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A Novel Role of XRCC1 in the Functions of a DNA Polymerase β Variant[†]

Nandan Bhattacharyya and Sipra Banerjee*

Department of Cancer Biology, NB40, Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195

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ABSTRACT: In the base excision repair pathway, wild-type DNA polymerase β (WT polβ) provides most of the gap filling synthesis. A truncated polβ protein (pol$\beta\Delta$), expressed in primary colorectal and breast tumors and in a primary culture of renal cell carcinoma, inhibits the gap filling synthesis and DNA binding activities of WT polβ. However, a purified recombinant pol$\beta\Delta$ does not inhibit a purified WT polβ. To determine the dominant inhibitory activity of pol$\beta\Delta$, we examined interactions of purified pol$\beta\Delta$ with X-ray cross complementing group 1 (XRCC1), poly(ADP-ribose) polymerase (PARP), and apurinic endonuclease (Ape) proteins. All of these proteins interact with pol$\beta\Delta$ in vitro and in vivo. The pol$\beta\Delta$ protein can fill one nucleotide gap by inserting a base at the AP site, whereas a presumed binary complex of pol$\beta\Delta$ and XRCC1 cannot. However, this binary complex not only suppresses gap filling synthesis activity of WT polβ but also binds more strongly to gapped DNA than WT polβ bound to XRCC1. These results are the first to suggest that XRCC1 is directly involved in the dominant negative activity of truncated polβ, possibly leading to the genomic instability characteristic of tumor cells.

DNA polymerase β (polβ)[1] is a major contributor to single-nucleotide gap filling synthesis of DNA involved in the eukaryotic short patch base excision repair (BER) pathway (*1*−*4*). In the long patch BER pathway, polβ is also competent in filling a gap of 2−13 nucleotides. Proteins other than polβ involved in BER processes are DNA glycosylases, Ape, XRCC1, proliferating cell nuclear antigen, DNA polymerases δ and ϵ, DNA ligases I and III, and flap endonuclease 1 (*1*−*4*). DNA polβ is essential for the embryonic viability of mice (*5*). It is interesting to note that during apoptosis, cell lines replace polα with polβ for DNA synthesis (*6*). An embryonic fibroblast cell line, homozygous for a polβ deletion mutation, is reported to be defective in uracil-mediated BER (*7*). Additional reports provide evidence that polβ has a contributing role in mammalian DNA replication, recombination, and meiosis and in conferring a drug-resistant phenotype (*8*−*10*). Furthermore, Reichenberger and Pfeiffer (*11*) have reported that polβ has a role in nonhomologous DNA-end joining in *Xenopus laevis*. DNA polβ is a single-copy gene that encodes a 39 kDa protein consisting of 335 amino acids with two distinct functional domains of 8 and 31 kDa (*4*). The catalytic property of polβ resides in the carboxyl-terminal region of the 31 kDa domain (~260 amino acid residues), whereas the amino-terminal 8 kDa domain (75 residues) shows a strong affinity for the ssDNA template and deoxyribose phosphate lyase activity (*12*, *13*). The catalytic domain is subdivided into finger (6 kDa), palm (10 kDa), and thumb (12 kDa) regions (*14*). The finger domain presumably is the dsDNA binding site. An important function of polβ, nucleotidyl transferase activity, has been mapped to the palm region of polβ, whereas the dNTP selection function resides in the thumb region (*4*). We provided the first evidence for the possible involvement of polβ in human colorectal, breast, prostate, and lung cancers (*15*−*18*). An 87 bp deletion in the coding sequence of polβ occurs in colorectal, breast, and lung tumors, but not in corresponding normal tissues. This deletion encodes amino acid residues 208−236 in the palm region of the catalytic domain of the polβ enzyme. Both WT and pol$\beta\Delta$ proteins are expressed in colorectal and breast tumors and primary culture of renal cell carcinomas (*18*−*20*). The levels of the primary functions of the polβ enzyme, BER and DNA binding activities, are markedly reduced in the 16.3ΔP cell line compared with the parent cell, 16.3 (*19*). The mouse embryonic fibroblast cell line, 16.3, expressing wild-type polβ was derived from a control mouse in a polβ knockout experiment (*7*). The 16.3ΔP cell line was established by stable transfection of 16.3 cells with pol$\beta_{\Delta 208-236}$ cDNA (*19*). Furthermore, the 16.3ΔP cells that overexpress the pol$\beta\Delta$ protein are hypersensitive to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) compared to the parent 16.3 cells in vivo. More importantly, the pol$\beta\Delta$ protein interferes with the functions of the WT enzyme, suggesting that the pol$\beta\Delta$ acts as a dominant negative mutant (*19*). These results led us to elucidate the functions of pol$\beta\Delta$ and possible mechanisms underlying its inhibitory effect on WT polβ.

Several mechanisms for the effects of pol$\beta\Delta$ are possible. The truncated protein may interact with the WT protein to form a heterodimer, which may block the functions of the WT enzyme, as shown in model I of Figure 1. The second mechanism would involve binding of the dominant negative polβ protein to a damaged DNA template, thus preventing the binding of the WT protein to the damaged template

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* To whom correspondence should be addressed. Phone: (216) 444-0631. E-mail: banerjs@ccf.org. Fax: (216) 445-6269.

[1] Abbreviations: polβ, DNA polymerase β; pol$\beta\Delta$, truncated DNA polymerase β; XRCC1, X-ray cross complementing group 1; PARP, poly(ADP-ribose)polymerase; Ape, apurinic endonuclease; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; BER, base excision repair; UDG, uracil DNA glycosylase.

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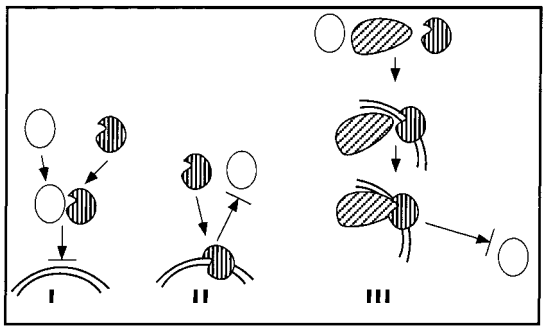


FIGURE 1: Models of potential mechanisms of the dominant negative function of the truncated polβ enzyme: (parallel arcs) DNA, (white oval) WTpolβ, (vertically striped notched oval) pol$\beta\Delta$, and (diagonally striped egg) XRCC1. (I) Heterodimer formation. (II) Competition for the DNA binding site at the damaged template. (III) Inhibition by the pol$\beta\Delta$ and XRCC1 complex.

