DNA polymerase β is the major dRP lyase involved in repair of oxidative base lesions in DNA by mammalian cell extracts

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The repair of oxidative base lesions in DNA is a co-ordinated chain of reactions that includes removal of the damaged base, incision of the phosphodiester backbone at the abasic sugar residue, incorporation of an undamaged nucleotide and sealing of the DNA strand break. Although removal of a damaged base in mammalian cells is initiated primarily by a damage-specific DNA glycosylase, several lyases and DNA polymerases may contribute to the later stages of repair. DNA polymerase β (Pol β) was implicated recently as the major polymerase involved in repair of oxidative base lesions; however, the identity of the lyase participating in the repair of oxidative lesions is unclear. We studied the mechanism by which mammalian cell extracts process DNA substrates containing a single 8-oxoguanine or 5,6-dihydrouracil at a defined position. We find that, when repair synthesis proceeds through a Pol β -dependent single nucleotide replacement mechanism, the 5'-deoxyribosephosphate lyase activity of Pol β is essential for repair of both lesions.

Keywords: base excision repair/cell extracts/DNA polymerase β /NTH1/OGG1

Introduction

Reactive oxygen species are formed continuously as a consequence of normal cellular metabolism and are also generated by a number of external factors including UV and ionizing radiation (reviewed in Lindahl, 1993; Demple and Harrison, 1994; Wallace, 1997). The reaction of active oxygen species with DNA results in numerous forms of base damage. An increased level of oxidized DNA bases has been observed after treatment of cells with UV, ionizing radiation or chemical mutagens that generate oxygen radicals (reviewed in Dizdaroglu, 1992; Demple and Harrison, 1994). Formation of oxidative lesions can have profound consequences for genomic stability due to the effects that these lesions can have on polymerase fidelity and processivity. Some DNA oxidation products are mutagenic, promoting misincorporation of nucleotides by polymerases. For example, 8-oxoguanine, one of the most commonly occurring oxidative lesions, can form a stable Hoogsteen base pair with adenine and thereby induce transversion mutations (Shibutani *et al.*, 1991). Other oxidized DNA bases, such as thymine glycol,

effectively terminate DNA replication and transcription and are cytotoxic (Ide *et al.*, 1985; Clark and Beardsley, 1987). It is therefore essential that oxidized bases be removed from DNA efficiently in order to maintain genome integrity.

Base excision repair (BER) is the major repair system that contributes to the processing of oxidized purines and pyrimidines in mammalian cell extracts (Dianov *et al.*, 1998, 2000). In mammalian cells, the two major DNA glycosylases implicated in BER of oxidative DNA damage are 8-oxoguanine-DNA glycosylase 1 (OGG1), which excises 8-oxoguanine and imidazole-ring opened purines, and NTH1 protein (the mammalian homologue of bacterial endonuclease III), which removes oxidized and fragmented pyrimidines (Wood *et al.*, 2001). Glycosylases remove a damaged base through hydrolysis of the glycosylic bond linking the damaged base to the sugar, generating an abasic (AP) site (Lindahl and Wood, 1999). Normally, repair of an AP site proceeds through hydrolysis by AP endonuclease of the phosphodiester bond 5' to the abasic site (Demple *et al.*, 1991; Robson and Hickson, 1991; reviewed in Wilson and Barsky, 2001), followed by a coordinated reaction in which DNA polymerase β (Pol β) adds one nucleotide to the 3' end of the incised AP site, simultaneously removing a 5'-sugar phosphate (5'-deoxyribosephosphate; dRP) residue by β -elimination (Matsumoto and Kim, 1995; Prasad *et al.*, 1998a; Lindahl and Wood, 1999). Recently, human (Arai *et al.*, 1997; Radicella *et al.*, 1997; Roldan-Arjona *et al.*, 1997) and mouse (Rosenquist *et al.*, 1997) 8-oxoguanine-DNA glycosylases and human NTH1 (Aspinwall *et al.*, 1997; Ikeda *et al.*, 1998) have been cloned and characterized. Each demonstrated an AP lyase activity in addition to the glycosylase activity (Shinmura *et al.*, 1997; Ikeda *et al.*, 1998; Zharkov *et al.*, 2000), suggesting a different pathway for repair of AP sites generated after removal of oxidative base lesions. In this scenario, the AP lyase activity of the glycosylase cleaves the phosphodiester bond 3' to the AP site by catalysing a β -elimination reaction (Klungland *et al.*, 1999). The subsequent action of APE1 generates a single nucleotide gap that may be filled by any polymerase because theoretically there is no need for the dRP lyase activity of Pol β at this reaction stage (Figure 1, pathway B). Although it is generally accepted that Pol β is involved in repair of 8-oxoguanine and oxidized pyrimidines in DNA (Dianov *et al.*, 1998, 2000; Fortini *et al.*, 1999; Klungland *et al.*, 1999; Boiteux and Radicella, 2000; Nilsen and Krokan, 2001; Scharer and Jiricny, 2001), there is no evidence for the identity of the AP lyase activity involved. To identify the AP lyase activity involved in processing of oxidative DNA lesions, we characterized the repair in mammalian cell extracts of closed-circular DNA constructs containing a single 8-oxoguanine or dihydrouracil at a defined site. We find

