural differences between the ternary crystal structures of fpolα (or dpolα)-single-nucleotide-gapped DNA:dNTP and tpolα-single-nucleotide-gapped DNA:dNTP (11), especially in the presence of a mismatched incoming nucleotide.

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