A mechanism for the exclusion of low-fidelity human Y-family DNA polymerases from base excision repair

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The human Y-family DNA polymerases, Polα, Polη, and Polκ, function in promoting replication through DNA lesions. However, because of their low fidelity, any involvement of these polymerases in DNA synthesis during base excision repair (BER) would be highly mutagenic. Mechanisms, therefore, must exist to exclude their participation in BER. Here, we show that although Polα, Polη, and Polκ are all able to form a covalent Schiff base intermediate with the 5′-deoxyribose phosphate (5′-dRP) residue that results from the incision of DNA at an abasic site by an AP endonuclease, they all lack the ability for the subsequent catalytic removal of the 5′-dRP group. Instead, the covalent trapping of these polymerases by the 5′-dRP residue inhibits their DNA synthetic activity during BER. The unprecedented ability of these polymerases for robust Schiff base formation without the release of the 5′-dRP product provides a means of preventing their participation in the DNA synthetic step of BER, thereby avoiding the high incidence of mutagenesis and carcinogenesis that would otherwise occur.

[Keywords: Y-family DNA polymerases; base excision repair; abasic site; 5′-deoxyribose phosphate; 5′-dRP lyase]

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Abasic (AP) sites, resulting from spontaneous hydrolysis of the N-glycosylic bond, arise in DNA relatively frequently, as hydrolytic depurination causes the loss of ∼9000 bases per day from the DNA of a mammalian cell [Nakamura et al. 1998]. AP sites are also formed in DNA as intermediates in the removal of damaged bases by base excision repair (BER; Wallace 1997). A plethora of endogenous and exogenous agents yield modified or damaged bases. For example, the DNA bases cytosine and 5-methyl cytosine are subject to hydrolytic deamination to uracil and thymine, respectively, and oxygen-free radicals produced during normal cellular metabolism are an important source of endogenous damage to DNA bases that results in oxidized purines such as 8-hydroxyguanine, and ring-saturated derivatives of pyrimidines such as thymine glycol [Wallace 1994, 1997; Seeberg et al. 1995]. The different types of modified or damaged bases are removed by specific DNA glycosylases by cleavage of the N-glycosylic bond between the base and the deoxyribose moiety of the nucleotide. The resulting AP site is then acted on by a class II AP endonuclease, which cleaves the phosphodiester backbone on the 5′ side of the AP site, leaving a 3′-hydroxyl group and 5′-baseless deoxyribose-5′-phosphate (5′-dRP) residue [Wallace 1994, 1997; Seeberg et al. 1995]. The 5′-dRP residue is then removed by a dRP lyase, such as that contained in DNA polymerase (Pol) β [Matsumoto and Kim 1995; Piersen et al. 1996], and the resulting single nucleotide gap is filled in by Polβ [Wilson 1998]. Because of the high frequency with which endogenous base modifications, base damages, and AP sites are generated, the repair synthesis step of BER needs to be fairly accurate to keep the incidence of mutations low.

Polβ is especially adapted to function in BER because it can efficiently catalyze both the removal of the 5′-dRP group via β-elimination and the subsequent single-nucleotide gap-filling reaction. Limited proteolysis studies have revealed two independently folded domains in Polβ, the 8-kD N-terminal portion, which contains the 5′-dRP lyase activity, and the 31-kD C-terminal portion, which contains the polymerase activity. Lys 72 in the conserved helix–turn–helix motif of the 8-kD domain acts as the nucleophile that initiates the 5′-dRP removal reaction [Prasad et al. 1999b; Deterding et al. 2000]. First, Lys 72 initiates an attack on the C3 position of the 5′-dRP group. Next, a transient covalent intermediate is formed in which the DNA substrate is linked covalently to the enzyme as a Schiff base that can be stabilized by reduction with sodium borohydride. Finally, the 5′-dRP group is released from Polβ [Matsumoto et al. 1998; Prasad et al. 1998a]. Polβ-null
mice cells are hypersensitive to the cytotoxic effects of the alkylating agent methyl methanesulphonate (MMS), indicating an in vivo requirement for Polβ in the repair of alkylated bases [Sobol et al. 1996]. Only the 5′-dRP lyase activity of Polβ, however, is critical for resistance to MMS, as Polβ-null mice cells stably transfected with a Polβ minigene carrying mutations in the 5′-dRP lyase active site still retain their sensitivity to MMS, whereas transfection with a Polβ minigene carrying mutations in the DNA polymerase active site complement the MMS sensitivity of Polβ-null mice cells [Sobol et al. 2000]. Thus, in mammalian cells, Polβ is primarily responsible for the rate-limiting removal of the 5′-dRP-group, whereas the other DNA polymerases can functionally substitute for the polymerase activity of Polβ in BER.