that the dRP lyase activity of Pol β is essential for its role in the repair of oxidative base lesions.

Results

Characterization of the substrates

In this study, we used closed-circular double-stranded DNA substrates bearing a single 8-oxoguanine/cytosine or dihydrouracil/guanine base pair at a defined position

Fig. 1. Model for repair pathways involved in processing of 8-oxoguanine. Repair of 8-oxoguanine is initiated by 8-oxoguanine-DNA glycosylase; however, there are two possible scenarios for further steps. Processing through pathway A would involve cleavage of the AP site by AP endonuclease, removal of the 5'-dRP residue by dRP lyase activity of the Pol β , incorporation of one nucleotide by the same polymerase and ligation of the DNA ends broken during repair. Processing through pathway B would involve generation of a single nucleotide gap by the combined action of the AP lyase activity of DNA glycosylase and AP endonuclease. DNA polymerase then would fill the gap and finally DNA ligase would seal the DNA ends.

(Figures 2A and 3A). The damage-containing strand of each substrate was 32 P-labelled upstream of the damage site. *Hind*III cleavage of the DNA substrates released a 59mer labelled fragment containing the damage (Figures 2B, lane 1 and 3B, lane 1). In the case of the 8-oxoguanine-containing substrate, this fragment has two *Hae*III restriction sites, but one of them is blocked by 8-oxoguanine and thus simultaneous cleavage with *Hae*III and *Hind*III restriction endonucleases generated only a 52mer labelled product, containing 8-oxoguanine (Figure 2B, lane 2). Following repair of the 8-oxoguanine-containing substrate, the second *Hae*III site will be restored and should give rise to a 48mer (repaired fragment). Similarly, in the dihydrouracil-containing substrate, the *Hpa*II restriction site is blocked by dihydrouracil and thus simultaneous cleavage with *Hpa*II and *Hind*III restriction endonucleases generated only a 59mer labelled product, containing dihydrouracil (Figure 3B, lane 2). However, once repair of the dihydrouracil-containing substrate has taken place, *Hind*III-*Hpa*II cleavage will generate a 49mer fragment.

When the 8-oxoguanine-containing substrate DNA was incubated with whole-cell extract (WCE) for 2 h and subsequently cleaved with *Hind*III after isolation from the reaction mixture, we observed majority release of a 59mer labelled product (containing both repaired and unrepaired DNA). Accumulation of 47 or 48mer fragments after *Hind*III cleavage of repaired DNA would indicate incomplete repair blocked after incision (47mer) or after addition of the first nucleotide during repair synthesis (48mer). Only a very small amount of 47mer repair incision intermediate was detected (Figure 2B, lane 3). However, simultaneous cleavage with *Hind*III and *Hae*III of the extract-treated substrate generated approximately equal amounts of 52mer (unrepaired) and 48mer (repaired) products (Figure 2B, lane 4).