(model II). In this case, the binding activity of the dominant negative protein is expected to be higher than that of the WT polβ protein. The pol$\beta\Delta$ protein in 16.3ΔP cells has no binding activity with respect to dsDNA, as determined by a gel mobility shift assay and dsDNA cellulose chromatography (*19*). Results presented in this report suggest that accessory proteins are involved in the inhibitory activity of the pol$\beta\Delta$ protein. Several reports have demonstrated that WT polβ interacts with human proteins such as XRCC1, PARP, and Ape (*21*−*23*). However, it is possible that such interactions may play a role in the functions of the polβ enzyme. If pol$\beta\Delta$ binds to the associated proteins, it may interfere with the binding of WT polβ to form a protein−protein complex and consequently prevent the gap filling synthesis catalyzed by WT polβ, proposed in model III. To understand the mechanisms of the dominant negative activity of the pol$\beta\Delta$, we have examined possible interactions of pol$\beta\Delta$ with XRCC1, PARP, and Ape. Using a reconstituted BER system consisting of purified recombinant WT and pol$\beta\Delta$ proteins, the results reveal that a polypeptide complex of pol$\beta\Delta$ and XRCC1 inhibits the functions of the WT polβ enzyme.

EXPERIMENTAL PROCEDURES

Cell Line. A mouse embryonic fibroblast cell line, 16.3ΔP, was established in our laboratory (*19*). The stable 19.4ΔP and 19.4WT cell lines were made by transfecting 19.4 cells with pol$\beta_{\Delta 208-236}$ and 1004 bp cDNA, respectively. The mouse embryo fibroblast 19.4 cell line was established from a polβ knockout mouse (*7*). The 19.4 cells are polβ deficient. The positive clones were selected against Geneticin (700 μg/mL) for 6 weeks. They were tested for polβ cDNA sequence by nucleotide sequencing and for expression by RT-PCR and Western blot analyses (*19*).

Co-Immunoprecipitation and Western Blot Analysis. For the co-immunoprecipitation assay, four cell lines were used: 16.3 (expressing WT polβ), 16.3ΔP (expressesing WT polβ and overexpressing pol$\beta\Delta$), 19.4 (lacking polβ expression), and 19.4ΔP (overexpressing pol$\beta\Delta$). Cell lysates were prepared according to the protocol described previously (*19*). Five hundred micrograms of the whole cell extract was incubated with 20 μL of anti-polβ antibody for 1 h. Protein A/G (50 μL) and agarose beads (Pharmacia, Piscataway, NJ) were added to the suspension, and the mixture was incubated at 4 °C overnight with gentle mixing. Agarose beads were pelleted at 13000*g* for 1 min at 4 °C. The pellet was washed 10 times with 10 mL of lysis buffer. The agarose beads were added with 70 μL of Laemmli buffer containing 10% β-mercaptoethanol. Then it was boiled for 5 min and centrifuged briefly. The supernatant containing the soluble protein was collected and separated by SDS−PAGE. The proteins were then transferred to membrane and immunodetected by Western blotting. Each blot was stripped and reprobed with polβ antibody.

Purification of Recombinant DNA Repair Proteins. Recombinant human polβ, pol$\beta\Delta$, Ape, and XRCC1 proteins were purified from pET15b polβ, pET15bpol$\beta\Delta$ (constructed in our laboratory), pQE30Ape, and pET16bXH9 expression vectors, respectively. The proteins were purified according to the procedure described by Gao et al. (*24*) with some modification. The bacterial BL21 (DE3) cells containing WT polβ, pol$\beta_{\Delta 208-236}$, Ape, or XRCC1 cDNA were grown at 37 °C until OD at 595 nm reached 0.2. The proteins were induced for 4 h after 1 mM IPTG induction. The pelleted bacterial cells were resuspended in lysis buffer containing 10 mM Tris-HCl (pH 8), 1 mM EDTA, 400 mM NaCl, 2 mM β-ME, 1 mM PMSF, and 1 mg/mL lysozyme. The solution was incubated at 4 °C for 30 min with intermittent vortexing. $MgCl_2$ and DNase I were added at final concentrations of 0.01 M and 30 units/mL, respectively. Triton X-100 was added at a final concentration of 1%. The solution was incubated at 4 °C for 15 min and was centrifuged at 10000*g* for 10 min at 4 °C. The supernatants were saved. The XRCC1, polβ, pol$\beta\Delta$, and Ape proteins were precipitated from these supernatants by selective precipitation with ammonium sulfate. The pellets were dissolved in a buffer containing 20 mM Tris-HCl (pH 8), 1 M NaCl, and 10 mM imidazole (pH 8). Proteins were then purified using Ni−NTA−agarose beads (Qiagen) following the manufacturer's instruction and identified by Western blot analyses using purified anti-monoclonal polβ, anti-monoclonal XRCC1 (Lab Vision Corp.), anti-monoclonal PARP (BIOMOL Research Laboratories, Inc., Plymouth, PA), and anti-polyclonal Ape (Lab Vision Corp.) antibodies.

Affinity Precipitation. An affinity precipitation assay was carried out following the protocol described by Kubota et al. (*22*). PARP and Ape proteins without the histidine tag were from BIOMOL Research Laboratories, Inc. The XRCC1 protein was translated in vitro using an XRCC1 construct without a histidine tag and the in vitro translation system (Promega Corp., Madison, WI). In these experiments, 4 μg each of WT polβ and pol$\beta\Delta$ proteins with the histidine tag were incubated with 1 μg each of Ape, PARP, and XRCC1 proteins and 0.5 μg of BSA separately for 1 h at room temperature. Twenty-five microliters of Ni−NTA−agarose beads (Qiagen) in 1 mM imidazole (pH 8) was added to the mixtures, and the mixtures were constantly shaken for an additional 1 h. The nonspecifically bound proteins were removed by washing with excess wash buffer containing 25 mM imidazole. The bound proteins were eluted with elution buffer containing 250 mM imidazole. Eluted proteins were separated by SDS−PAGE and stained with silver stain.

Oligonucleotide Substrate for BER. A 51 bp oligonucleotide with a U residue at position 22 was labeled at the 5′-end with T_4 polynucleotide kinase (Roche Molecular Biochemicals, Indianapolis, IN) and [γ-^{32}P]ATP (Amersham,

Piscataway, NJ). This 5′-end-labeled oligonucleotide was annealed to a complementary strand with the G residue opposite the U residue. Annealed, double-stranded oligonucleotides were separated with a 15% native PAGE gel, and the band corresponding to 51 bp was purified (*19*). After treatment with uracil glycosylase and Ape, AP sites were generated in an aliquot of oligonucleotide (*22*).

Gel Mobility Shift Assay. A 51-mer gapped DNA was used as a substrate for the gel mobility shift assay. The sequence of this substrate is

P1 (51 bp)

5′-TCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGC-3′

3′-AGCTCGAGCCATGGGCCCCTAGGAGATCT AGCTGGACGTCCGTACGTTCG-5′

P2 (29 bp) **P3 (21 bp)**

P1−P3 are 51, 29, and 21 bp oligos, respectively. The P1 primer was end-labeled at the 5′ position as described in an earlier section and annealed to P2, and Pβ by heating at 95 °C for 5 min and then slowly cooling to room temperature to form a 51 bp gapped DNA. Gel mobility shift assays were performed as described previously (*25*). In the DNA binding experiments, 0.05 pmol of duplex gapped oligonucleotide substrate was incubated with polβ/pol$\beta\Delta$ substrate at 0 °C for 15 min in gel mobility shift buffer [10 mM Tris (pH 8.0), 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1% CHAPS, 10 mM β-mercaptoethanol, and 7.5% glycerol] containing 400 ng of BSA. The final reaction volumes were 10 μL. The protein−DNA complexes were separated with a 5 to 8% nondenaturing polyacrylamide gel, dried, visualized, and quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) system. For competition experiments, different concentrations of cold oligonucleotides were preincubated with either the polβ or pol$\beta\Delta$ protein at 4 °C before the ^{32}P-labeled probe was added.