The human Y-family DNA polymerases, Polη, Polκ, and Polκ, promote replication through DNA lesions that present a block to the normal replication machinery. Polη replicates through UV-induced thymine–thymine [TT] dimers with the same efficiency and accuracy as it replicates through undamaged Ts [Johnson et al. 1999b, 2000c; Washington et al. 2000], and genetic studies in yeast have implicated Polη in the error-free bypass of cyclobutane dimers formed at TC and CC sites [Yu et al. 2001]. Because of the involvement of Polη in the error-free bypass of cyclobutane pyrimidine dimers, its mutational inactivation in humans causes an increase in the incidence of UV mutagenesis and leads to the cancer-prone syndrome, the variant form of xeroderma pigmentosum [Johnson et al. 1999a; Masutani et al. 1999]. Polη can efficiently replicate through other DNA lesions as well such as 7,8-dihydro-8-oxoguanine (8-oxoG) and O6-methyl guanine [m6G; Haracska et al. 2000a,b]. In contrast to Polη, the ability of which to replicate through DNA lesions derives both from its ability to proficiently incorporate nucleotides opposite the lesion site and to proficiently extend from the inserted nucleotide, the bypass of certain DNA lesions requires the sequential action of two DNA polymerases, wherein one inserts the nucleotide opposite the lesion site and the other extends from the inserted nucleotide [Prakash and Prakash 2002]. Polκ is able to incorporate nucleotides opposite the 3′-T of a (6–4)T–T photoproduct or opposite an AP site, but it does not extend from the nucleotide opposite these lesion sites [Johnson et al. 2000b]. Polκ, on the other hand, is unable to insert nucleotides opposite DNA lesions, as, for example, opposite the 3′-T of a TT dimer or opposite an m6G lesion, but it can efficiently extend from the nucleotides opposite these lesion sites [Haracska et al. 2002a; Washington et al. 2002]. The Y-family polymerases possess an active site that is more tolerant of geometric distortions in the DNA than the replicative DNA polymerases that are unable to bypass DNA lesions. The cost of the ability of Polη, Polκ, and Polκ to promote lesion bypass, however, is borne by their low fidelity. Steady-state kinetic analyses have indicated that Polη and Polκ misincorporate nucleotides with a frequency of $10^{-2}$ to $10^{-3}$ and $10^{-4}$ to $10^{-5}$, respectively [Washington et al. 1999; Johnson et al. 2000a,c]. The fidelity of Polκ is much poorer than that of Polη, particu-

larity for incorporating nucleotides opposite the template pyrimidines [Johnson et al. 2000b; Tissier et al. 2000].

Previously, a role for human Polκ has been implicated in BER [Bebenek et al. 2001]. However, considering that Polκ is an extremely low-fidelity DNA polymerase, DNA synthesis by this enzyme in BER would be very highly mutagenic. Here, we address whether the human Y-family DNA polymerases Polη, Polκ, and Polκ can, in fact, play a role in BER, or whether these low-fidelity polymerases are somehow excluded from the DNA synthesis step of BER. Interestingly, we found that although each of these human polymerases can form a covalent Schiff base complex with the 5′-dRP group in DNA, they are unable to carry out the subsequent removal of the 5′-dRP group. Furthermore, and in striking contrast to Polη, the DNA synthetic activity of Polκ, Polκ, and Polκ is inhibited upon their binding to the 5′-dRP group. From these and other observations presented here, we conclude that nonproductive binding to the 5′-dRP group represents a means to exclude these DNA polymerases from the DNA synthetic step of BER.

