DNA Polymerase β Promotes Recruitment of DNA Ligase IIIα−XRCC1 to Sites of Base Excision Repair

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Received January 14, 2005; Revised Manuscript Received May 6, 2005

ABSTRACT: Base excision repair is a major pathway for the removal of simple lesions in DNA including base damage and base loss (abasic site). Base excision repair requires the coordinated action of several repair and ancillary proteins, the impairment of which can lead to genetic instability. Using a protein−DNA cross-linking assay during repair in human whole cell extracts, we monitored proteins involved in the initial steps of repair of a substrate containing a site-specific abasic site to address the molecular events following incision of the abasic site by AP endonuclease. We find that after dissociation of AP endonuclease from the incised abasic site, both DNA polymerase β (Pol β) and the DNA ligase IIIα−XRCC1 heterodimer efficiently bind/cross-link to the substrate DNA. We also find that the cross-linking efficacy of the DNA ligase IIIα−XRCC1 heterodimer was decreased about 2-fold in the Pol β-deficient cell extract but was rescued by addition of purified wild type but not a mutant Pol β protein that does not interact with the DNA ligase IIIα−XRCC1 heterodimer. We further demonstrate that Pol β and the DNA ligase IIIα−XRCC1 heterodimer are present at equimolar concentrations in whole cell extracts and that Pol β has a 7-fold higher affinity to the incised abasic site containing substrate than DNA ligase IIIα. Using gel filtration of whole cell extracts prepared at physiological salt conditions (0.15 M NaCl), we find no evidence for a stable preexisting complex of DNA Pol β with the DNA ligase IIIα−XRCC1 heterodimer. Taken together, these data suggest that following incision by AP endonuclease, DNA Pol β recognizes and binds to the incised abasic site and promotes recruitment of the DNA ligase IIIα−XRCC1 heterodimer through its interaction with XRCC1.

Spontaneously derived DNA lesions, such as apurinic/apyrimidinic sites (AP sites, abasic sites), and products of base deamination, oxidation, and alkylation are removed by the base excision repair (BER)[1] pathway (*1*). In human cells, following removal of the damaged base by a DNA glycosylase, the AP site generated is recognized by an AP endonuclease (APE1) that cleaves the phosphodiester backbone 5′ to the lesion, creating a 3′-OH and 5′-deoxyribose phosphate (dRP) terminus (*2*, *3*). Repair of the incised AP site mainly proceeds via so-called "short-patch" repair (*4*) whereby DNA polymerase β (Pol β) inserts the correct nucleotide to fill the gap and furthermore removes the 5′-dRP lesion through a β-elimination mechanism (*5*). The DNA ligase IIIα−XRCC1 (Lig IIIα−XRCC1; X-ray cross-complementing protein 1) heterodimer then seals the remaining nick in the DNA backbone (*6*−*8*).

Multiple known interactions between BER proteins (reviewed in ref *9*) instigated the proposal of a "passing the baton" mechanism of BER whereby each protein passes the repair intermediate to the next protein in the pathway in a coordinated manner (*10*, *11*). From this hypothesis, a damaged DNA base would be passed from a DNA glycosylase to APE1 to Pol β and finally to Lig IIIα−XRCC1 during the course of repair. This mechanism would prevent exposure of repair intermediates to the cellular environment and ensure that they are occupied by one repair protein at any point in the repair process. Subsequently, Pol β has been shown to interact with APE1 bound to DNA (*12*) and also with the Lig IIIα−XRCC1 heterodimer (*6*, *13*). Although well supported by structural studies of BER enzymes and their substrate and product complexes (*14*), this model requires direct biochemical evidence since the accepted order of recruitment of BER proteins during the repair reaction is complicated by the fact that many of the BER proteins have multiple interactions that are not accounted for by this model. For example, the Lig IIIα−XRCC1 heterodimer was proposed to be involved at the early steps of BER, since it was found to interact with APE1 and OGG1 (*15*, *16*).

Cross-linking is widely used in studies of protein−protein and protein−DNA interactions (*17*, *18*). Several pioneering studies proposed the use of UV or chemical cross-linking of proteins to their substrates during the course of repair. In these studies, immunoprecipitation followed by PCR (*19*) or Western blot analysis (*20*) was used to identify the proteins cross-linked during single- or double-strand break repair in yeast or human cell extracts. In this present study, we used formaldehyde cross-linking to examine the post-incision steps of BER and in particular the interrelations between Pol β and the Lig IIIα−XRCC1 heterodimer.

