Localization of the Deoxyribose Phosphate Lyase Active Site in Human DNA Polymerase \( \iota \) by Controlled Proteolysis*

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Human DNA polymerase \( \iota \) (pol \( \iota \)) is a member of the Y-family of low-fidelity lesion bypass DNA polymerases. In addition to a probable role in DNA lesion bypass, this enzyme has recently been shown to be required for somatic hypermutation in human B-cells. We found earlier that human pol \( \iota \) has deoxyribose phosphate (dRP) lyase activity and unusual specificity for activity during DNA synthesis, suggesting involvement in specialized forms of base excision repair (BER). Here, mapping of the domain structure of human pol \( \iota \) by controlled proteolysis revealed that the enzyme has a 48-kDa NH\(_2\)-terminal domain and a protease resistant 40-kDa "core domain" spanning residues Met\(^{79}\) to Met\(^{445}\). A covalently cross-linked pol \( \iota \)-DNA complex, representing a trapped intermediate in the dRP lyase reaction, was subjected to controlled proteolysis. Cross-linking was mapped to the 40-kDa core domain, indicating that the dRP lyase active site is in this region. To further evaluate the BER capacity of the enzyme, the dRP lyase and DNA polymerase activities were characterized on DNA substrates representing BER intermediates, and we found that pol \( \iota \) was able to complement the in vitro single-nucleotide BER deficiency of a DNA polymerase \( \beta \) null cell extract.

Human DNA polymerase \( \iota \) (pol \( \iota \))\(^1\) is a member of the Y-family of DNA polymerases (UmuC/DnB/Rev1/Rad30) (1–5). These polymerases are thought to facilitate DNA replication beyond certain types of damaged DNA blocking lesions by conducting translesion DNA synthesis (6–12). The enzymes in the Y-family share little or no primary structural homology with DNA polymerases of the other families (13–15). Among the Y-family DNA polymerases, DNA polymerase \( \eta \) (pol \( \eta \)), which is encoded by the POLH gene (also referred to as XPV/RAD30A), is present in both Saccharomyces cerevisiae and humans, whereas two other Y-family polymerases, pol \( \iota \) and DNA polymerase \( \kappa \), are absent in S. cerevisiae (12). A physiological function of pol \( \eta \) in protecting humans against UV light exposure was established by the finding that individuals with Xeroderma pigmentosum complementation group V carry muta-

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‡ The abbreviations used are: pol \( \iota \), DNA polymerase \( \iota \); BER, base excision repair; GST, glutathione S-transferase; UDG, uracil-DNA glycosylase; dRP, 5'-deoxyribose phosphate; AP endonuclease, apurinic/apyrimidinic endonuclease; DTT, dithiothreitol; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(2-hydroxyethyl)propane-1,3-diol.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic oligodeoxyribonucleotides purified by high pressure liquid chromatography were from Oligos Etc., Inc. (Wilson-
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After 60-h incubation, the cells were collected by centrifugation. Cells were infected with a high titer stock of pJM299-derived baculovirus and cross-linked pol° indicated fraction from a RESOURCE Q column (Fig. 1) and incubated that contained a 32P-labeled uracil at position 16 and a nick between the slow turnover number for the pol° were analyzed using ImageQuant software. Due to dRP instability and reaction products, gels were scanned on a PhosphorImager and the data precast gel) for 30 min at constant voltage (200 V). To quantify the final step of purification a RESOURCE Q column (fast protein liquid chromatography.

3°-End Labeling—A 34-mer oligodeoxyribonucleotide containing uracil at position 16 was labeled on its 3°-end by terminal deoxynucleotidyltransferase using [α-32P]dATP. This strand was then annealed with a complementary 34-mer template strand with a G at a position that pairs with U, by heating the mixture at 90 °C for 3 min, followed by slow cooling to 25 °C. 32P-Labeled duplex oligonucleotide was separated from unincorporated [α-32P]dATP using a MicroSpin G-25 column (Amersham Biosciences). The radiolabeled oligonucleotide duplex was stored at −30 °C.