**Results**

**Human DNA polymerases η, κ, and λ form a covalent complex with the 5′-dRP group**

We purified human Polκ, Polη, and Polκ as native as well as N-terminal GST-fusion proteins [Fig. 1A], and first examined their DNA synthesis activities. As shown in Figure 1B, the DNA synthesis activity of the GST-fusion polymerases were nearly identical to the corresponding native enzymes. Thus, the GST-fusion does not interfere with the activity of Polκ, Polη, or Polκ.

Next, we examined whether Polκ, Polη, and Polκ can form a Schiff-base covalent complex with the 5′-dRP-containing DNA substrate. A Schiff base can be formed between a properly positioned N-terminal α NH₂ group [Dodson et al. 1993] or an ε NH₂ group [Matsumoto et al. 1998; Prasad et al. 1998a] of a protein and the –CHO group of an open abasic residue in DNA, and the covalent linkage between DNA and protein can be trapped by the reduction of this Schiff base with sodium borohydride [Piersen et al. 1996]. As outlined in Figure 2A, we generated a DNA substrate containing a 5′-dRP residue at an internal nick by the sequential treatment of a single-uracil-containing DNA duplex with uracil DNA glycosylase and a class II AP endonuclease. Figure 2B shows that, similarly to Polθ, Polκ, Polη, and Polκ are active in the borohydride trapping assay. Because of the cross-linking of the polymerase to the 5′-dRP-containing DNA, the trapped products exhibit a slower mobility on an SDS–polyacrylamide gel than the unmodified polymerases [cf. Figs. 2B and 1A]. We were able to cross-link the GST-fusion proteins as well as the native polymerases to the DNA, which indicates that the GST-fusion part is not required for the Schiff base formation. In conclusion, Polκ, Polη, and Polκ are able to react with the 5′-dRP residues in DNA to form a Schiff-base covalent complex.
Exclusion of Y-family DNA Pols from BER

Figure 1. Purity and DNA synthetic activity of human Polı, Polıı, Polııı, and Polıııı. [A] Purified native and N-terminal GST-fusion Polı, Polıı, Polııı, and native Polıııı were analyzed on an 8% denaturing polyacrylamide gel and stained with Coomassie blue. [Lane 1] Molecular weight standards containing 500 ng of protein in each band; [lane 2] 300 ng of Polı; [lane 3] 500 ng of GST-Polı; [lane 4] 300 ng of Polıı; [lane 5] 500 ng of GST-Polıı; [lane 6] 500 ng of Polııı; [lane 7] 500 ng of GST-Polııı; [lane 8] 300 ng of Polıııı. [B] The DNA synthetic activity of purified GST-fusion and native Polı, Polıı, Polııı, and native Polıııı was assayed using a 75-nt template oligonucleotide primed with a 40-nt 5'-32P-labeled oligonucleotide [S1 substrate] in the presence of all four deoxynucleotides [100 µM] in buffer A containing 40 mM Tris-HCl [pH 7.5], 8 mM MgCl2, 1 mM dithiothreitol, 100 µg/mL bovine serum albumin, and 10% glycerol. The DNA substrate [20 nM] was incubated with 2 nM of DNA polymerase at 37°C for 10 min. The reaction products were analyzed on an 8 M urea, 8% polyacrylamide gel, and the DNA bands were visualized by autoradiography.