Reconstitution of the Gap Filling Synthesis Assay. Initially, nicked AP sites were generated using UDG and Ape in a reaction mixture containing 40 mM HEPES-KOH (pH 7.8), 70 mM KCl, 7 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 2 mM ATP, 20 μM dATP, 20 μM dTTP, 20 μM dGTP, 20 μM dCTP (Roche), and 500 μg/mL DNase-free BSA (Sigma), as described by Kubota et al. (*22*). Polβ or pol$\beta\Delta$ was added to the reaction mixture and incubated for 10 min followed by the addition of ligase I. The incubation was continued for a further 5 min. Where indicated, polβ or pol$\beta\Delta$ and XRCC1 were preincubated at 37 °C for 10 min and were added to the reaction mixture containing AP substrate followed by the addition of pol$\beta\Delta$ or polβ.

RESULTS

Purification of the Pol$\beta\Delta$, Ape, and XRCC1 Proteins. To investigate interactions between pol$\beta\Delta$ and accessory proteins, we purified the recombinant wTpolβ, pol$\beta\Delta$, Ape, and XRCC1 proteins. Coomassie blue-stained WT polβ (2 μg), pol$\beta\Delta$ (5 μg), Ape (5 μg), and XRCC1 (1 μg) proteins are shown in lanes 1−4 of Figure 2A. Panel B shows Western blot analysis of these purified proteins. The WT and pol$\beta\Delta$ proteins are 39 and 36 kDa, respectively. The Ape and XRCC1 proteins are 34 and 70 kDa, respectively.

DNA Binding Activity of the Pol$\beta\Delta$ Protein. DNA binding activity of a purified, recombinant WT polβ protein steadily increased with the increase in protein concentration from 0.1 to 2.5 nM (lanes 2−7, Figure 3A). Similarly, the pol$\beta\Delta$ forms a protein−DNA complex with the gapped substrate, and the level of pol$\beta\Delta$−DNA complex was enhanced in the presence of increasing protein concentration, from 0.1 to 2.5 nM (lanes 8−12). The concentrations of the pol$\beta\Delta$−DNA complexes were measured, and the K_d values of WT polβ and pol$\beta\Delta$ were determined (Figure 3C). Using a Scatchard plot shown in Figure 3C, the K_d of polβ is 0.46 nM when the K_d of WT polβ is 0.61 nM under the conditions used in this experiment. To evaluate whether pol$\beta\Delta$ inhibits functions of the WT enzyme, we titrated DNA binding activities of both proteins under unsaturated conditions of WT polβ (0.1 nM), and the signal of protein−DNA complex was measured in the presence of increasing concentrations of the pol$\beta\Delta$ protein, from 0.1 to 2.5 nM. Both WT polβ and pol$\beta\Delta$ bind to the gapped DNA to form complexes with similar mobility. For this reason, the titration experiment with pol$\beta\Delta$ was performed in the presence of an unsaturated concentration of WT polβ (0.1 nM). The polβ−pol$\beta\Delta$−DNA binding complexes were quantified with the ImageQuant program. As shown in Figure 3B, lanes 13−16 indicate that an amount of the protein−DNA complex was enhanced in an additive fashion due to the increased pol$\beta\Delta$ concentration. Thus, it appears that the intensified signals of DNA−protein complexes arise from the pol$\beta\Delta$ protein and not from the WT polβ protein. These data indicate that both forms of polβ bind to the gapped template. In addition to the experiment described above, DNA binding activities of the WT polβ enzyme were determined in the presence of a preincubated (5, 10, and 15 min) complex of pol$\beta\Delta$ and DNA. Results showed that the complex did not suppress the binding ability of the WT protein (data not shown). Therefore, these results suggest that pol$\beta\Delta$ by itself does not interfere with the DNA binding activity of the WT protein.

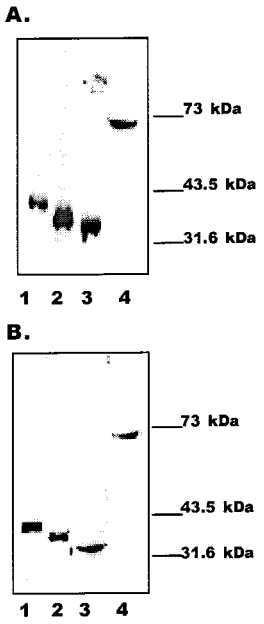
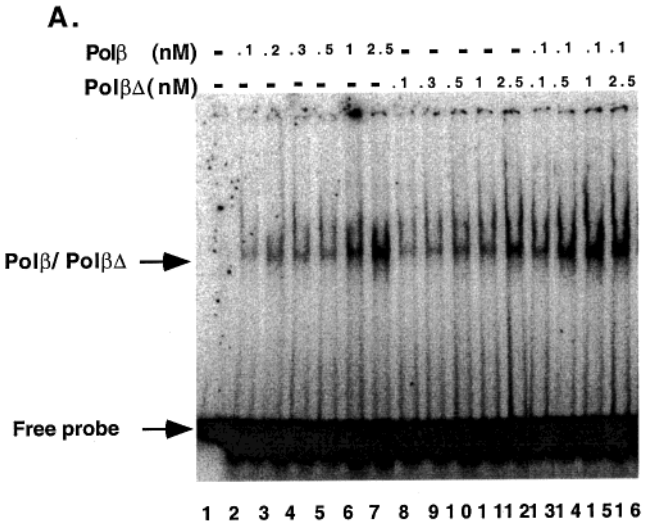


FIGURE 2: Purification of the recombinant WTpolβ, pol$\beta\Delta$, Ape, and XRCC1 proteins. (A) Lanes 1−4 show Coomassie blue-stained gels of purified recombinant WT polβ, pol$\beta\Delta$, Ape, and XRCC1 proteins, respectively. (B) Lanes 1−4 show the Western blot analysis of the same fractions using the respective antibodies.