Fig. 2. Repair of 8-oxoguanine-containing substrate. (A) Schematic presentation of 8-oxoguanine-containing substrate. The sites of cleavage by the restriction enzymes *Hind*III and *Hae*III and the position of the 32 P label are shown. The site of incision by AP endonuclease subsequent to glycosylase processing of 8-oxoguanine (8-oxo) is indicated with an arrow. (B) Restriction digestion of untreated substrate DNA (lanes 1 and 2) and substrate incubated with 100 μ g of human WCE at 37°C for 2 h (lanes 3 and 4). (C) Time-dependent repair of 8-oxoguanine by human WCE. Reactions contained 50 ng of plasmid DNA and 100 μ g of WCE and were incubated at 37°C for the indicated time prior to isolation of the substrate DNA followed by digestion with *Hind*III and *Hae*III. Reaction products were analysed by electrophoresis in a 10% denaturing polyacrylamide gel.

The kinetics of processing of 8-oxoguanine are shown in Figure 2C. Around 50% of the 8-oxoguanine lesions were repaired by human WCE within 2 h and repair proceeded to completion within 4–5 h. Repair of the dihydrouracil-containing substrate was noticeably faster, with ~80% of dihydrouracil being removed within 2 h (Figure 3B, lane 4). Interestingly, only a minor amount of repair incision intermediates accumulate during repair of 8-oxoguanine, indicating that the sequential repair steps are highly coordinated (Figure 2C). In contrast, more repair intermediates accumulate during repair of dihydrouracil (Figure 3B, lane 3).

Repair of 8-oxoguanine by normal cell extracts

A combination of either APE1–Pol β or OGG1–APE1 may be involved in processing of the AP site, thus determining

the repair pathway (Figure 1). To discriminate between these pathways, we used an 8-oxoguanine-containing substrate to develop reaction conditions under which only Pol β -dependent single nucleotide replacement repair was allowed, and then addressed the role of dRP lyase activity of Pol β under these conditions. Mouse WCEs, at concentrations similar to those used for human cell extracts, rapidly removed 8-oxoguanine from DNA. Repair was almost complete after only 30 min of incubation, as revealed by *Hind*III–*Hae*III hydrolysis (Figure 4A, lanes 4–6). The Pol β dependence of the repair process was controlled by using the DNA synthesis inhibitor aphidicolin. Three major mammalian polymerases involved in DNA replication, α , δ and ϵ , are very sensitive to aphidicolin. In contrast, Pol β is only slightly sensitive to aphidicolin (Wang, 1996); thus, in the

Fig. 3. Repair of dihydrouracil-containing substrate. (A) Schematic presentation of dihydrouracil-containing substrate. The sites of cleavage by the restriction enzymes *Hind*III and *Hpa*II and the position of the 32 P label are shown. The site of incision by AP endonuclease subsequent to glycosylase processing of dihydrouracil is indicated with an arrow. (B) Restriction digestion of untreated substrate DNA (lanes 1 and 2) and substrate incubated with 100 μ g of human WCE at 37°C for 2 h (lanes 3 and 4).

Fig. 4. Repair of 8-oxoguanine by wild-type mouse cell extract. (A) Time-dependent repair of 8-oxoguanine in the presence of dNTPs. Reaction mixtures (50 μ l) contained 50 ng of substrate DNA, 100 μ g of mouse WCE, 100 μ g/ml aphidicolin and 20 μ M dNTPs and were incubated at 37°C for the indicated time periods. The substrate DNA was subsequently purified and then treated with either *Hind*III (lanes 1–3) or *Hind*III and *Hae*III (lanes 4–6). (B) Repair is able to proceed to completion via single nucleotide insertion. The composition of reactions was altered such that dNTPs were replaced with 50 μ M dGTP, ddCTP, ddATP and ddTTP. The reactions were incubated at 37°C for the indicated time periods. The substrate DNA subsequently was purified and then treated with either *Hind*III (lane 1) or *Hind*III and *Hae*III (lane 2). Reaction products were analysed in a 10% denaturing polyacrylamide gel.