Lack of 5'-dRP lyase activity in human DNA polymerases ı, ıı, and ııı

Although Schiffs base formation is a prerequisite for a nonhydrolytic dRP lyase reaction, it does not necessitate a strong subsequent b-elimination reaction. To test whether the Schiff base formed by Polı, Polıı, and Polııı with DNA was an intermediate in the 5'-dRP excision reaction, we examined whether Polı, Polıı, and Polııı could excise the 5'-dRP group. We directly compared the 5'-dRP lyase [Fig. 2C] and DNA polymerase [Fig. 2D] activities of Polı, Polıı, and Polııı at different enzyme concentrations, and as a control, we used Polıııı. Whereas Polıııı displayed a strong activity for removing the 5'-dRP group from the AP endonuclease-incised oligonucleotide [19 nt + dRP band], thereby generating the 19-nt product that lacks the 5'-dRP group and therefore has a faster mobility [Fig. 2C, lanes 10,11], we were unable to detect any 5'-dRP lyase activity associated with Polı, Polıı, or Polııı [Fig. 2C, lanes 4-9]. Despite the fact that at 2 nM concentration, Polı, Polıı, and Polııı had as robust a DNA polymerase activity as Polıııı [Fig. 2D], neither Polı, Polıı, nor Polııı removed any 5'-dRP group even at as high a concentration as 50 nM. With Polıııı, however, as little as 2 nM enzyme removed 70% of the 5'-dRP group, and 10 nM Polıııı could remove all of the 5'-dRP groups from the DNA. Thus, although the human Y-family DNA polymerases are all able to form a covalent complex with the 5'dRP-group in DNA, the resulting polymerase–DNA complexes do not act as intermediates for the 5'-dRP excision reaction.

To further verify the absence of a 5'-dRP lyase activity in DNA polymerases ı, ıı, and ııı, we examined the kinetics of borohydride cross-linking with these enzymes. Depending on whether the polymerase–5'-dRP–DNA complex is an intermediate in the dRP lyase reaction or not, the borohydride cross-linking should occur with different kinetics, and therefore, the amount of the cross-linked product should change if the polymerase were first preincubated with the 5'-dRP substrate for varied intervals before the addition of borohydride. For an enzyme such as Polııııı, which has a robust 5'-dRP lyase activity, the cross-linked protein–DNA complex would diminish following preincubation with the enzyme because the enzyme would have removed the 5'-dRP group from the DNA. However, for an enzyme that has no 5'-dRP lyase activity, the results indicate for Polı, Polıı, and Polııı, preincubation of these enzymes with the 5'-dRP substrate should cause no decrease in the amount of cross-linked protein–DNA complex. Whereas Polı, Polıı, and Polııı, as well as Polııııı, rapidly formed a complex with the 5'-dRP substrate, as even without any preincubation, we were able to trap their complex with borohydride [Fig. 3, lane 1], striking differences were observed between Polıııı and the other polymerases upon their preincubation with the 5'-dRP substrate before borohydride addition. Thus, after preincubation for 10 min, the intensity of the Polıııı–5'-dRP complex diminished, while the intensity of the Polı–5'-dRP, Polıı–5'-dRP, and Polııı–5'-dRP–DNA complexes increased [Fig. 3, cf. lanes 1 and 2]. With a 30-min preincubation, the amounts of Polı, Polıı, and Polııı covalent complexes showed a further increase, but no cross-linked product could be seen with Polıııı [Fig. 3, lane 3]. These results show that in contrast to Polıııı, which has a potent 5'-dRP lyase activity, Polı, Polıı, and Polııı did not excise the 5'-dRP group after complex formation with the 5'-dRP in DNA, and instead, with these DNA polymerases, the amount of cross-linked product increased with the increase in preincubation time. These are the results expected for an enzyme that has no 5'-dRP lyase activity but can form a covalent complex with the 5'-dRP group in DNA.

Exclusion of DNA Polymerases ı, ıı, and ııı from DNA synthesis at an incised abasic site