Effects of the Purified Pol$\beta\Delta$ Protein on BER Activity of 16.3 Nuclear Extracts. We reported earlier that on adding increasing amounts of 16.3ΔP proteins to nuclear extracts



B

Polβ (nM)	Polβ∆ (nM)	Lane number	Arbitrary Unit
0.1	-	2	2.89
-	0.1	8	2.95
0.1	0.1	13	5.32
0.1	-	2	2.89
-	0.5	10	3.47
0.1	0.5	14	6.65
0.1	-	2	2.89
-	1	11	3.9
0.1	1	15	6.9
0.1	-	2	2.89
-	2.5	12	6.3
0.1	2.5	16	9.27

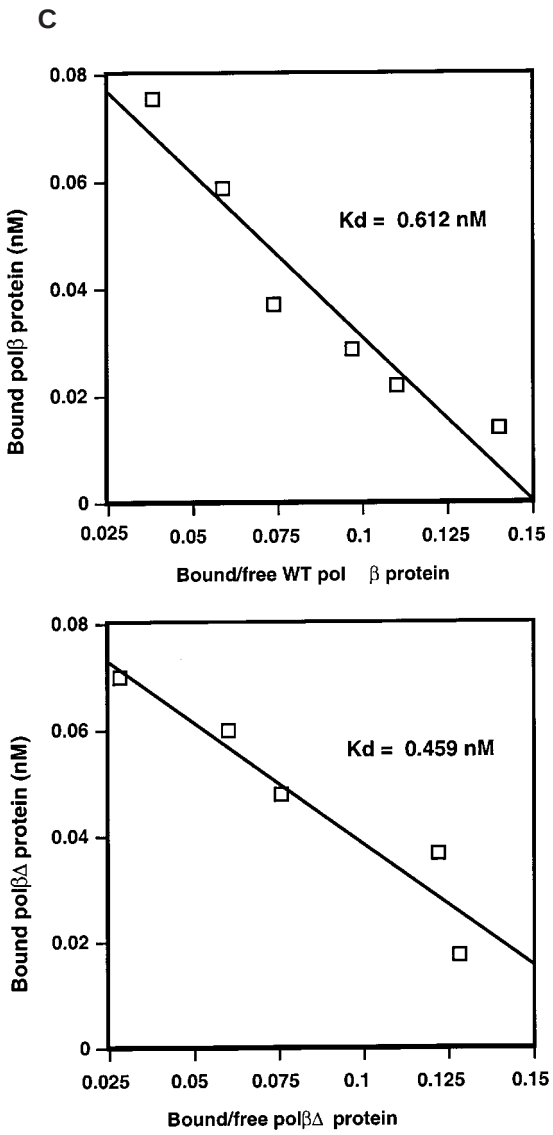


FIGURE 3: (A) DNA binding activity of a recombinant purified pol$\beta\Delta$ protein: lanes 2−7, DNA binding activities of WT polβ; lanes 8−12, activities of pol$\beta\Delta$; and lanes 13−16, activities of WT polβ (0.1 nM) and pol$\beta\Delta$ (0.1−2.5 nM). Lane 1 represents a negative control without any protein. (B) Table showing the additive effect of polβ and pol$\beta\Delta$. The intensities of the polβ or pol$\beta\Delta$ and DNA complexes were measured by using the ImageQuant program. The measured intensity was expressed in arbitrary units. (C) K_d values of WT polβ and pol$\beta\Delta$.

of the parent 16.3 cells, the levels of functions of 16.3 cells were progressively reduced (*19*). To establish further the inhibitory role of the dominant negative pol$\beta\Delta$, the BER activity of a nuclear extract of 16.3 cells was reevaluated after adding increasing amounts of purified recombinant pol$\beta\Delta$. The BER activity in nuclear extract without pol$\beta\Delta$ is shown in first lane on the left side of Figure 4. In the presence of increasing concentrations (1−1000 pg) of purified pol$\beta\Delta$, BER activities progressively decreased. At 25 pg of purified pol$\beta\Delta$, BER activity was reduced more than 50% (ImageQuant program, Molecular Dynamics) as shown in the bar diagram. When the protein level was increased to 75 pg, BER activity was almost same as the background. These data demonstrate that the pol$\beta\Delta$ protein antagonizes the function of the WT enzyme expressed in 16.3 cells, supporting our previous results (*19*). The results from this experiment (Figure 4) clearly indicate that pol$\beta\Delta$ inhibits the BER activity in the nuclear extract of 16.3 cells.

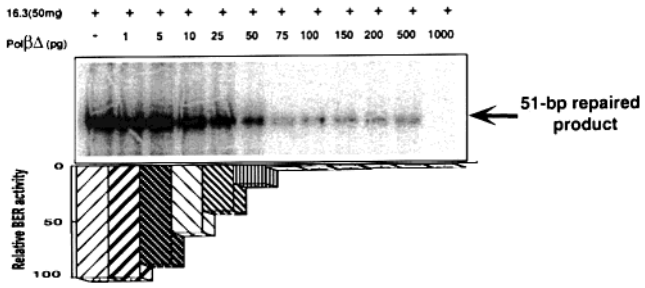


FIGURE 4: Effects of purified pol$\beta\Delta$ on BER activity of 16.3 nuclear extracts. Each data bar represents the mean ± standard deviation of three separate experiments.

Results presented in Figures 3A and 4 suggest accessory proteins in nuclear extracts perhaps are involved in the dominant negative activity of pol$\beta\Delta$.

Interaction of Pol$\beta\Delta$ with XRCC1, PARP, and Ape Proteins in Vitro and in Vivo. To test whether the pol$\beta\Delta$

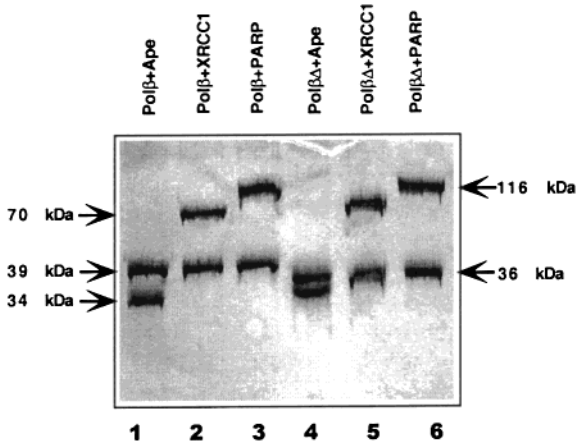


FIGURE 5: Interaction of pol$\beta\Delta$ with XRCC1, PARP, and Ape proteins in vitro determined by the affinity precipitation assay: lane 1, a 39 kDa WT polβ and a 34 kDa Ape protein in the eluate; lane 2, 70 kDa XRCC1 and 39 kDa polβ proteins; lane 3, the 116 kDa PARP protein separated from the 39 kDa polβ; and lanes 4−6, 36 kDa pol$\beta\Delta$ and a 34 kDa Ape protein, 70 kDa XRCC1 and 36 kDa pol$\beta\Delta$, and 116 kDa PARP and a 36 kDa pol$\beta\Delta$ protein, respectively, in the same eluate.

protein binds directly to XRCC1, PARP, and Ape, we took advantage of histidine-tagged pol$\beta\Delta$ using an affinity precipitation assay. A 36 kDa protein (presumably the pol$\beta\Delta$ protein) and a 70 kDa XRCC1 protein were identified in an eluate fraction (lane 5, Figure 5). A similar result was obtained from the interaction of WT polβ with XRCC1 (lane 2). The pol$\beta\Delta$ was incubated with PARP or Ape and affinity precipitated, revealing a pol$\beta\Delta$ protein (36 kDa), PARP (116 kDa), and Ape (34 kDa), shown in lanes 6 and 4, respectively. These results suggest that pol$\beta\Delta$ indeed directly interacts with XRCC1, PARP, and Ape. XRCC1, PARP, and Ape are known to form a multiprotein complex with WT polβ (*21*−*23*). It is unknown, however, whether pol$\beta\Delta$ interacts with these proteins in vivo.