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[1] Abbreviations: BER, base excision repair; APE1, apurinic/apyrimidinic endonuclease 1; Pol β, DNA polymerase β; Lig IIIα, DNA ligase IIIα; XRCC1, X-ray cross-complementing protein 1; WCE, whole cell extract.

10.1021/bi050085m CCC: $30.25
Published on Web 07/15/2005

FIGURE 1: Structures of oligonucleotides used in the cross-linking and electrophoretic gel mobility shift (EMSA) assays. To construct 3′-biotinylated hairpin substrates for cross-linking, oligonucleotides were designed to contain the complementary sequence with a TTTT hairpin loop and a 3′-biotinylated moiety (designated *). Substrates **1** and **2** contain an AP site (Φ) and cytosine, respectively, which are base paired with guanine. For EMSA, a 39-mer uracil-containing oligonucleotide was 5′-labeled with [γ-^{32}P]ATP and an incised AP site containing duplex oligonucleotide was constructed (**3**).

EXPERIMENTAL PROCEDURES

Materials. Synthetic oligodeoxyribonucleotides were purchased from MWG-Biotech and gel purified on a 20% polyacrylamide gel. Oligonucleotide substrates used in this study are shown in Figure 1. [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from PerkinElmer Life Sciences. Streptavidin magnetic beads and a magnetic separation rack were purchased from New England Biolabs.

Proteins. Recombinant human uracil−DNA glycosylase (UDG) was purified as described (*21*). Histidine-tagged human Pol β, Pol β triple mutant, and DNA ligase IIIα were purified on Ni-NTA agarose (Qiagen) as recommended by the manufacturer. The vector for expression of the L301R/V303R/V306R human Pol β triple mutant was generated from the parent vector (pET28Polβ) using the QuikChange site-directed mutagenesis kit (Stratagene). The Pol β K72A mutant protein was kindly provided by Drs. S. Wilson and R. Prasad.

Antibodies. XRCC1 (ab144) and DNA ligase III (ab587) antibodies were purchased from Abcam Ltd. Antibodies against rat Pol β and human APE1 were raised in rabbit and affinity purified as described (*22*).

Cells. Pol β knockout mouse fibroblasts MB19tsA and isogenic wild-type cell line MB16tsA were obtained from Drs. S. Wilson and R. Sobol and were grown as described (*23*). HeLa cell pellets were purchased from Paragon. Whole cell extracts (WCE) were prepared by the method of Manley et al. (*24*) and dialyzed overnight against buffer containing 25 mM Hepes−KOH, pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol, and 2 mM DTT. Extracts were aliquoted and stored at −80 °C.

Cross-Linking Assay. Streptavidin magnetic beads were blocked in 5% milk and subsequently washed with binding buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 1 mM EDTA) using the magnetic separator rack. Beads were then incubated with hairpin substrates, containing a 3′-biotinylated moiety, at room temperature with agitation for 30 min in binding buffer, and the DNA beads were subsequently washed with wash buffer (25 mM Hepes, pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, and 2 mM DTT). The DNA beads (250 fmol of DNA per reaction) were then included in a reaction containing 100 μg of WCE in 50 μL of reaction buffer containing 50 mM Hepes−KOH, pH 7.8, 50 mM KCl, 10 mM EDTA, 1.5 mM DTT, 2.5% glycerol, 20 μM dCTP, 20 μM dATP, 20 μM dGTP, 20 μM dTTP, 2 mM ATP, 25 mM phosphocreatine (diTris salt; Sigma), 2.5 μg of creatine phosphokinase (type I; Sigma), 0.25 mM NAD^+, and 1 μg of carrier DNA (single-stranded 30-mer oligonucleotide). Pol β null cell extracts (100 μg) were also complemented with recombinant wild-type, Pol β K72A mutant, or L301R/V303R/V306R triple thumb mutant Pol β (200 fmol) prior to incubation with the substrate. Reactions were incubated for the time indicated at 30 °C and stopped by addition of formaldehyde (0.5%, final concentration). After incubation for 10 min at 30 °C the beads were washed twice with 50 μL of wash buffer and resuspended in 20 μL of SDS−PAGE sample buffer (25 mM Tris-HCl, pH 6.8, 2.5% mercaptoethanol, 1% SDS, 5% glycerol, 1 mM EDTA, and 0.15 mg/mL bromophenol blue). Cross-links were reversed by heating for at least 2 h at 65 °C and proteins separated on a 10% SDS−polyacrylamide gel followed by transfer to a PVDF membrane and immunoblot analysis with the indicated antibodies. For direct comparison, proteins cross-linked from different extracts or substrates were analyzed on the same immunoblot. To generate an AP site containing substrate (oligonucleotide **1**, Figure 1), the uracil hairpin oligonucleotide (250 fmol) was bound to streptavidin beads and incubated with purified UDG (2 pmol) for 30 min at 37 °C in 40 mM Hepes (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM DTT, followed by washes with wash buffer just prior to use.