The ability of Polı, Polıı, and Polııı, to form a covalent complex with the 5'-dRP group without carrying out the subsequent lyase reaction raised the possibility that binding to the 5'-dRP group precludes these polymerases from carrying out the DNA synthesis step during BER. To test this possibility, first we compared the DNA synthetic activity of Polı and Polııı on two DNA substrates,
one of which was incised at the AP site and the other had a single nucleotide gap [Fig. 4A]. To examine DNA synthesis, and to determine which nucleotide is inserted opposite the template residue T, which is situated opposite the 5′-incised AP site or opposite the single nucleotide gap, we monitored the extension of the 5′-32P-labeled 15-nt standing start primer, the 3′-end of which is situated right before the gap or the AP site, in the presence of all four deoxynucleotides. We compared the electrophoretic mobilities of the products of DNA synthesis with 16-nt oligonucleotide markers containing the 15-nt primer plus a G or an A residue at position 16, on a 20% polyacrylamide gel [Fig. 4A, lanes 1, 2]. As expected, whereas Polβ inserted an A opposite the template T [Fig. 4A, lanes 6, 7], Pol inserted predominantly a G opposite the template T [Fig. 4A, lanes 4, 5]. In striking contrast to Polβ, which displayed nearly identical DNA synthetic activity on the two DNA substrates [Fig. 4A, lanes 6, 7], the DNA synthetic activity of Pol was severely inhibited on the nicked AP-site-containing DNA [Fig. 4A, cf. lanes 4 and 5]. Thus, not only does Pol have an extremely low fidelity in single nucleotide gap filling, it is also very poor in carrying out the DNA synthesis reaction on the 5′-nick AP-site-containing DNA substrate.

To test if the 5′-dRP group inhibits the DNA synthesis activity of all three Y-family DNA polymerases, we compared the activity of Pol, Polη, Polκ, and Polβ on two DNA substrates containing a 5′-C or a 5′-dRP residue at the nick [Fig. 4B]. Compared with DNA synthesis on the nicked DNA substrate without the 5′-dRP residue [Fig. 4B, lanes 2–7], the DNA synthesis activities of Pol, Polη, and Polκ were severely inhibited on the DNA substrate containing the 5′-dRP moiety [Fig. 4B, lanes 10–15], whereas no such inhibition was observed with Polβ [Fig. 4B, cf. lanes 8, 9, and 16, 17]. Thus, relative to primer extension on the nicked DNA substrate lacking the 5′-dRP residue, the inhibition of the DNA synthetic activity on the 5′-dRP-containing DNA substrate was ~10-fold for Pol, ~5-fold for Polη, and ~10-fold for Polκ, whereas Polβ was ~20% more efficient at synthesizing DNA on the 5′-dRP substrate than on the nicked DNA substrate lacking this lesion. In conclusion, binding of Pol, Polη, and Polκ to the 5′-dRP group in DNA severely inhibits their DNA synthetic activity.
Exclusion of Y-family DNA Pols from BER

Translesion DNA synthesis is one of the main roles of human Polβ, Polδ, and Polκ (Prakash and Prakash 2002). These DNA polymerases, however, replicate DNA with a low fidelity, and the fidelity of Polδ is the poorest, particularly for incorporating nucleotides opposite pyrimidine templates. Therefore, to prevent increased mutagenesis, it is critical that the DNA synthetic activity of these polymerases be restricted. Because of the preponderance of a variety of base damages, and because of the high incidence of AP sites, which are generated as a result of spontaneous base loss and as intermediates in BER, it is particularly important that high-fidelity DNA synthesis prevail during BER.

Figure 3. Time course of polymerase–DNA cross-linking by borohydride. Polε [panel I], Polδ [panel II], Polκ [panel III], or Polβ [panel IV; each at 10 nM] were mixed at 0°C with the internal 5′-dRP-containing 3′-32P-labeled DNA [50 nM] shown in Figure 2A, followed by incubation at 37°C for 0, 10, or 30 min (lanes 1–3, respectively) before the addition of 20 mM NaBH₄ and further incubation at 0°C for 30 min. After the addition of SDS-containing loading buffer, the samples were resolved on an 8% SDS–polyacrylamide gel, and the cross-linked polymerase–DNA products, indicated by arrowson the left of panels, were analyzed by autoradiography.