5°-End Labeling—A uracil-containing 19-mer oligodeoxyribonucleotide was 5°-phosphorylated with T4 polynucleotide kinase and γ-[32P]ATP. The 34-mer template strand (described above) was then annealed with complementary 15-mer and 19-mer 32P-labeled oligonucleotides. The duplex was purified by a MicroSpin G-25 column and stored as described above.

dRP Lyase Assay for Column Fractions—dRP lyase activity of pol° was measured using a 34-base pair substrate DNA as described previously (28). Briefly, the 32P-labeled uracil-containing duplex DNA was pretreated with 10 nM UDG and AP endonuclease. The reaction mixture (10 μl) contained 50 mM Hepes, pH 7.5, 20 mM KCl, 5 mM MgCl2, 0.5 mM EDTA, 200 nM 32P-labeled AP site-containing DNA. The reaction was initiated by adding 1 μl of the indicated fraction from a RESOURCE Q column (Fig. 1) and incubated at 37 °C for 30 min. The reaction was terminated by transfer to 0–1 °C, and the DNA product was stabilized by addition of 2 μM sodium borohydride (NaBH4) to a final concentration of 30 mM. Incubation was continued for 30 min on ice. The stabilized DNA products were recovered by ethanol precipitation in the presence of 0.1 μg/ml tRNA and resuspended in 10 μl of gel-loading dye buffer (95% formamide, 20 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol). After incubation at 75 °C for 2 min, the reaction products were separated by electrophoresis in a 15% polyacrylamide gel containing 8 M urea in 89 mM Tris-HCl, pH 8.8, 89 mM boric acid, and 2 mM EDTA. Gels were scanned on a PhosphorImager 450 (Amersham Biosciences), and the data were analyzed using ImageQuant software.

Kinetic Measurement of dRP Lyase Activity—Kinetic analysis of dRP lyase activity of pol° was performed using a 34-base pair substrate DNA that contained a 32P-labeled uracil at position 16 and a nick between positions 15 and 16. The duplex DNA (100 nM) was pretreated at 20 min at 30 °C with 20 nM UDG in a buffer containing 50 mM Hepes, pH 7.5, 20 mM KCl, 0.5 mM EDTA, and 2 mM DTT. The dRP lyase reaction mixture (50 μl) was assembled at 0–1 °C in the above buffer. Reactions were initiated by adding appropriate dilutions of pol° and incubated at 37 °C. Aliquots were withdrawn at different time intervals and transferred to −11 °C to stop the reaction. The DNA product was stabilized by addition of 20 mM NaBH4 and incubated 30 min on ice. Then, an equal volume of gel-loading buffer was added, and the reaction mixture was incubated at 75 °C for 2 min. The reaction products were separated by electrophoresis in a 15% polyacrylamide TBE-Urea gel (Invitrogen, precast gel) for 30 min at constant voltage (200 V). To quantify the reaction products, gels were scanned on a PhosphorImager and the data were analyzed using ImageQuant software. Due to dRP instability and the slow turnover number for the pol°dependent dRP lyase reaction, high enzyme concentrations were utilized. Under these conditions, the first-order rate constants of the exponential time courses are dependent on enzyme concentration. A secondary plot of the concentration dependence was fitted to the Michaelis–Menten equation.

Covariant Cross-linking of DNA to Pol°—To prepare the covalently cross-linked pol°DNA complex, the NaBH4 trapping technique was used (29). Briefly, the reaction mixture (10 μl) contained 50 mM Hepes, pH 7.5, 20 mM KCl, 0.5 mM EDTA, 2 mM DTT, 200 mM 32P-labeled

UDG-treated duplex DNA, and 1 μM pol°. NaBH4 was added immediately to the reaction mixture (1 mM final concentration) and incubated for 30 min on ice. SDS-PAGE sample buffer (10 μl) was then added. The sample was boiled for 2 min, and the covalent complexes were resolved by NuPAGE, 4%–12% BisTris gel (Invitrogen), and the gel was scanned on a PhosphorImager.

Proteolysis of the Pol°DNA Complex with Chymotrypsin—After NaBH4 reduction to achieve cross-linking of pol° to 32P-labeled DNA, the reaction mixture was supplemented with 10 mM CaCl2, and the complex was treated with microsomal nuclease (0.5 μg/ml). After a 20-min incubation at 30 °C, the pH of the reaction mixture was adjusted to 8.0 by adding 1 mM Tris-HCl, pH 8.0, to a final concentration of 100 mM. Cross-linked complex was then treated with chymotrypsin by mixing chymotrypsin to complex (w/v; 1:6) and incubating at 30 °C. Aliquots were withdrawn at 1, 2, 5, 10, and 20-min intervals, mixed with

UDG-treated duplex DNA, and 1 μM pol°. NaBH4 was added immediately to the reaction mixture (1 mM final concentration) and incubated for 30 min on ice. SDS-PAGE sample buffer (10 μl) was then added. The sample was boiled for 2 min, and the covalent complexes were resolved by NuPAGE, 4%–12% BisTris gel (Invitrogen), and the gel was scanned on a PhosphorImager.