Repair Assay. Oligonucleotides were 5′-end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, and unincorporated label was removed on a Sephadex G-25 spin column. Substrates were then bound to streptavidin beads and incubated (250 fmol of DNA per reaction) with 100 μg of HeLa cell extract in 50 μL of reaction buffer for the time indicated at 30 °C, prior to washing the beads twice with 50 μL of 10 mM Tris (pH 8.0) and 100 mM EDTA and resuspending in 20 μL of formamide loading dye. DNA was separated on a 20% denaturing polyacrylamide gel and the gel exposed to storage phosphor screens at 4 °C prior to analysis and quantification by phosphorimaging.

Electrophoretic Gel Mobility Shift Assay (EMSA). A uracil-containing oligonucleotide was 5′-end labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase and unincorporated label removed on a Sephadex G-25 spin column. The labeled oligonucleotide was incubated with a 2-fold molar excess of the complementary strand in 200 mM NaCl at 90 °C for 3−5 min prior to slow cooling to room temperature. To generate an incised AP site containing substrate (oligonucleotide **3**, Figure 1), the oligonucleotide (200 fmol) was incubated with purified UDG (2 pmol) and APE1 (1 pmol) for 30 min at 37 °C in 40 mM Hepes (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM DTT, followed by phenol−chloroform extraction and ethanol precipitation. Recombinant Pol β or Lig IIIα was incubated with 200 fmol of radiolabeled incised abasic site substrate in 20 μL of buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 20 μg/mL BSA, 7.5% glycerol, and 0.1% Triton X-100 for 15 min on ice. Five microliters of loading dye (30% glycerol, 0.25% xylene cyanol, 0.25% bromophenol blue) was added, and the samples were analyzed on a 12% nondenaturing polyacry-

lamide gel at 4 °C in 0.5× TBE. The gels were dried and exposed to storage phosphor screens prior to analysis by phosphorimaging and quantification using the Quantity One software (Bio-Rad). A Hill plot was used to calculate the binding affinities (K_d) of Pol β and Lig IIIα for the oligonucleotide substrate.

Gel Filtration Analysis. Whole cell extracts (10 mg) were loaded onto a Superose-12 HR 10/30 column (Amersham Pharmacia), and proteins were eluted with 50 mM Hepes, pH 7.5, 150 mM KCl, and 1 mM EDTA. Fractions (0.5 mL) were collected, and 10 μL of individual fractions was mixed with an equal amount of 2× SDS−PAGE sample buffer and heated for 5 min at 90 °C prior to separation of the proteins on a 10% SDS−polyacrylamide gel, followed by transfer to a PVDF membrane and immunoblot analysis with the indicated antibodies.

RESULTS

Outline of Cross-Linking Assay. To study the interactions and recruitment of Pol β and Lig IIIα−XRCC1 during BER, a new protocol was designed to reveal the engagement of BER proteins during repair of damaged DNA. This protocol employs oligonucleotides containing a 3′-biotinylated end that are used to form an AP site containing and a control duplex oligonucleotide complete with a hairpin loop to prevent nuclease digestion of the oligonucleotide (Figure 1; oligonucleotides **1** and **2**). The oligonucleotides are subsequently bound to streptavidin magnetic beads and incubated with whole cell extracts (WCE). After incubation for the times indicated, proteins involved in repair were cross-linked to the DNA and to each other, by the addition of formaldehyde. The beads were subsequently washed prior to reversal of the cross-links and analysis of the released proteins by gel electrophoresis and immunoblotting with the corresponding antibodies. The cross-linking efficiency of individual proteins during ongoing BER most probably would reflect a velocity of turnover of these enzymes on the AP site: the faster the reaction rate, the lower the cross-linking efficiency, and this will therefore mask the role of protein−protein interactions. We thus decided to reduce the reaction velocity, although attempts to do this by lowering the reaction temperature did not result in elevated cross-linking (data not shown).