Fig. 5. Pol β -null mouse cell extracts are unable to complete single nucleotide patch repair. (A) Time-dependent repair of 8-oxoguanine in the presence of dNTPs. Reaction mixtures (50 μ l) contained 50 ng of substrate DNA, 100 μ g of Pol β -deficient mouse WCE and 20 μ M dNTPs and were incubated at 37°C for the indicated time periods. The substrate DNAs subsequently were purified and then treated with either *Hind*III (lanes 1–3) or *Hind*III and *Hae*III (lanes 4–6). (B) Repair of 8-oxoguanine by Pol β -deficient cell extracts is blocked by aphidicolin. Reaction mixtures (50 μ l) contained 20 ng of substrate DNA, 100 μ g of mouse *pol* $\beta^{-/-}$ WCE, 100 μ g/ml aphidicolin and 20 μ M dNTPs and were incubated at 37°C for the indicated time periods. The substrate DNA subsequently was purified and then treated with either *Hind*III (lanes 1–3) or *Hind*III and *Hae*III (lanes 4–6). (C) Single nucleotide repair synthesis. Reactions were carried out as in (B) with the exception of the dNTPs being replaced with dGTP/ddCTP/ddATP/ddTTP. The reactions were incubated at 37°C for the indicated time periods. The substrate DNA subsequently was purified and then treated with either *Hind*III (lane 1) or *Hind*III and *Hae*III (lane 2). Reaction products were analysed in a 10% denaturing polyacrylamide gel.

presence of aphidicolin, mainly Pol β -dependent repair will be monitored. Repair synthesis in mouse cell extracts was not affected by aphidicolin and, as revealed after *Hind*III cleavage of the DNA, very few repair intermediates were accumulated (Figure 4A, lanes 1–3). We next changed repair reaction conditions so that only Pol β -dependent short-patch repair was allowed [aphidicolin, dGTP and dideoxynucleotide triphosphates (ddNTPs)]. Under these conditions, any extension of the repair gap beyond one nucleotide will lead to incorporation of ddCMP (see Figure 1) and termination of both DNA synthesis and ligation, detectable by accumulation of incised, but unligated, products after *Hind*III hydrolysis. As in human cell extracts (Dianov *et al.*, 1998; Fortini *et al.*, 1999), repair in normal mouse cell extracts was accomplished mostly through insertion of a single nucleotide, as it was not blocked under these ‘short-patch repair’ conditions (Figure 4B).

Repair of 8-oxoguanine by Pol β -deficient mouse cell extracts

If Pol β is an essential enzyme for single nucleotide BER of oxidative DNA lesions, then disruption of this gene should lead to the disruption of this pathway and engagement of other means for DNA repair. In the absence of aphidicolin, Pol β -deficient extracts were able to carry out repair (Figure 5A), most probably through a long-patch repair mechanism supported by an aphidicolin-sensitive DNA polymerase (Fortini *et al.*, 2000). Indeed, in the presence of aphidicolin and normal dNTPs, WCE derived from Pol β -deficient mouse cells showed the time-dependent accumulation of repair intermediates (Figure 5B, lanes 1–3). Simultaneous digestion with *Hind*III and *Hae*III confirmed that under these conditions, very little of the substrate had undergone full repair since approximately the same amount of the 48mer fragment accumulated after *Hind*III cleavage alone (Figure 5B, 48mer at lanes 3 and 6). When reactions were carried out under conditions that completely block the long-patch pathway (aphidicolin, dGTP and the rest ddNTPs), repair was stalled within 15 min after incision and insertion of the first nucleotide (Figure 5C, lane 1) and, after 30 min of incubation in cell extract, most of the substrate had

undergone further degradation (Figure 5C, lane 2). These data demonstrate that, although there is some residual aphidicolin-resistant repair synthesis in the absence of Pol β , under these conditions Pol β -deficient WCEs are unable to support efficient BER via the characteristic single nucleotide insertion pathway.

Complementation of Pol β -deficient mouse cell extracts: essential role of Pol β dRP lyase activity