To investigate such potential interactions in vivo, we used four mouse embryonic fibroblast cell lines: a 16.3 cell line expressing WT polβ, a 19.4 cell lines established from polβ knockout mouse experiment that do not express polβ, a 16.3ΔP cell line that was established in our laboratory by transfecting 16.3 cells with 87 bp deleted polβ cDNA expressing the truncated polβ and endogenous polβ (19), and a 19.4ΔP cell line that was also established in our laboratory by transfecting 19.4 cells with 87 bp deleted polβ cDNA. Results from Co-IP-Western blot analyses demonstrate that the 34 kDa Ape binds to WT polβ expressed in 16.3 cells (lane 1, Figure 6A, middle of the panel). Ape also binds to pol$\beta\Delta$ at a comparable level in 19.4ΔP cells (lane 4). Ape interacts with pol$\beta\Delta$ and WT polβ in 16.3ΔP cells (lane 3, Figure 6A). A 116 kDa PARP protein binds to both WT polβ (lane 1) and pol$\beta\Delta$ (lanes 1 and 4, Figure 6B, middle of the panel). XRCC1 binds to both WT polβ and pol$\beta\Delta$ (lane 3, Figure 6C). The pol$\beta\Delta$ alone interacts with XRCC1 (lane 4) in 19.4ΔP cells (expressing only pol$\beta\Delta$). Represented in the bar diagram (right side of panels A−C of Figure 6), our data provide evidence that Ape or PARP binds to pol$\beta\Delta$ at a similar level. However, interactions of XRCC1 with pol$\beta\Delta$ are markedly higher than those with Ape or PARP in 19.4ΔP cells. The XRCC1 protein binds 5−6 times more to pol$\beta\Delta$ expressed in 19.4ΔP cells (lane 4) than to

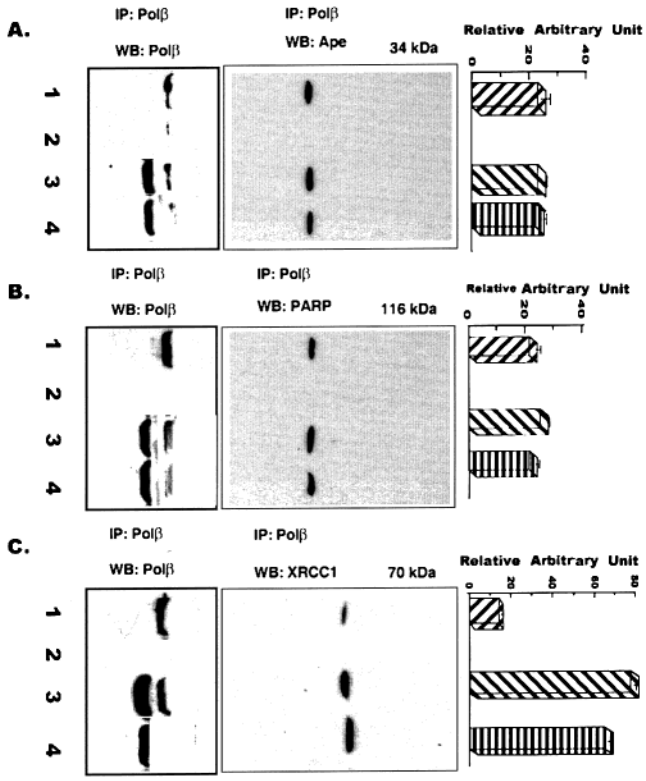


FIGURE 6: Interactions of pol$\beta\Delta$ with XRCC1, PARP, and Ape proteins in vivo determined by Co-IP-Western blot analysis of pol$\beta\Delta$, Ape, PARP, and XRCC1 in mouse embryo fibroblast cell lines. Lanes 1−4 contained cell lysates of 16.3, 19.4, 16.3ΔP, and 19.4ΔP cell lines, respectively. In the middle of panel A is shown Western blot analysis of the immunoprecipitated polβ with the anti-Ape antibody. The 34 kDa Ape protein is shown in this panel. At the left side of panel A is shown Western blot analysis of the immunoprecipitated polβ protein with the polβ antibody. At the right side of panel A is shown a representation of the data in the middle of the panel by bar diagram. Results are the mean ± standard deviation of three independent preparations of cell extracts. In the middle of panel B is shown Western blot analysis of the immunoprecipitated polβ with the anti-PARP antibody; the size of PARP is 116 kDa. At the left side of panel B is shown Western blot analysis of the immunoprecipitated polβ protein with the polβ antibody. At the right side of panel B is shown a representation of the data in the middle of the panel by bar diagram. Results are the mean ± standard deviation of three independent preparations of cell extracts. In the middle of panel C is shown Western blot analysis of immunoprecipitated polβ with the anti-XRCC1 antibody; the size of XRCC1 is 70 kDa. At the left side of panel C is shown Western blot analysis of the immunoprecipitated polβ protein with the polβ antibody. At the right side of panel C is shown a representation of the data in the middle of the panel by bar diagram. Results are the mean ± standard deviation of three independent preparations of cell extracts.

the WT polβ expressed in 16.3 cells (lane 1, bar diagram), as detected by the NIH Image program. As shown in lane 3 of Figure 6C (bar diagram), an enhancement of binding activities of WT and pol$\beta\Delta$ proteins to XRCC1 is evidenced in 16.3ΔP cells. Thus, these results suggest that in vivo pol$\beta\Delta$ interacts with XRCC1, PARP, and Ape, and interestingly, it most avidly binds to XRCC1.