To reduce the velocity of repair reactions, we chelated Mg^{2+} by supplementing the reaction mixture with 10 mM EDTA. It has been previously shown that the reaction of APE1 with AP sites is a very rapid process in the presence of magnesium but APE1 is still active in the presence of EDTA (*25*). Additionally, these reaction conditions will subsequently inhibit magnesium-dependent gap filling by Pol β and further inhibit ligation by Lig IIIα−XRCC1, thus reducing dissociation of these proteins from the substrate and allowing us to address the question of their recruitment during the initial stages of BER.

Dynamics of BER Protein Cross-Linking during AP Site Repair. Using HeLa WCE and an AP site containing hairpin substrate in the absence of magnesium, we were able to cross-link all of the BER proteins analyzed, including both components of the Lig IIIα−XRCC1 heterodimer, Pol β and APE1 (Figure 2A, left panel). Under these conditions, we were able to efficiently cross-link APE1 to the substrate DNA starting as early as 15 s after incubation with cell extract (Figure 2A, left panel). When 5′-labeled AP site containing

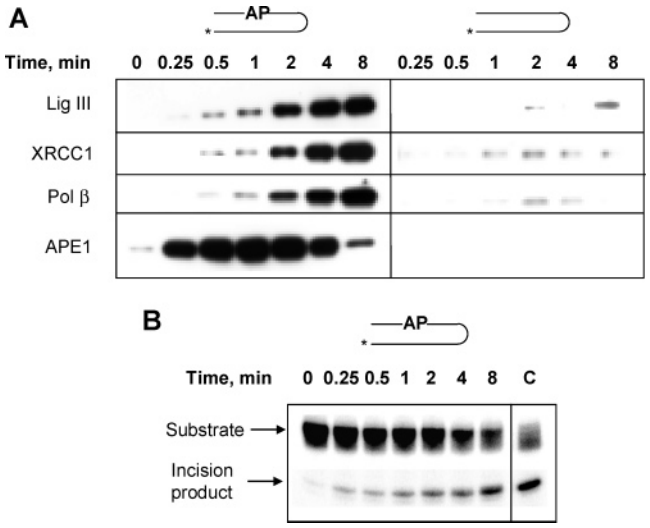

FIGURE 2: Dynamics of repair of an AP site-containing substrate by HeLa WCE. (A) An AP site containing (left panel) or the corresponding control (right panel) biotinylated hairpin substrate was bound to magnetic streptavidin beads and incubated with 100 μg of HeLa cell extract for the times indicated in the presence of 10 mM EDTA and cross-linked with 0.5% formaldehyde. The beads were subsequently washed, the cross-links were reversed, and proteins were separated by 10% SDS−PAGE, transferred to PVDF membranes, and analyzed by immunoblotting with the indicated antibodies. Time zero equates to cross-linking immediately after extract addition. (B) An AP site containing biotinylated hairpin substrate was 5′-end labeled with [γ-^{32}P]ATP, bound to magnetic streptavidin beads, and incubated with 100 μg of HeLa cell extract for the times indicated or with 3 pmol of recombinant APE1 for 20 min (lane C). DNA beads were subsequently purified and washed, and formamide loading dye was added. DNA was separated by 10% denaturing polyacrylamide gel electrophoresis, and the phosphorimage of the corresponding gel is shown.

oligonucleotide attached to the beads was incubated with WCE, as expected, we observed that in the absence of magnesium the incision rate was considerably reduced. AP site incision products can be observed as early as 15 s, and slow accumulation of incised products occurred during the incubation period, reaching approximately 60% of the control cleavage performed with excess purified APE1 at 8 min (Figure 2B, lane C). These data suggest that the majority of APE1 should dissociate from the substrate between 2 and 8 min of incubation at which the bulk of incision has been performed. Cross-linking of APE1 correlates well with the kinetics of incision activity of the oligonucleotide substrate. After 2 min of incubation, APE1 is gradually released from the substrate and is replaced by Pol β and the Lig IIIα−XRCC1 heterodimer that enter the incised AP site simultaneously (Figure 2A, left panel). In contrast, very little cross-linking of either Pol β, APE1, or either component of the Lig IIIα−XRCC1 heterodimer was observed using a control oligonucleotide hairpin substrate (Figure 2A, right panel). Taken together, these experiments demonstrate that Pol β and the Lig IIIα−XRCC1 heterodimer efficiently cross-link to the DNA after APE1 dissociates from the incised AP site.