As we mentioned above, a combination of either APE1–Pol β or glycosylase–APE1 may be involved in processing of the AP site generated after removal of an oxidative base lesion (Figure 1). We hypothesized that if the removal of the AP site is initiated by the AP lyase activity of the glycosylase, then the inability of Pol β -deficient cell extracts to carry out short-patch repair could be complemented by addition of a mutant of Pol β , proficient in DNA synthesis but deficient in dRP lyase activity. Such a polymerase should be able efficiently to fill the gap generated by sequential action of AP lyase and APE1 (Figure 1). To test this hypothesis, we carried out repair reactions under conditions where only Pol β -dependent short-patch repair was allowed (dGTP and the rest ddNTPs, in the presence of aphidicolin) and complemented Pol β -deficient extracts with purified, recombinant Pol β or Pol β K72A, a dRP lyase-deficient mutant (Prasad *et al.*, 1998b). We analysed the efficiency of repair by monitoring the accumulation of repair intermediates after *Hind*III cleavage of substrate DNA repaired in a Pol β -deficient mouse cell extract. Under these conditions, as described in detail above (Figure 5C), we detected accumulation of a 48mer intermediate in Pol β -deficient cell extracts but the ligation step was blocked (Figure 6A, lane 1). When the extract was complemented with Pol β K72A, we observed addition of a second nucleotide to the 3' end of the incised AP site (49mer). However, because the second nucleotide was ddCMP, further repair was blocked at this stage (Figure 6A, lane 3). These data indicated that Pol β K72A was shifting repair to the long-patch pathway and was not able to stimulate repair via a short-patch mechanism. However, the addition of Pol β completely restored repair capability to Pol β -deficient extracts (Figure 6A, lane 2). Removal of 8-oxoguanine

Fig. 6. Complementation of Pol β -deficient cell extracts with Pol β and Pol β K72A mutant. Pol β -deficient mouse WCEs (100 μ g) were incubated with 50 ng of DNA substrate and 50 μ M dGTP/ddCTP/ddATP/ddTTP in the case of 8-oxoguanine-containing substrate (**A**) and 50 μ M dCTP/ddGTP/ddATP/ddTTP in the case of dihydrouracil-containing substrate (**B**) in the presence of aphidicolin (100 μ g/ml). Pol β and Pol β K72A (2 ng) were included in the reactions shown in lanes 2 and 3, respectively. The substrate DNA was purified and then treated with *Hind*III. To demonstrate complete restoration of repair after addition of Pol β , a sample identical to that in lane 2 was cleaved simultaneously with *Hind*III and *Hae*III (**A**, lane 4) or with *Hind*III and *Hpa*II (**B**, lane 4). Reaction products were analysed in a 10% denaturing polyacrylamide gel.

from substrate DNA in the reaction complemented with Pol β was confirmed by simultaneous *Hind*III–*Hae*III cleavage (Figure 6A, lane 4).

In a similar experiment with dihydrouracil-containing substrate, we also observed failure to carry out complete short-patch repair by Pol β -deficient cell extract. Again, repair activity could be restored by complementation with Pol β but not by dRP lyase-deficient Pol β K72A (Figure 6B). The requirement for the dRP lyase function of Pol β in the complementation reactions indicates that dRP is an intermediate product during repair of both 8-oxoguanine and dihydrouracil, substrates for the bifunctional glycosylases OGG1 and NTH1, respectively. We therefore conclude that processing of the AP sites generated after removal of these lesions is initiated by APE1, directing the repair process through pathway A (Figure 1).

Repair of 8-oxoguanine-containing substrate in a reconstituted system

The presence of a 5'-dRP incision intermediate and the essential role of the dRP lyase activity of Pol β were confirmed further by experiments with purified enzymes.

Fig. 7. Repair of 8-oxoguanine reconstituted with purified enzymes. mOGG1 (1 ng, 25 fmol), APE 1 (2 ng, 58 fmol), Pol β or Pol β K72A (0.5 ng, 12.5 fmol) and DNA ligase I (10 ng, 80 fmol) were mixed and incubated for 5 min on ice. The buffer containing magnesium, dNTPs and 50 ng of substrate DNA was then added and incubation was carried out for 20 min at 37°C. The substrate DNA was purified as described in Materials and methods, treated with *Hind*III and analysed by electrophoresis in a 10% denaturing polyacrylamide gel. The sample in lane 5 was digested with *Hae*III in addition to *Hind*III.