Reconstituted Base Excision Repair Function of Pol$\beta\Delta$. The 5′-end-labeled, 21 bp AP substrate is shown in Figure 7A (lane 1). Purified recombinant polβ repairs an AP site substrate by gap filling synthesis, and the expected 22 bp product has been generated (lane 2). Using a reconstituted repair system, the 22 bp product is ligated to a 51 bp product

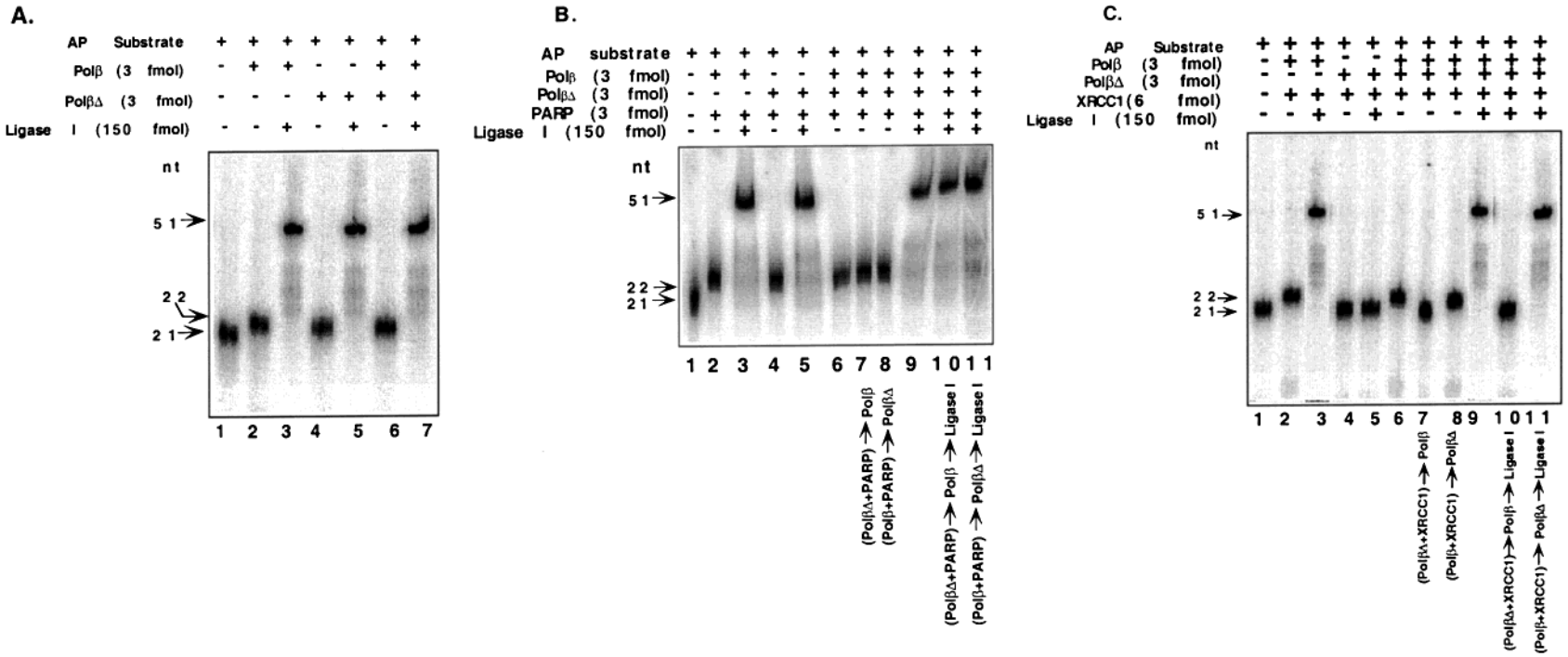


FIGURE 7: (A) Gap filling synthesis activity of WT polβ and the pol$\beta\Delta$ protein: lane 1, a 21 bp AP substrate with an incised site substrate; lane 2, a 22 bp repair product catalyzed by WT polβ; lane 3, a 51 bp ligated product in the presence of WT polβ and ligase I; lanes 4 and 5, 22 and 51 bp repaired and ligated products catalyzed by pol$\beta\Delta$ in the absence and presence of ligase I, respectively; lane 6, a 22 bp repaired product generated by polβ and pol$\beta\Delta$; and lane 7, a 51 bp ligated product generated by ligase I in the presence of polβ and pol$\beta\Delta$. (B) Effects of PARP on the gap filling synthesis of pol$\beta\Delta$: lane 1, a 21 bp AP substrate; lanes 2 and 3, gap filling synthesis activity of WT polβ in the presence of PARP and ligase I, respectively; lanes 4 and 5, activities of pol$\beta\Delta$ in the presence of PARP and ligase I, respectively; lane 6, activity of pol$\beta\Delta$ in the presence of polβ, pol$\beta\Delta$, and PARP; lane 7, gap filling synthesis of polβ in the presence of a preincubated pol$\beta\Delta$ and PARP complex; lane 8, activity of pol$\beta\Delta$ in the presence of a preincubated polβ and PARP complex; lane 9, a 51 bp ligated product formed in the presence of polβ, pol$\beta\Delta$, PARP, and ligase I; lane 10, BER product from a reaction mixture containing polβ, the preincubated pol$\beta\Delta$, and PARP complex and ligase I; and lane 11, a ligated product from a reaction mixture containing pol$\beta\Delta$ and the preincubated polβ and PARP complex. (C) Gap filling synthesis activity of WT polβ and the pol$\beta\Delta$ bound to XRCC1: lane 1, a 21 bp AP substrate; lanes 2 and 3, gap filling synthesis activity of WT polβ in the presence of XRCC1 and ligase I, respectively; lanes 4 and 5, activities of pol$\beta\Delta$ in the presence of XRCC1 and ligase I, respectively; lane 6, activity of the pol$\beta\Delta$ in the presence of polβ and XRCC1; lane 7, gap filling synthesis of polβ in the presence of a preincubated pol$\beta\Delta$ and XRCC1 complex; lane 8, activity of pol$\beta\Delta$ in the presence of a preincubated polβ and XRCC1 complex; lane 9, a 51 bp ligated product formed in the presence of polβ, pol$\beta\Delta$, XRCC1, and ligase I; lane 10, BER product from a reaction mixture containing polβ and the preincubated pol$\beta\Delta$ and XRCC1 complex; and lane 11, a ligated product from the reaction mixture containing pol$\beta\Delta$ and the preincubated polβ and XRCC1 complex.

(lane 3). More importantly, after the purified polβΔ fills in one nucleotide, converting a 21 bp substrate to a 22 bp product (lane 4), ligase I converted it to a 51 bp product (lane 5). When equimolar amounts of polβ and polβΔ were added to the reaction mixture containing AP substrate, a repair product was generated (lane 6) which was ligated by ligase I to an expected product (lane 7). These results indicate that polβΔ can efficiently catalyze the removal of the 5′-deoxyribonucleotide phosphate from the AP site and fill a single-nucleotide gap, establishing that the purified truncated protein is functionally active. Furthermore, when both WT polβ and polβΔ are added to a BER reaction mixture, a 22 bp repaired product is formed (lane 6) and ligated efficiently to a 51 bp product in the presence of ligase I (lane 7). It appears from these results that polβΔ does not disrupt the catalytic function of the WT enzyme. Hence, we conclude that other associated proteins are required for the dominant negative activity of polβΔ.