Pol β Stimulates Binding of the Lig IIIα−XRCC1 Heterodimer to the Incised AP Site. Although Pol β and the Lig IIIα−XRCC1 heterodimer are cross-linked to the substrate DNA in a synchronized way (Figure 2A, left panel), from these experiments it is not clear whether they are cross-linked to the same DNA molecule and thus forming a complex or whether they are cross-linked to different DNA molecules

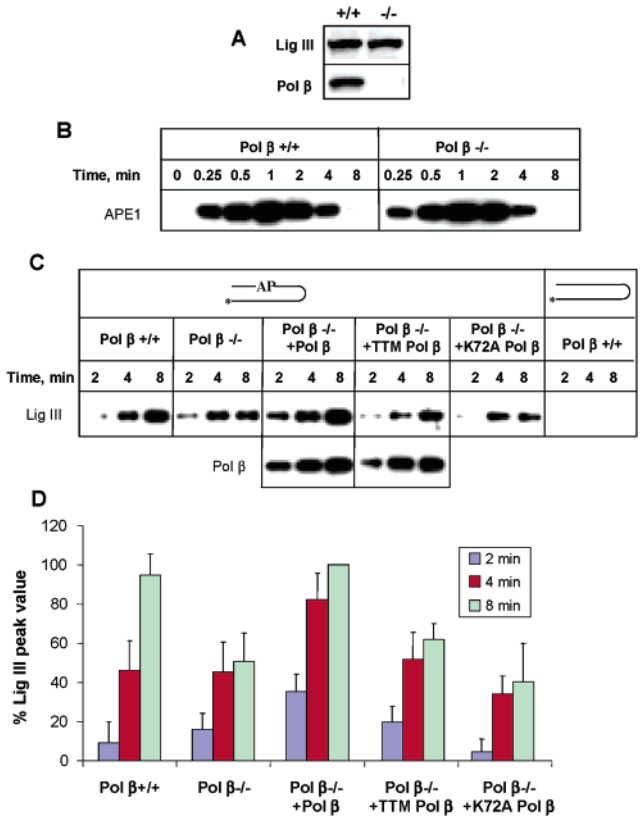

FIGURE 3: Dynamics of repair of an AP site containing substrate by Pol β null WCE in the absence of magnesium and complementation with human Pol β. (A) Pol β and Lig III levels in wild-type (+/+) and Pol β null (−/−) cell extracts were examined by subjecting 30 μg of WCE to electrophoresis on a 10% polyacrylamide gel, transferring the proteins onto a PVDF membrane, and immunoblotting with the indicated antibodies. (B) An AP site containing biotinylated hairpin substrate was bound to magnetic streptavidin beads and incubated with 100 μg of wild-type (left panel) or Pol β null (right panel) cell extract or (C) 100 μg of wild type (first panel), Pol β null (second panel), or Pol β null cell extract complemented with 200 fmol of recombinant wild type (third panel), Pol β triple thumb mutant (TTM, fourth panel), or Pol β K72A mutant (fifth panel) for the times indicated in the presence of 10 mM EDTA and cross-linked with 0.5% formaldehyde. A control biotinylated hairpin substrate was also incubated with 100 μg of WCE prior to cross-linking (sixth panel). The beads were subsequently washed, the cross-links were reversed, and proteins were separated by 10% SDS−PAGE, transferred to PVDF membranes, and analyzed by immunoblotting with the indicated antibodies. (D) Cross-linking results of Lig IIIα are shown graphically, plotted as the percentage of the Lig IIIα peak value obtained from at least three independent experiments.