Discussion

We show here that Polε, Polδ, and Polκ can each form a Schiff base with 5′-dRP-containing DNA. These enzyme–DNA complexes, however, are not intermediates of a 5′-dRP lyase reaction, as we found no 5′-dRP lyase activity in our purified Polε, Polδ, and Polκ preparations, which are fully active in the DNA synthesis reaction. Thus, in contrast to Polβ, where as little as 2 nM of the enzyme removed ~70% of the 5′-dRP group from the substrate, Polε, Polδ, and Polκ had no 5′-dRP lyase activity, even at a concentration as high as 50 nM. The absence of a 5′-dRP lyase activity from Polε, Polδ, and Polκ was further supported from studies in which these enzymes were preincubated with the 5′-dRP DNA substrate prior to borohydride reduction of the polymerase–DNA complexes. Although preincubation with Polβ before the addition of borohydride decreased the Polβ–DNA cross-linked product, consistent with a robust 5′-dRP lyase activity in Polβ, such preincubation with Polε, Polδ, and Polκ resulted in an increase in the amount of Pol–DNA cross-linked product. The persistence of 5′-dRP residues, even after prolonged preincubation with Polε, Polδ, and Polκ, has provided unambiguous evidence for the lack of any 5′-dRP lyase activity in these enzymes.

The ability of Polδ, Polκ, and Polκ to form a covalent protein–DNA Schiff base intermediate, but not to promote the subsequent 5′-dRP lyase reaction, could arise...
from the differences in the chemistry for the two reactions. When the environment of a particular –NH2 group in a protein lowers its pK_a, it becomes a good nucleophile at physiological pH and is able to form a Schiff base with the –CHO group at the C1 position of an AP site. However, for the protein to display a strong 5'-dRP lyase activity, at this intermediate stage, the pK_a of the imine group needs to be high enough for it to be protonated and to carry out an efficient β-elimination reaction, otherwise, the 5'-dRP group removal will be highly inefficient, occurring via a spontaneous β-elimination reaction. Thus, Schiff base formation is a prerequisite for the lyase activity, but it is not sufficient for the subsequent β-elimination reaction to occur.

Cova lent binding to the 5’-dRP residue in DNA inhibits the DNA synthetic activity of Pol, Polη, and Polκ. This inhibition of synthesis may arise because covalent binding to the 5’-dRP group results in a physical orientation so that the polymerase can no longer access the 3’-primer end, alternatively, a transient conformational change may occur in the polymerase that inhibits its binding to the 3’-primer end or inhibits its catalytic activity. Because the covalent complexes of Pol, Polη, and Polκ with the 5’-dRP-containing DNA can be stabilized by borohydride, structural studies of such complexes could help unravel the mechanism of inhibition of their DNA synthetic activity.

Previously, a 5’-dRP lyase activity has been reported for human Pol (Bebenek et al. 2001; Prasad et al. 2003). This observation is inconsistent with our results. In these studies, however, no convincing evidence was provided to establish that the lyase activity is, in fact, intrinsic to Pol. The dRP lyase activity was inferred to be intrinsic to Pol because a covalent Pol–DNA Schiff-base intermediate could be trapped by reduction with sodium borohydride (Bebenek et al. 2001), and this site in Pol has been recently mapped to a 40-kD domain spanning residues Met 79 to ∼460 (Prasad et al. 2003). Our results confirm the formation of a covalent Pol–DNA Schiff-base intermediate, but we have found no evidence for a 5’-dRP lyase activity in Pol. We have examined many different highly purified native Pol and GST-Pol preparations that support robust DNA synthesis, but even with long incubation periods and with high enzyme concentrations, we have found no evidence of any significant 5’-dRP lyase activity in Pol. Moreover, as we show here, Polη and Polκ also form a Schiff base intermediate, but they have no 5’-dRP lyase activity. And, for Polη and Polκ also, we expect the region corresponding to Pol’s 40-kD domain to be involved in Schiff base formation. In view of our observations, the formation of a Schiff base intermediate cannot be taken as evidence to support the existence of a 5’-dRP lyase activity in Pol. Furthermore, in the absence of additional supporting evidence, the coelution of Pol’s polymerase activity with 5’-dRP lyase cannot be considered definitive, as that could be fortuitous (Prasad et al. 2003). Although we cannot be sure of the source of the 5’-dRP lyase activity that has been reported for Pol, any number of factors, as, for example, the presence of an extraneous peptide or a protein in the Pol preparation, could have been the source of that activity. Because of the extreme lability of a 5’-terminal abasic site, the removal of a 5’-dRP group can occur spontaneously in the absence of β-elimination catalysts (Bailly and Verly 1989), and basic proteins such as histones and polyamines stimulate this reaction by inducing β-elimination (Bailly and Verly 1988). Moreover, even short basic peptides such as Lys–Trp–Lys and Lys–Tyr–Lys, can promote this reaction (Helene et al. 1982).