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Fig. 2. Proteolysis of pol 1 with chymotrypsin. A, a photograph of a Coomasie Blue-stained gel, illustrating the results of chymotrypsin digestion of pol 1, is shown. Purified GST-pol 1 was digested with chymotrypsin as described under “Experimental Procedures.” The digested samples were analyzed by 4–12% NuPAGE gel, and the proteins were transferred onto polyvinylidene difluoride membrane for sequencing. The positions of pol 1 and chymotryptic peptides are indicated. B, amino-terminal sequencing was performed using an Applied Biosystems 477A protein sequencer. The amino-terminal sequence of the polypeptides (I–IV) is shown. C, summary of the domain organization of pol 1 and designations used in this report.

SDS-PAGE sample buffer, and boiled for 3 min. The digested products were analyzed by NuPAGE 4–12% Bis-Tris gel. When pol 1 alone was digested with chymotrypsin, a ratio of 1:50 (chymotrypsin to pol 1) was used.

DNA Polymerase Assay—Radiolabeled template-primer DNA was prepared by annealing the 5'-32P-labeled 21-mer primer to the 40-mer oligodeoxyribonucleotide template at a molar ratio of 1:1. The standard DNA polymerase reaction mixture (10 μl) contained 40 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 5 mM DTT, 100 μg/ml bovine serum albumin, 100 μM dNTPs, 2.5% glycerol, and 200 nM 32P-labeled DNA. The reactions were initiated by adding the appropriate dilution of pol 1. After incubation at 37 °C for 10 min, reactions were terminated by the addition of gel-loading buffer and the reaction products were analyzed as above.

Steady-state and Single Turnover Measurements of Pol 1 Utilizing Gapped DNA—Steady-state (enzyme + substrate DNA) and single turnover (enzyme + substrate DNA) measurements were performed with a templating G residue in a single-nucleotide gapped DNA substrate. The concentrations of pol 1 and DNA substrate were 5 and 200 nM, respectively, in contrast to the single turnover time course where the ratio of GST-pol 1/DNA was 5 (i.e. 250 nM/50 nM). Products were separated by 12% denaturing polyacrylamide gel electrophoresis and the reaction products were analyzed as above. Data were fitted to the appropriate equations by nonlinear least squares methods.

Base Excision Repair Assay—The reaction mixture (10 μl) contained 50 mM Hepes, pH 7.5, 20 mM KCl, 10 mM MgCl2, 2 mM DTT, 0.5 mM EDTA, 4 mM ATP, 5 mM dTris-phosphocreatine, 100 μg/ml creatine phosphokinase, 0.5 mM NAD, a 20 μM concentration each of dATP, dGTP, dTTP, and 2 μM [α-32P]dCTP (specific activity: 1 × 106 dpm/μmol), 250 nM 35-bp DNA substrate with a uracil at position 15, and 10 μg of cell extract with or without supplemental pol 1 (100 nM). After incubation for 30 min at 37 °C, the reactions were stopped by adding 10 μl gel-loading dye, and the reaction products containing radiolabeled dCMP were processed as above.

RESULTS AND DISCUSSION

Enzymatic Activities—To further examine the relationship between the polymerase and lyase activities of the recombinant pol 1 protein, we purified the protein to near-homogeneity and then subjected the preparation to RESOURCE Q column chromatography (Fig. 1A). Fractions from the RESOURCE Q column containing most of the protein (fractions 9–13) were pooled and represented the final purified enzyme. There is a single peak for both the lyase and polymerase activities observed in these fractions (Fig. 1, B and C). Since most of the 108
kDa pol i protein elutes in these fractions, the results are consistent with the interpretation that these enzymatic activities are intrinsic to pol i.

**Domain Mapping of Pol i**—To examine the domain organization of pol i, we subjected the purified enzyme (i.e. GST-Pol i fusion protein) to controlled proteolysis with endoproteinase Glu-C, endoproteinase Lys-C, chymotrypsin, or trypsin. Results after chymotrypsin digestion are described in the legend to Fig. 2A. After 5 or 10 min, this digestion produced four major polypeptides (designated I–IV; Fig. 2A) with molecular masses of about 8-, 28-, 40-, and 48-kDa. Amino-terminal sequence analysis of these four polypeptides was undertaken (Fig. 2B). The results revealed that the 8- and 48-kDa polypeptides corresponded to the NH₂ terminus of the pol i sequence, whereas the 40-kDa polypeptide started at residue Met⁷⁹ of pol i. Sequencing of the 28-kDa proteolytic fragment revealed it to be the GST tag. With longer incubation, the 48-kDa polypeptide was no longer observed, whereas the 40-kDa pol i polypeptide and the 28-kDa GST tag polypeptide persisted. These results indicated that the 40-kDa polypeptide is a protease-resistant domain of pol i derived from the NH₂-terminal and central regions of the 80-kDa enzyme. The 40-kDa region is designated as the core domain (Fig. 2C). This 40-kDa core domain was also observed after digestion with other proteases, e.g. trypsin and endoproteinase GluC (not shown). Finally, we note that no stable proteolytic fragments were obtained from the COOH-terminal region, ~35% of the pol i sequence, indicating that
DNA containing a uracil residue at position 16 was incubated without (H9259) or with (H18528) purified pol of BER assay were as described under Experimental Procedures. The positions of ligated (35-mer) and unligated single-nucleotide (15-mer) products are indicated. A photograph of a PhosphorImager scan, illustrating the incorporation of [32P]dCTP into DNA, is shown. A 35-bp duplex DNA containing a uracil residue at position 16 was incubated without (lane 1) or with (lane 2) purified pol, [α-32P]dCTP, β-pol null extract for 30 min at 37 °C. The DNA products were separated by 12% denaturing polyacrylamide gel electrophoresis and analyzed by using ImageQuant software.