To pursue this question, we first examined the gap filling synthesis of WT polβ in the presence of the polβΔ and PARP polypeptide complex. When equimolar amounts (3 fmol) of polβΔ and PARP were added to the reaction mixture, a repaired product was formed (lane 4, Figure 7B). Similarly, in the presence of PARP, the control WT polβ by filling in the gap can generate a 22 bp repaired product (lane 2, Figure 7B). Additions of ligase to these reaction mixture leads to the formation of a 51 bp repaired and ligated product (lanes 3 and 5). Addition of polβ, polβΔ, and PARP to the reaction mixture can generate a 22 bp repaired product (lane 6). Addition of a preincubated polβΔ and PARP complex or polβ and PARP complex to either the polβ or polβΔ reaction mixture resulted in the formation of repaired product shown in lanes 7 and 8, respectively. These data suggest that the preincubated polβΔ and PARP complex has no dominant negative effect on the repair activity of WT polβ. Addition of ligase I to the reaction mixture containing polβ, polβΔ, and PARP in different combinations results in 51 bp repaired product (lanes 9−11). However, the results presented in Figure 7B indicate that the PARP−polβΔ complex probably does not have such an effect on WT polβ.

To determine whether XRCC1 has a role in the dominant negative activity of the truncated polβ, a reconstituted gap filling synthesis assay was carried out in the presence of polβΔ. In the presence of XRCC1 (6 fmol), the WT enzyme fills one nucleotide gap and generates a 22 bp repaired product (lane 2, Figure 7C). In contrast, in the presence of XRCC1, polβΔ alone cannot repair the gap (lane 4). Interestingly, in the absence of XRCC1, the truncated protein is as efficient as WT polβ in repairing gapped DNA (lane 4, Figure 7A). Furthermore, when ligase I is added to this reaction mixture, a ligated product is undetectable (lane 5, Figure 7C), indicating that the AP substrate containing one nucleotide gap remains unrepaired. Unlike polβΔ, the WT enzyme can fill in the gap at the AP substrate in the presence of XRCC1, and ligase I can convert the repaired product to a 51 bp product (lane 3). Upon incubation of WT polβ, polβΔ, and XRCC1 together, a repaired product becomes visible (lane 6). When preincubated polβΔ and XRCC1 were added to WT polβ, a 21 bp product was seen (lane 7). This observation strongly suggests that polβΔ bound to XRCC1 interferes with the BER function of the WT enzyme. In contrast, in the presence of polβΔ, WT polβ preincubated with XRCC1 can repair a gapped DNA to a 22 bp product (lane 8), suggesting that the complex of polβ and XRCC1 does not inhibit the BER activity of polβΔ. To determine further the effect of the complex of polβΔ and XRCC1 on the gap filling synthesis of polβ and ligation steps, we added preincubated polβΔ with XRCC1 to the WT polβ assay containing ligase I. In this case, a 21 bp product was identified, without a ligated product (lane 10). However, a 51 bp ligated product was detected in a BER assay consisting of a WT polβ and XRCC1 complex, polβΔ and ligase I (lane 11), indicating that the WT polβ and XRCC1 complex is potentially capable of repairing efficiently.

DNA Binding Activity of polβΔ in the Presence of XRCC1 and Oligonucleotides. To examine whether a complex of polβΔ and XRCC1 binds to DNA substrate, the gel mobility assay was performed with an increase in the XRCC1 concentration. It is known that XRCC1 at a concentration of 240 nM forms a supershifted complex with 1.2 nM WT polβ and a gapped DNA (*25*). Following this protocol (*25*), we have examined interactions of 1.25 nM polβΔ with 25−250 nM XRCC1 and binding to a gapped DNA. XRCC1 and the polβΔ protein (1.25 nM) form a supershifted complex with gapped DNA (Figure 8A, lanes 3−6). Two protein−DNA bands were visible in the gel when the concentration of XRCC1 was increased from 200 to 250 nM. It is tempting to speculate that the upper slow-moving band perhaps represents an equimolar complex of polβΔ, XRCC1, and DNA; on the other hand, the slow-moving band may suggest formation of the polβΔ−XRCC1−DNA complex at a high level of XRCC1 such as 250 nM. The order of protein additions to the reaction mixture containing substrate was as shown in panels A and B of Figure 8 starting from top to bottom. We next addressed the possibility of a tighter binding of the polβΔ and XRCC1 complex to gapped DNA compared with the binding of a complex of WT polβ and XRCC1. Two bands are visible when either WT polβ (lane 1, Figure 8B) or polβΔ (lane 8) forms a protein−DNA complex. When the complex is challenged with cold oligos, one shifted band (second from the top of the gel) is visible when the other one was abolished (lanes 2−7 and 9−14). Exactly similar observations of effects of cold oligos on WT polβ−DNA binding were reported by Kubota et al. (*22*).

The levels of formation of protein−DNA complexes mediated by the WT polβ shown in lanes 2−7 (Figure 8B) or polβΔ (lanes 9−14) in the presence of XRCC1 progressively declined when the amount of cold oligonucleotides was increased from 0.05 to 1.5 fmol or from 0.25 to 2 fmol. At 0.05 fmol, the binding activity of the WT polβ−XRCC1 complex appears to be ∼70% reduced, whereas the DNA binding activity of the polβΔ−XRCC1 complex was reduced to ∼60% by oligos at 0.25 fmol, an amount 5 times higher than the amount used in the WT polβ reaction. In the presence of 1.5 fmol of oligos, the DNA binding activity of WT polβ and XRCC1 has been almost abolished (lane 7). However, at the same concentration of oligos, the polβΔ−XRCC1 complex exhibits ∼18% activity (lane 13). Results show that the DNA binding affinity of the polβΔ−XRCC1 complex is 3−5 times higher (quantitated by the ImageQuant program) than that of the WT polβ−XRCC1 complex. Thus, the presumable binary polβΔ-bound XRCC1 complex binds more strongly to gapped DNA than does the WT polβ-bound XRCC1 complex.