In summary, with respect to their interaction with a 5’-dRP group in DNA, all three human polymerases, Pol, Polη, and Polκ, behave very similarly. They all form a covalent Schiff base intermediate with the 5’-dRP group, but have no detectable 5’-dRP lyase activity [Fig. 5]. To our knowledge, such robust Schiff base formation without the release of any significant 5’-dRP product is unprecedented, as all the other DNA polymerases that yield a covalent Schiff base intermediate also exhibit 5’-dRP lyase activity (Matsumoto et al. 1998; Pinz and Bogenhagen 2000; Garcia-Diaz et al. 2001). Our observation that the DNA synthetic activity of Pol, Polη, and Polκ is greatly reduced on a 5’-dRP-containing DNA substrate strongly supports the inference that covalent binding to the 5’-dRP residue represents a means by which these low-fidelity polymerases are excluded from the DNA synthetic step of BER [Fig. 5], thereby avoiding the high incidence of mutagenesis and carcinogenesis that would otherwise result.

Materials and methods

DNA substrates

Oligonucleotides were synthesized by Midland Certified Reagent Co. The S1 substrate, used for the DNA synthesis reactions shown in Figures 1B and 2D, was generated by annealing a 75-nt oligonucleotide template, 5’-ACGATCCATGCTGAGTCAGAAATTCGTAGATGCTCAGCTACTGGGTACCCGGGAGATCCTGAGTCGGTGAGTACCGGTACCCGGGGAGATCCTGGGTACCCGGGTAC-3’ (S1 substrate); or the 19-nt oligonucleotide primer, 5’-GTGTTCAGAGTGAGTCGGTGAGTACCGGTACCCGGGGAGATCCTGGGTACCCGGGTAC-3’ (S2 substrate); or the 34-nt oligonucleotide, 5’-CTCGAGCTGTCGACGCUGTACGGATCCCCGGGTAC-3’, was annealed to the 34 nt template oligonucleotide, 5’-GTAACCGGGATCCGTACCCGGAGATCCTGAGTCGGTGAGTACCGGTACCCGGGGAGATCCTGGGTACCCGGGTAC-3’, and then 3’-pUGTACGGATCCCCGGGTAC-3’ was annealed to the 34 nt template oligonucleotide, 5’-GTACCCGGGGATCCGTACCCGGAGATCCTGAGTCGGTGAGTACCGGTACCCGGGGAGATCCTGGGTACCCGGGTAC-3’, and then 3’-pUGTACGGATCCCCGGGTAC-3’ was annealed to the 34 nt template oligonucleotide, 5’-pUGTACGGATCCCCGGGTAC-3’ (S3 substrate); or the 19-nt oligonucleotide, 5’-pUGTACGGATCCCCGGGTAC-3’ (S4 substrate). The DNA substrates shown in Figure 4B were generated by annealing three oligonucleotides together, the 34-nt template oligonucleotide used also for generating the S2 sub-
Figure 5. A model for the exclusion of Y-family polymerases from DNA synthesis during BER. An AP site is generated in DNA either by the action of a DNA glycosylase on the damaged base (shown in bold) or via spontaneous hydrolysis of purines (data not shown). Nicking of the phosphodiester backbone on the 5’-side of the AP lesion by a class II AP endonuclease generates a 3’-OH terminus and a 5’-dRP moiety, which is removed by the potent 5’-dRP lyase activity of Polβ, followed by a single nucleotide gap filling by this enzyme (left). The thick arrow on the left denotes that because of its high affinity for the lesion site, Polβ would be the predominant enzyme to carry out the reactions shown. The low-fidelity Y-family polymerases, however, would be excluded from the DNA synthesis reaction during BER because of their unproductive binding to the 5’-dRP residue, which inhibits their ability to synthesize DNA on such a substrate (right). The bidirectional thin arrows on the right signify the less efficient and transient binding of Y-family polymerases to the lesion site. Dissociation of these polymerases from the 5’-dRP residue would free up the residue for removal by the 5’-dRP lyase activity of Polβ and for repair synthesis, as is shown on the left.