and thus compete for binding to the incised AP site. To address this question, we performed cross-linking experiments using a Pol β null cell extract and investigated cross-linking of the Lig IIIα−XRCC1 heterodimer by probing for Lig IIIα. We hypothesize that if these proteins compete for the substrate, then removal of Pol β should give rise to increased cross-linking of the Lig IIIα−XRCC1 heterodimer. First of all, by Western blot analysis, we confirmed the absence of Pol β but the presence of equal amounts of the Lig IIIα−XRCC1 complex in the null cell extract compared to the corresponding wild-type extract (Figure 3A). In cross-linking assays, using the AP site containing oligonucleotide substrate, the gradual accumulation of Lig IIIα, hence the Lig IIIα−XRCC1 complex, can be observed using the wild-type extracts (Figure 3C, first panel) from 2 min onward, at a point when APE1 is dissociating from the incised AP site (Figure 3B). In comparison, although after 4 min nearly equal amounts of Lig IIIα were cross-linked in both wild-type and Pol β-deficient cell extracts, there is about a 2-fold reduced amount of Lig IIIα accumulated after 8 min of repair. This indicates, most probably, that after initial attempted binding the Lig IIIα−XRCC1 heterodimer dissociates from the incised AP site since it is not able to ligate it and processing of the AP site by Pol β is required for productive Lig IIIα−XRCC1 heterodimer binding. We next complemented the Pol β null WCE with recombinant human Pol β and found that added Pol β effectively cross-linked to the substrate and enhanced the amount of Lig IIIα cross-linking at all time points (Figure 3C, third panel). Most probably, removal of the 5′-sugar phosphate is necessary for efficient Lig IIIα−XRCC1 binding since using a Pol β mutant defective in AP lyase activity (*26*, K72A Pol β) was unable to stimulate Lig IIIα−XRCC1 binding (Figure 3C, fifth panel). We next addressed the question whether interaction between Pol β and the Lig IIIα−XRCC1 heterodimer is required for efficient recruitment of the Lig IIIα−XRCC1 heterodimer onto substrate DNA. We found that complementation of Pol β null WCE with a triple thumb mutant (TTM) of Pol β that does not interact with XRCC1 (*27*) but still retains equally active DNA polymerase and AP lyase activities (data not shown) and only slightly reduced DNA binding/cross-linking had no stimulatory effect on the cross-linking of the Lig IIIα−XRCC1 heterodimer to the incised AP site (Figure 3B, fourth panel). This indicates that the interaction between Pol β and XRCC1 is important for efficient recruitment of the Lig IIIα−XRCC1 heterodimer to the sites of repair. No substantial cross-linking of the Lig IIIα−XRCC1 heterodimer was observed with the control oligonucleotide substrate (Figure 3C, sixth panel). Shown graphically, these data further demonstrate that Pol β stimulates Lig IIIα−XRCC1 binding to the incised AP site and that the removal of the 5′-sugar phosphate and interaction of Pol β with XRCC1 are important components for the recruitment of the Lig IIIα−XRCC1 complex (Figure 3D).

Pol β Has a Higher Affinity for an Incised Abasic Site Than Lig IIIα. To provide further evidence for priority of Pol β over the Lig IIIα−XRCC1 heterodimer in binding of an incised AP site, we used the electrophoretic gel mobility shift assay (EMSA). We have been unable to demonstrate binding of purified full-length XRCC1 to damaged DNA (data not shown), in contrast to work published using the N-terminal domain of XRCC1 (*28*, *29*). However, Lig IIIα is believed to be a weak nick sensor, although it contains a zinc finger which has been shown to bind to secondary DNA structures (*30*, *31*). In agreement with these data, using the EMSA, we found that the binding affinities to an incised AP site of recombinant Pol β ($K_d = 57.6 \pm 5.6$ nM) and Lig IIIα ($K_d = 383.6 \pm 22.8$ nM) were approximately 7-fold different in favor of Pol β (representative gels are shown in Figure 4). However, using quantitative Western blot analysis (data not shown) we find in HeLa whole cell extracts about the same amount of Pol β and DNA ligase IIIα (3.5 and 2.5 pmol/mg of cell extract protein, respectively). This further supports the model where Pol β binds first to an incised abasic site after APE1 dissociation and is involved in the recruitment of the Lig IIIα−XRCC1 complex to facilitate repair.