The reconstitution of repair of the 8-oxoguanine-containing substrate was performed with mOGG1, APE1, Pol β and DNA ligase I. Substrate DNA was incubated with the indicated enzymes and then treated with the restriction endonuclease *Hind*III to release a 59mer labelled fragment from the substrate DNA. Incubation of the substrate DNA with mOGG1 and APE1 generated a 47mer incision product (Figure 7, lane 2). Pol β added one nucleotide to the incised DNA (Figure 7, lane 3) and finally DNA ligase sealed the ends to restore the 59mer repaired product (Figure 7, lane 4). Removal of 8-oxoguanine in the complete reaction was confirmed by simultaneous *Hind*III–*Hae*III cleavage (Figure 7, lane 5). However, although Pol β K72A (dRP lyase mutant) in a similar reaction was able to add one nucleotide to the incised substrate DNA (Figure 7, lane 6), DNA ligase was not able to seal the ends (Figure 6, lane 7). These data demonstrate the essential role of the dRP lyase activity of Pol β in removal of the dRP residue prior to ligation, indicating the presence of the dRP residue at the 5' end of the incised substrate and thus confirming bypass of the AP lyase activity of OGG1. We therefore conclude that, in agreement with our experiments performed with cell extracts, the reconstituted repair of 8-oxoguanine proceeds through incision of the AP site by APE1 (Figure 1, pathway A).

Discussion

Pol β is a major repair DNA polymerase involved in both short- and long-patch repair of AP sites generated by monofunctional glycosylases (Wiebauer and Jiricny, 1990; Dianov *et al.*, 1992, 1999; Singhal *et al.*, 1995; Sobol *et al.*, 1996; Podlutzky *et al.*, 2001). The role of this enzyme in repair of G/T mismatches and single strand breaks has also been well documented (Wiebauer and Jiricny, 1990; Whitehouse *et al.*, 2001). However, there is

some controversy about the role of Pol β and its dRP lyase activity in the repair of oxidative lesions. In eukaryotes, the 8-oxoguanine-DNA glycosylase OGG1 and NTH1 proteins are central players in the repair of oxidative base lesions (Lindahl and Wood, 1999). In addition to their glycosylase activity, each has also been shown to catalyse a β -elimination (lyase) reaction, incising the phosphodiester backbone 3' to the abasic site (McCullough *et al.*, 1999). The resultant 3'-unsaturated aldehyde terminus can be removed by APE1 (Klungland, 1999), leaving a one-nucleotide gap with a 3'-hydroxyl and a 5'-phosphate, although the 3'-phosphodiesterase activity of APE1 is significantly (~100-fold) weaker than its AP endonuclease activity (Wilson and Barsky, 2001). Theoretically, repair could then proceed simply through insertion of a single nucleotide followed by ligation. This would then imply that there were two different pathways for single nucleotide patch repair, one dRP lyase-dependent pathway initiated by monofunctional glycosylases (Figure 1, pathway A) and another dRP lyase-independent pathway initiated by bifunctional glycosylases (Figure 1, pathway B). However, there has been a recent accumulation of evidence demonstrating the stimulation of hOGG1 (Hill *et al.*, 2001; Saitoh *et al.*, 2001; Vidal *et al.*, 2001) activity by AP endonuclease and it has been reported that this stimulation of glycosylase activity may occur at the expense of AP lyase activity (Vidal *et al.*, 2001). We therefore set out to address the question of whether Pol β /dRP lyase is involved in processing of the AP site arising after removal of either 8-oxoguanine or dihydrouracil, or whether the OGG1/NTH1 AP lyase activity entails a different mechanism for bifunctional glycosylases in which the dRP lyase activity of Pol β is redundant.

We constructed ^{32}P -labelled closed-circular plasmid substrates, containing either a single site-specific 8-oxoguanine or dihydrouracil residue, which subsequently were treated with mammalian WCEs in the presence of the relevant cofactors. Restriction analysis of the substrates allowed us to follow the progress of the repair reactions. Addition of aphidicolin and limitation of the pool of dNTPs available for strand resynthesis to the first nucleotide alone, with the remaining nucleotides present as dideoxyribonucleoside triphosphates, prevented completion of repair through any pathway other than Pol β -dependent single nucleotide patch repair. Our experiments showed that wild-type mouse WCE is capable of carrying out full BER of 8-oxoguanine and dihydrouracil under such conditions, with only a trace amount of product corresponding to insertion of more than one nucleotide detectable. We therefore concluded that under these conditions, the majority of oxidative base lesion repair in normal WCEs proceeds through the single nucleotide insertion mechanism and is supported by Pol β . This result is in good accordance with previously reported experiments where restriction analysis of repair incorporation was performed (Dianov *et al.*, 1998; Fortini *et al.*, 1999). In contrast, repair in extracts derived from Pol β knockout mouse cells was inhibited substantially in the presence of aphidicolin, even under conditions where a complete pool of dNTPs was provided (Figure 5). However, in Pol β -deficient cell extracts, we always observed some residual DNA polymerase activity even in the presence of aphidicolin, suggesting that in the absence of Pol β other