To localize the dRP lyase active site in pol, we subjected the cross-linked enzyme-DNA complex first to micrococcal nuclease digestion and then to chymotrypsin digestion (Fig. 3A). Micrococcal nuclease digestion was used to remove extraneous substrate DNA, and hence this preserves the gel migration properties of the labeled enzyme (32). With shorter periods of proteolytic digestion, label was observed mainly in the 48-kDa domain was capable of dRP lyase activity and that its 8-kDa NH2-terminal segment, which was removed by prolonged proteolytic digestion, was required for this domain peptide to be active.

Base Excision Repair Activities of Pol — We examined several features of purified human pol η related to BER. First, kinetic features of single-nucleotide gap-filling DNA synthesis and dRP lyase were measured and compared with those of human pol β. To study kinetic parameters of dRP removal by pol η, a duplex DNA (34-bp) that contained uracil at position 16 and a nick between positions 15 and 16 was prepared by annealing a 15-mer and 5′-end 32P-labeled 19-mer to the 34-mer complementary DNA strand; the resulting duplex DNA was treated with UDG to create a dRP-containing DNA substrate. Thus, this DNA substrate contained a 32P-labeled dRP flap in a single-nucleotide gap (Fig. 4A; 18-mer+32P-dRP). The time course of dRP removal from this substrate by pol η was assayed as a function of enzyme concentration. The apparent Km for the dRP lyase of pol η were 0.4 μM and 0.002 s⁻¹, respectively (Fig. 4), resulting in a catalytic efficiency (kcat/Km) of 5 nm⁻¹ s⁻¹. Utilizing an optimized protocol to measure dRP lyase activity, this is ~30-fold lower than that found earlier for pol β (28).

To determine the steady-state rate of single-nucleotide gap-filling by pol η, a time course was performed using a saturating concentration of DNA and dCTP (Fig. 5A). The observed rate (i.e., kcat) of insertion was 0.02 s⁻¹. A time course performed under single turnover conditions (i.e., enzyme/DNA = 5) at saturating dCTP concentration yielded a rate of nucleotide insertion (kcat) of 0.05 s⁻¹ (Fig. 5B). The similarity between
these values for $k_{\text{cat}}$ and $k_{\text{pol}}$ indicated that for single-nucleotide gap-filling by pol $i$ product release was not strongly rate-limiting, such that $K_{\text{pol, dCTP}}$ reported for this polymerase should be equivalent to the equilibrium dissociation constant of the nucleotide (18–20, 30). With the turnover numbers for dRP lyase and polymerase activities in hand, it is possible to compare the ratio of these activities (e.g. $k_{\text{lyase}}/k_{\text{pol}}$, where $k_{\text{lyase}}$ and $k_{\text{pol}}$ represent the rate-limiting steps for dRP excision and nucleotide insertion, respectively) for pol $i$ and pol $b$ (28, 33). The ratios are 0.04 and 0.008 for pol $i$ and pol $b$, respectively, indicating that pol $i$ has a stronger dRP lyase activity relative to its polymerase activity than pol $b$. This is primarily due to the weak polymerase activity of pol $i$ (34).

Next, in view of the single-nucleotide DNA synthesis and gap tailoring activities of pol $i$, we asked whether the purified enzyme could complement the in vitro BER deficiency of an extract from pol $b$ null cells. The results of an experiment to address this question are shown in Fig. 5C. It is well known that the pol $b$ null extract is deficient in single-nucleotide BER activity on uracil-containing oligonucleotide substrate (35). The addition of 100 nM pol $i$ strongly stimulated BER activity in this extract, and the amount of activity observed in the presence of pol $i$ was similar to that observed with the wild type extract. These results indicate that pol $i$ is capable of functioning in BER in a cell extract-based system. The in vitro enzymatic activities of purified pol $i$ are therefore clearly consistent with such a role in the cell.

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