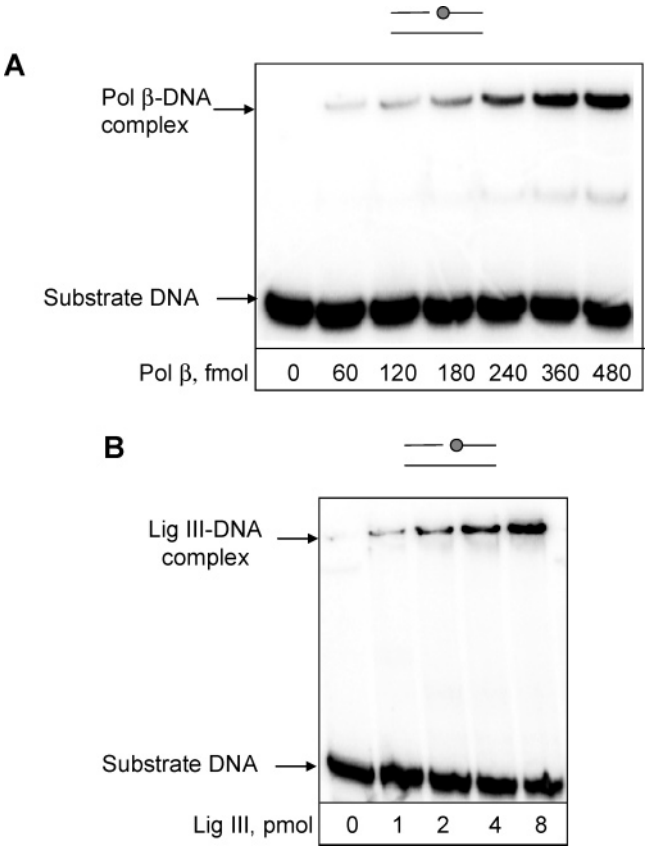

FIGURE 4: Binding affinity of purified Pol β and Lig IIIα for an incised abasic site containing oligonucleotide determined by the electrophoretic gel mobility shift assay (EMSA). (A) Purified Pol β or (B) Lig IIIα was incubated with an incised abasic site containing oligonucleotide for 15 min on ice prior to separation by 12% nondenaturing gel electrophoresis at 4 °C. The gel was dried and exposed to storage phosphor screen at 4 °C prior to analysis by phosphorimaging.

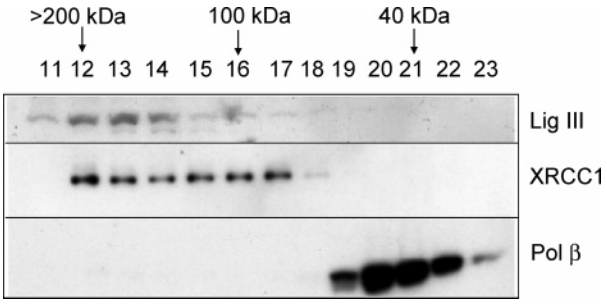

FIGURE 5: Fractionation of whole cell extracts by gel filtration. Whole cell extracts were fractionated on a Superose-12 column, and 10 μL of collected fractions was analyzed by immunoblotting with the indicated antibodies.

Gel Filtration of Whole Cell Extracts Reveals No Stable Complexes between Pol β and the XRCC1−DNA Ligase III Heterodimer. Although we demonstrated the dependence of the Lig IIIα−XRCC1 heterodimer binding on Pol β, it is not clear whether the Pol β−DNA ligase IIIα−XRCC1 complex is recruited to the damaged DNA as a preformed complex or is assembled on the damaged DNA. To address this question, whole cell extracts generated at a physiological salt concentration (0.15M NaCl) were subjected to gel filtration on a calibrated Sepharose-12 column. BER proteins in collected fractions were identified by gel electrophoresis followed by Western blotting with the corresponding antibodies. As expected, XRCC1 (69 kDa) and DNA ligase IIIα (103 kDa), that form a stable heterodimer (*7*), are coeluted as a high molecular weight product (Figure 5, fractions 12−14). The majority of DNA ligase IIIα is in complex with XRCC1, although some of the XRCC1 migrated as a monomer (Figure 5, fractions 15−17). However, we did not find any Pol β (39 kDa) stably complexed with the DNA ligase IIIα−XRCC1 heterodimer, as all of the protein migrated into fractions 19−22, where proteins with a molecular mass of about 40 kDa are eluted (Figure 5).

DISCUSSION

Although the major players in BER of AP sites are well established and biochemically characterized (reviewed in ref *1*), there is no common view on the molecular mechanism involved. On the basis of the known interactions between BER proteins, several modifications of the BER pathway have been suggested that engage different sequential involvement of BER proteins or protein complexes in the repair process (*11*, *12*, *15*, *32*). Following cleavage of an AP site by APE1, Pol β then incorporates the correct nucleotide and simultaneously removes the 5′-dRP residue to generate a nick that is ligated by the Lig IIIα−XRCC1 complex. Therefore, according to the passing the baton model (*10*, *11*), Pol β binding to an incised AP site should precede binding by the Lig IIIα−XRCC1 heterodimer. However, other reports have suggested that XRCC1 serves as a docking platform for BER proteins and that it may bind to damaged DNA during the initial stages of BER and recruit other proteins required (*7*, *15*, *32*). However, none of these models have been verified experimentally. We have recently demonstrated that interaction between Pol β and the Lig IIIα−XRCC1 heterodimer is required for efficient base excision repair (*33*). In this study we further address the role of this interaction at the early stages of AP site processing.