DNA polymerases may become involved in repair synthesis. The repair defect in Pol β -deficient cells is evident: they are highly sensitive to hydrogen peroxide (Fortini *et al.*, 2000) and alkylating agents (Sobol *et al.*, 1996) and have increased rates of chromosomal abnormalities (Ochs *et al.*, 1999). Although our data demonstrate that the short-patch BER pathway does not operate in Pol β -null cells, the viability of these cells indicates that some alternative DNA repair pathways (most probably long-patch BER; Fortini *et al.*, 2000; G.L.Dianov, unpublished data) must operate. Nevertheless, since the knockout mice are not viable (Gu *et al.*, 1994; Sugo *et al.*, 2000), it is likely that the efficiency or quality of this backup repair is insufficient to maintain genome integrity during development of a multicellular organism. However, as we demonstrate, in cell extracts this repair defect can be complemented by wild-type Pol β and these cells may therefore be used for complementation studies.

In order to establish which of either the AP lyase activity of OGG1/NTH1 or the dRP lyase activity of Pol β was responsible for supporting single nucleotide patch repair, we complemented Pol β -deficient extract with either Pol β or Pol β K72A mutant deficient in dRP lyase activity. Whereas Pol β was able to restore repair capability to the extract, the mutant enzyme, which is unable to catalyse β -elimination of a 5'-dRP residue (Prasad *et al.*, 1998b), did not. It therefore seems reasonable to conclude that dRP remains as a 5'-block, with the implication that, under our experimental conditions, BER of 8-oxoguanine and dihydrouracil proceeds via the same fundamental mechanism as repair initiated by monofunctional glycosylases (Figure 1, pathway A). This conclusion is also strongly supported by our data obtained in a reconstituted repair reaction.

What is the role of a glycosylase's AP lyase activity? It has been proposed that BER proceeds via a highly orchestrated mechanism in which the enzyme involved at one step directly communicates with the enzyme of the next (Lindahl and Wood, 1999; Mol *et al.*, 2000; Wilson and Kunkel, 2000). The possibility exists that the covalent interaction between bifunctional DNA glycosylase and the DNA substrate, which necessarily precedes lyase activity, is required for some other function such as ensuring that abasic sites are not left unprotected during BER.

It has also been hypothesized that the AP lyase pathway for repair of oxidative lesions may play a role in prevention of double-strand break formation during repair of clustered lesions induced by ionizing radiation (Klungland *et al.*, 1999). Since the mechanism for repair of clustered oxidative lesions may be distinct from that of repair of single base lesions (Dianov *et al.*, 2001), the AP lyase activity of OGG1 may play a role in repair of such lesions.

In conclusion, our data suggest that BER involved in repair of simple base lesions has one major pathway strictly depending on the dRP lyase activity of Pol β . As has been shown before, elimination of the dRP lyase activity of Pol β , or Pol β itself, leads to increased sensitivity to DNA-damaging agents (Sobol *et al.*, 2000), genetic instability (Ochs *et al.*, 1999) and premature termination of embryonic development (Gu *et al.*, 1994; Sugo *et al.*, 2000).

Materials and methods

Materials

Synthetic oligodeoxyribonucleotides purified by high-performance liquid chromatography were obtained from Midland (8-oxoguanine) or Synthesgen (5,6-dihydrouracil). [γ - 32 P]ATP (3000 Ci/mmol) was purchased from NEN Life Science Products.

Proteins

Recombinant human wild-type Pol β and K72A mutant deficient in dRP lyase activity were kindly provided by Drs S. Wilson and R. Prasad. Mouse 8-oxoguanine-DNA glycosylase (mOGG1) was a gift from Dr D. Zharkov, and human DNA ligase I was a gift from Dr A. Tomkinson. Histidine-tagged human APE1 was purified on Ni $^{2+}$ -charged His-Bind resin (Novagen, Cambridge, MA) as recommended by the manufacturer.