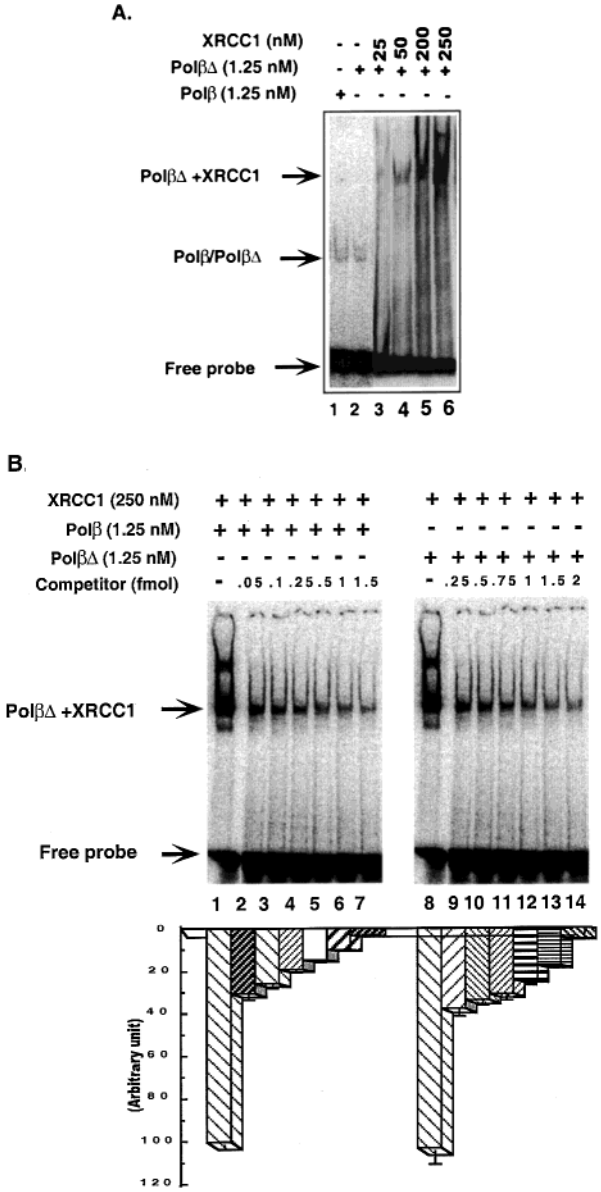


FIGURE 8: (A) Gel mobility shift assay using increasing concentrations of XRCC1 and 1.25 nM pol$\beta\Delta$ (lanes 3−6). Lanes 1 and 2 represent DNA binding of WTpolβ and pol$\beta\Delta$, which was used as a control. (B) Effect of oligonucleotides on the DNA binding activity of the pol$\beta\Delta$ and XRCC1 complex: lane 1, the WT polβ and XRCC1 complex; lanes 2−7, the WT polβ and XRCC1 complex in the presence of 0.05−1.5 fmol of consensus oligos (competitor); lane 8, the pol$\beta\Delta$ and XRCC1 complex; and lanes 9−14, the pol$\beta\Delta$ and XRCC1 complex in the presence of 0.25−2.0 fmol of consensus oligos. Data represented by a bar diagram are means ± standard deviation of three independent experiments.

DISCUSSION

In elucidating the mechanisms by which pol$\beta\Delta$ inhibits functions of WT polβ, we provide evidence that pol$\beta\Delta$ by itself does not disrupt the DNA binding or gap filling synthesis activity of WT polβ. Data also provide the first indication that a pol$\beta\Delta$−XRCC1 protein−protein complex is involved in the inhibitory activity of the truncated protein. Complexes of either pol$\beta\Delta$ and Ape or PARP did not exhibit such negative activity.

The precise mechanisms underlying the dominant negative interaction of the pol$\beta\Delta$ and XRCC1 complex with WT polβ remain unclear at present. A truncated and WT polβ protein complex could lead to dominant negative inhibition by the truncated protein, as proposed in model I (Figure 1). This complex could bind to the substrate, which would prevent WT polβ from binding to DNA. If two proteins formed a heterodimer, the mobility of the protein−DNA complex should have shifted. Results presented in this study indicate that these two proteins do not form a heterodimer. It is conceivable that the truncated protein inhibits the function of WT polβ by competitive binding to the AP site of damaged DNA, as represented in model II. In this case, the pol$\beta\Delta$ protein should have higher binding affinity toward damaged DNA than WT polβ. However, pol$\beta\Delta$ itself does not inhibit DNA binding activity of WT polβ. Moreover, pol$\beta\Delta$ efficiently fills a single-nucleotide gap by incorporating one base at an AP site. These results eliminate the possibility that the pol$\beta\Delta$ protein does directly impair functions of the WT enzyme by competition. Therefore, model II seems not to be accountable for the inhibitory role of the pol$\beta\Delta$. Instead, our data support model III. In a reconstituted system, a protein−protein complex of pol$\beta\Delta$ and XRCC1 inhibits the gap filling synthesis activity of the WT enzyme. However, when pol$\beta\Delta$ is bound to XRCC1, the truncated protein fails to insert one base in the gapped DNA. More importantly, the complex of pol$\beta\Delta$ and XRCC1 binds more tightly to the DNA gap than does the WT polβ and XRCC1 complex. It is known now from a NMR study that the N-terminal domain of XRCC1 binds to the concave side of the 90° bend nicked and/or gapped DNA, whereas WT polβ binds to the convex side (*25*). It is possible that XRCC1 bound to DNA specifically obscures the active site of pol$\beta\Delta$. As the pol$\beta\Delta$, XRCC1, and gapped/AP substrate complex has very strong internal binding affinity, the complex may inhibit WT polβ from binding to the substrate.

XRCC1 consists of 633 amino acids. The polβ protein interacts with XRCC1 between amino acid residues 84 and 183 in the N-terminal region of XRCC1 (*22*). This region is located upstream of a nuclear localization signal domain and a BRCT1 region (*27*). The three-dimensional structure of the XRCC1 BRCT domain has been established (*28*). Recently, the N-terminal domain of XRCC1 has been characterized and shown to interact with the palm−thumb region of polβ in a ternary complex with DNA (*25*). XRCC1 also binds to PARP by its BRCT1 module (BRCA1 carboxyl-terminal), and has been implicated in the downregulation of PARP (*29*). XRCC1 interacts with ligase III via binding through its BRCT2 module (*22*, *29*, *30*). Marinchev et al. (*25*) established that XRCC1 has a novel ssbreak DNA binding function. Here we report that XRCC1 bound to pol$\beta\Delta$ not only can bind to DNA but also can inhibit the function of WT polβ. It is unknown whether XRCC1 binds to the amino- or carboxyl-terminal region of the polβ. However, an important role of the multiprotein consortium, which includes XRCC1, is recognized in cellular BER activity. Since the truncation of 29 amino acids in the polβ protein of tumors occurs in the palm domain at the carboxyl-terminal region of the enzyme, it is important to identify the precise region of truncated and WT polβ primary structures to which XRCC1 binds. The human XRCC1 gene efficiently repairs DNA base damage in CHO-EM9 and EM-C11 cells (XRCC1 mutant cells); these cells are hypersensitive to alkylating agents and ionizing radiation (*27*, *31*). XRCC1 interacts tightly with eukaryotic ligase III and has

been implicated in the BER pathway downstream from Ape and in polβ-mediated hydrolysis of a phosphodiester bond at the damaged strand of the DNA template (*32*, *33*). It is also possible that novel protein(s) bound to XRCC1 may contribute to the inhibitory activity of the pol$\beta\Delta$. Although the apparent catalytic activity of XRCC1 is unknown (*21*, *22*, *34*), the XRCC1 protein is thought to have an important role in the ligation step of the short patch BER pathway catalyzed by WT polβ (*33*). Like WT polβ, the XRCC1 gene is essential for the survival of knockout mice and for normal mouse development (*35*, *36*), and contributes to cellular sensitivity to X-ray irradiation (*37*).

Because it exerts a dominant inhibitory effect on functions of the WT polβ enzyme, the pol$\beta\Delta$, once expressed in cells, may cause increased cell sensitivity to environmental factors and endogenous stresses that damage DNA, leading to genomic instability and decreased cell viability. Cells expressing pol$\beta\Delta$ are more sensitive to chemicals and less likely to repair chemically induced DNA damage (*7*, *19*, *38*). The dominant negative pol$\beta\Delta$ may thus play a significant role in the increased susceptibility of individuals to toxicity, embryogenesis, teratogenesis, and cancer.

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