Exclusion of Y-family DNA Pols from BER

DNA polymerase assays

The DNA polymerase reaction mixture [10 µL] contained 20 nM DNA substrate; 100 µM dGTP, dATP, dCTP, and dTTP, in either buffer A containing 40 mM Tris-HCl (pH 7.5), 8 mM MgCl₂, 1 mM dithiothreitol, 100 µg/mL bovine serum albumin, and 10% glycerol [Fig. 1B], or in buffer B containing 50 mM HEPES [pH 7.5], 10 mM MgCl₂, 20 mM KCl, and 2 mM dithiothreitol [Figs. 2D, 4]. Polymerase reactions were started by the addition of Pols, Poλ, Polκ, or Polβ in concentration as indicated in the figure legends. After incubation at 37°C for 10 or 20 min as indicated in the figure legends, the reactions were terminated by the addition of 40 µL of formamide containing loading buffer [95% formamide, 20 mM EDTA, 0.3% bromophenol blue, and 0.3% cyanol blue]. The reaction products were resolved on 8% or 20% polyacrylamide gels containing 8 M urea, as indicated in the figure legends. Quantitation of the products was done using a Molecular Dynamics STORM Phosphorimager and ImageQuant software.

Preparation of 5’-dRP-containing DNA

The incised AP-site-containing DNA was generated by first pretreating the DNA duplex, which contained a single U in the 5’-dRP residue, with UDG (2 units/1 pmole DNA) in 50 mM HEPES [pH 7.5] at 30°C for 10 min, followed by treatment with human APE1, a class II AP endonuclease [2 units/1 pmole DNA] in 10 mM MgCl₂, 20 mM KCl, and 2 mM dithiothreitol [Figs. 2D, 4]. Polymerase reactions were started by the addition of Pols, Poλ, Polκ, or Polβ in concentration as indicated in the figure legends. After incubation at 37°C for 10 or 20 min as indicated in the figure legends, the reactions were terminated by the addition of 40 µL of formamide containing loading buffer [95% formamide, 20 mM EDTA, 0.3% bromophenol blue, and 0.3% cyanol blue]. The reaction products were resolved on 8% or 20% polyacrylamide gels containing 8 M urea, as indicated in the figure legends. Quantitation of the products was done using a Molecular Dynamics STORM Phosphorimager and ImageQuant software.

Purification of DNA polymerases

Human Pols, Poλ, and Polκ in fusion with glutathione S-transferase (GST) were expressed in yeast strain BJ5464 and bound to a glutathione-Sepharose 4B column as described [Haracska et al. 2001a,b, 2002b]. GST-Poλ, GST-Poλ, and GST-Polκ were eluted from one-half of the glutathione-Sepharose 4B beads with 20 mM reduced glutathione in elution buffer containing 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, and 10% glycerol. The other half of the glutathione-Sepharose 4B beads were incubated with PreScission protease (Amersham), which cleaves the GST fusion from the native proteins, releasing the native Pols, Poλ, and Polκ in elution buffer. Purified proteins were concentrated by using a Microcon 30 (Amicon) and frozen at −70°C. Human Poλ purified as described [Rajendran et al. 1998] was kindly provided by W. Bujalowski [University of Texas Medical Branch, Galveston, TX].

5’-dRP lyase assay

Reaction mixtures (10 µL) contained the DNA polymerases at two different concentrations, as indicated in the figure legends, and the 5’-dRP-containing DNA substrate (20 nM) in buffer B. Reactions were incubated at 37°C for 10 min. Thereafter, the reaction product was stabilized by the addition of 2M sodium borohydride to a final concentration of 340 mM, followed by incubation at 0°C for 30 min. Formamide-containing gel loading
buffer [40 µL] was then added, and the reaction products were resolved on 16% polyacrylamide gels containing 8 M urea followed by visualization by autoradiography.

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