DNA substrates

The oligonucleotides 5'-ATATACCGCG[8-oxo]GCCGATCAAGCTT-ATT-3' (30 pmol) and 5'-ATATACCGCGGCGATCAAGCTTATT-3' (where U stands for dihydrouracil, 30 pmol) were 5'-end labelled with 100 μ Ci (33 pmol) of [γ - 32 P]ATP and used for construction of substrates containing single 8-oxoguanine or dihydrouracil in closed-circular double-stranded DNA as previously described (Dianov *et al.*, 1998).

BER reactions

The BER reactions were carried out in a reaction mixture (50 μ l) containing 50 mM HEPES-KOH pH 7.8, 50 mM KCl, 10 mM MgCl $_2$, 0.5 mM EDTA, 1.5 mM dithiothreitol (DTT), 2 mM ATP, 0.4 mg/ml bovine serum albumin (BSA), 25 mM phosphocreatine (di-Tris salt, Sigma), 2.5 μ g creatine phosphokinase (type I, Sigma), 8.5% glycerol (Fluka), 20 μ M each of the indicated dNTPs or ddNTPs, 50 ng (10 fmol) of 32 P-labelled single 8-oxoguanine-containing DNA substrate and 400 ng of carrier plasmid DNA (pUC18). When aphidicolin (Sigma) was added to a final concentration of 100 μ g/ml, the buffer also contained 1% dimethylsulfoxide (DMSO). Reactions were initiated by the addition of WCEs (100 μ g) and incubated for the indicated time at 37°C. The reactions were stopped by addition of 2 μ l of 0.5 M EDTA. Substrate DNA was purified from the reaction mixture by phenol-chloroform extraction and ethanol precipitation, and treated with 10–40 U of the indicated restriction endonuclease(s) for 2 h at 37°C in buffers supplied by the manufacturer. An equal volume of gel loading buffer was then added (95% formamide, 20 mM EDTA, 0.02% bromophenol blue and 0.02% xylene cyanol). Following incubation at 90°C for 2–5 min, the reaction products were separated by electrophoresis in a 10% polyacrylamide gel containing 7 M urea in 89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA pH 8.0.

Reconstituted BER

The BER was reconstituted with purified enzymes in a reaction mixture (20 μ l) that contained 45 mM HEPES pH 7.8, 70 mM KCl, 7.5 mM MgCl $_2$, 0.5 mM EDTA, 1 mM DTT, 2 mM ATP, 20 μ M each of dATP, dGTP, dCTP and dTTP, 0.5 mg/ml BSA and a 32 P-labelled single 8-oxoguanine-containing substrate DNA (~50 ng, 10 fmol). The reaction was initiated by adding BER proteins at the amount indicated in the figure legends. After incubation for the indicated time at 37°C, the reaction was stopped by addition of 20 μ l of phenol-chloroform. Following extraction, the aqueous phase was collected and passed through a G-25 spin column. The samples were evaporated and the pellets were dissolved in 10 μ l of a HindIII buffer supplied by the manufacturer. DNA was treated with 40 U of the indicated restriction endonuclease(s) for 1 h at 37°C. Reactions were stopped by addition of 10 μ l of gel loading buffer (95% formamide, 20 mM EDTA, 0.02% bromophenol blue and 0.02% xylene cyanol). Following incubation at 80°C for 2 min, the reaction products were separated by electrophoresis in a 10% denaturing polyacrylamide gel.

Cells and extracts

Normal human lymphoid cells AG09387 were obtained from the Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ). Cells were grown in medium recommended by the Repository. The normal mouse fibroblast and DNA Pol β knockout mouse fibroblast cell lines were obtained from Dr R. Sobol and were grown as described (Sobol *et al.*, 1996). WCEs were prepared by the method of Manley *et al.* (1980) and dialysed overnight against buffer containing 25 mM HEPES-KOH pH 7.9, 2 mM DTT, 12 mM MgCl $_2$, 0.1 mM EDTA, 17% glycerol and 0.1 M KCl. Extracts were aliquoted and stored at -80°C.

All experiments were repeated at least 3–5 times and representative gels are shown.

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