To visualize the early stages of AP site repair, we chose to perform the repair reactions in the absence of magnesium. The major reason for this was our finding (data not shown) that during repair under physiological conditions (in the presence of magnesium), the efficiency of protein cross-linking negatively correlates with the previously reported (*34*) velocity of the catalyzed reaction: the faster the reaction rate, the lower the enzyme−substrate complex lifetime and, correspondingly, the lower the cross-linking efficiency. Addition of EDTA to the reaction mixture allowed us to significantly reduce reaction rates and cross-link robust amounts of proteins. APE1 was cross-linked at the very early stages of repair; however, other BER proteins (Pol β and the Lig IIIα−XRCC1 heterodimer) cross-linked to the damaged DNA only when APE1 dissociated from the incised AP site. Most probably no complexes, including other BER proteins, are formed on DNA containing an AP site before incision.

Subsequently, using Pol β null cell extracts we found about 2-fold less Lig IIIα−XRCC1 cross-linked to the incised AP site, indicating that dissociation of APE1 alone is not sufficient for efficient binding of Lig IIIα−XRCC1 to the incised AP site. However, the Lig IIIα−XRCC1 heterodimer was still able to form an "unproductive" complex with the incised AP site containing an unprocessed 5′-sugar phosphate preventing ligation. The formation of such complexes was expected since the Lig IIIα−XRCC1 heterodimer is involved

in the repair of DNA single-strand breaks but it probably is not able to discriminate between ligatable and unligatable strand breaks and in the absence of competitor (Pol β) it would bind to a certain degree to unligatable incised AP sites. Therefore, binding of Pol β may play two important functions: to process the incised AP site and to signal to the Lig IIIα−XRCC1 heterodimer that the substrate is ready for ligation. We found that wild-type Pol β protein, but not a triple thumb mutant Pol β that does not interact with XRCC1, was able to rescue Lig IIIα−XRCC1 heterodimer binding. Although we found that the triple thumb mutant Pol β had about a 2-fold reduced binding to the DNA substrate compared to the wild-type Pol β (K_d = 110 and 58 nM, respectively), we used at least 5-fold more Pol β in the complementation experiments than the amount of endogenous Pol β in 100 μg of cell extract to overcome the observed reduced affinity of the mutant. Since at this condition both polymerases are equally able to efficiently process the incised AP site, the inability of the mutant Pol β to rescue Lig III cross-linking suggests that the interaction between Pol β and the Lig IIIα−XRCC1 heterodimer facilitates the recruitment of Lig IIIα−XRCC1. Furthermore, by EMSA we have demonstrated that Pol β has a higher affinity to an incised abasic site than Lig IIIα although they are present at equimolar concentrations in the cell extract. This further supports the model that once APE1 has dissociated, Pol β binds before the Lig IIIα−XRCC1 heterodimer. The simultaneous cross-linking of Pol β and the Lig IIIα−XRCC1 heterodimer also suggests that, at this stage of BER, the passing the baton mechanism operates, through formation of a transient complex between Pol β and the Lig IIIα−XRCC1 heterodimer rather than through displacement of one enzyme by another.

We have previously shown that the XRCC1−Pol β interaction is required for efficient BER by demonstrating that disruption of this interaction, by a point mutation in XRCC1, affects the ligation efficiency of the mutant Lig IIIα−XRCC1 heterodimer and that EM-C11 cells transfected with the mutant XRCC1 gene have an increased sensitivity to hydrogen peroxide (*33*). We now provide further evidence that the interaction of Pol β and XRCC1 is required for efficient recruitment of the Lig IIIα−XRCC1 complex to the sites of repair. It should be mentioned, however, that this order of reactions applies to BER and that a different scenario may be engaged in the repair of DNA single-strand breaks.

The question of whether the Pol β−DNA ligase IIIα−XRCC1 complex is recruited to the damaged DNA as a preformed complex or is assembled on the damaged DNA was addressed by gel filtration chromatography. Although coprecipitation of two proteins by affinity resin, co-immunoprecipitation or a positive response in the two-hybrid system are indications that two proteins are interacting with each other at some point during the repair process, such experiments do not provide direct evidence that these proteins are forming a stable preexisting complex. A stable multiprotein complex should be able to survive purification at physiological conditions, at least during the gel filtration procedure. However, we were not able to detect the stable Pol β−DNA ligase IIIα−XRCC1 complex during gel filtration, indicating that most probably these proteins form only transient complexes on damaged DNA.

ACKNOWLEDGMENT

We thank Drs. S. Wilson, R. Sobol, and R. Prasad for providing Pol β null cells and Pol β K72A protein.

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BI050085M